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Rapid qPCR Detection of Mycobacterium Tuberculosis in Blood and Organ Tissues Using a Collection-to-Detection System

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Received Date: 23rd September 2017

Accepted Date: 28th October 2017

Published Date: 05th November 2017

Citation: Daum LT, Schuman RF, CJ Sei, Rikhi N, Masadieu A, Rodriguez JD, Chambers JP, and Fischer GW Fischer GW (2017) Rapid qPCR Detection of Mycobacterium Tuberculosis in Blood and Organ Tissues Using a Collection-to-Detection System. Enliven: Microbes and Microbial Techniques 4(1):002.

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Abstract

Introduction: *Mycobacterium tuberculosis* (MTB) is an important cause of sepsis in individuals with HIV and they often die within 18 days of presentation. Thus, there is an urgent need to rapidly detect the presence of MTB bacteremia.

Objective: To demonstrate the potential usefulness of a 'collection-to-detection' system for rapid qPCR detection of MTB in blood and organ tissue specimens transported in molecular medium to a distant lab.

Methods: A total of 39 blood and tissue specimens were collected from mice (n=6) given intraperitoneally either anti-MTB monoclonal antibodies (MABS) or PBS, and challenged 24 hours later with an intravenous injection of 10^5 ethanol-killed MTB. Whole blood and tissue specimens were transported to a distant laboratory for qPCR analysis.

Results: MTB was detected by qPCR in both blood and tissues transported over 1000 miles at ambient temperature within 6 hours of arrival. Differential MTB clearance was observed using two anti-MTB opsonic MABs.

Conclusion: This study supports the usefulness of transporting blood and tissue to a centralized or regional laboratory for expedient diagnosis of MTB bacteremia in high risk patients.

Keywords: Real-time PCR; Primestore; *Mycobacterium tuberculosis*; Diagnostic detection

Introduction

The convergence of human immunodeficiency virus (HIV) and tuberculosis (TB) in many areas of the world contributes to increased drug resistance and rapidly progressive disease [1,2]. In sub- saharan Africa, MTB is now a leading cause of community acquired bloodstream infection among hospitalized patients [3-6]. In HIV patients presenting with severe sepsis, MTB was the most prevalent pathogen detected in blood [5]. Patients with HIV and MTB bacteremia exhibited a 30 day mortality of approximately 50% [3,5], and in one study half of the deaths occurred within 18 days, making blood culturing impractical for detecting MTB bacteremia [5]. In contrast to culturing, real-time quantitative PCR (qPCR) could afford a more rapid diagnosis of MTB from blood. However, in low resource countries many hospitals lack qPCR capabilities. Thus, an easy to use 'on-site' means of collection in combination with safe transport to a regional or centralized laboratory facility capable of qPCR analysis would be very useful in resource limited environments.

A commonly exploited MTB target for sensitive MTB detection is the IS-6110 multi-copy gene (16-20 genomic copies). Previous reports have described PCR and hybridization assays targeting the IS-6110 gene for MTB diagnosis from blood [7,8]. However, strains containing few or no IS-6110 gene copies have been reported [9,10]. Conversely, the IS-1081 gene is less variable in sequence and copy number with most MTB genomes containing approximately six IS 1081 copies. A *Ready-to use*, multiplex MTB qPCR assay mixture containing buffers, primers, probe, and enzyme targeting both IS-6110 and IS-1081 has been developed [11]. In this study, using a mouse model we explored the utility of shipping blood and tissues in a molecular transport medium to a distant laboratory for rapid MTB detection using a *ready-to-use* multiplex qPCR assay mixture.

Materials and Methods

Mouse Bacteremia Model

Ethanol killed MTB (EK-MTB) Erdman strain (ATCC 35801) provided by Battelle Laboratories at 10^8 CFU/mL was washed three times in phosphate buffered saline (PBS), and diluted to $5x10^5$ CFU/mL. Female BALB/c mice (Harlan Laboratories, Indianapolis, IN) weighing approximately 30 grams (n=6) were given an intravenous injection of 0.2 mL EK- MTB (10^5 CFU/mouse). Monoclonal antibodies (MABs) that bind MTB as determined by ELISA, demonstrating opsonic activity using HL60 cells (Sei et al., unpublished data) were used to mediate MTB clearance from blood and tissues in order to detect MTB bacilli over a broad range. Two mice in each treatment group were given intra peritoneal injections of either 0.3 mL sterile phosphate buffered saline (PBS), or 0.3mL of PBS containing either 26 µg MAB AB9, or 205 µg MAB GG9 24 hours before MTB challenge. Mice were bled retro-orbitally at 15 minutes *post*-MTB challenge, and subsequently at 4 and 24 hours by cardiac puncture immediately *post-euthanasia*. This mouse protocol was approved by the Institutional Animal Care and Use Committee (IACUC protocol 73).

For qPCR, 0.2mL whole blood was transferred to tubes containing EDTA (Becton Dickinson, Sparks, MD), and after all mice were bled 100 µL samples were transferred to 1.0 mL Primestore Molecular Transport Medium® (PS-MTM). Murine tissues (~1 gram kidney, liver, lung and spleen) obtained at 4 and 24 hours *post* MTB-challenge were homogenized in PBS, and transferred to tubes containing 1.0 mL PS-MTM. A total of 39 specimens (15 blood and 24 tissue homogenates) were transported over night via Fed Ex at ambient temperature from Gaithersburg, MD to San Antonio, TX for qPCR analysis.

DNA Extraction and Multiplex qPCR:

Murine blood and tissue homogenates were subjected to nucleic acid extraction using PrimeXtract (Longhorn Vaccines and Diagnostics, San Antonio, TX). For DNA extraction, PS-MTM treated samples (e.g., blood and tissues) were thoroughly mixed for 1 minute by inversion after which time a 0.2 mL aliquot was removed, transferred to 0.2 mL Lysis Solution to which 0.2 mL 100% ethanol was added, and thoroughly mixed for five minutes at room temperature. Following mixing, samples were centrifuged for one minute at ~2000x g in order to pellet red blood cells, and the resulting supernatant was decanted, and transferred to spin columns per manufacturer's instruction. Elution of nucleic acid was achieved by application of 50 µL heated nuclease-free water to the column. Real-time qPCR was carried out in replicates of three for each sample following addition of 2.5µL total DNA(~1-10 ng/µL) to 7.5µL PrimeMix® Multiplex MTB (Longhorn Vaccines and Diagnostics, LLC, San Antonio, TX, USA) and applied to an ABI 7500 Instrument (Thermo Fisher Scientific, Waltham, MA, USA). Results were analyzed using a C_T threshold baseline of 0.1 with start cycle = 3, and end cycle = 15. Results were plotted as C_T mean values with standard error. The qPCR testing was completed within six hours of specimen arrival.

Results

MTB in mouse blood was detected across a broad concentration range according to qPCR (Figure 1). Blood samples obtained 15 minutes post MTB challenge were qPCR positive for MTB with C_T mean values of 29.8 (range 28.7-31.1), and 31.6 (range 30.6-33.0) for IS-6110 and IS-1081, respectively (approximately 10^3 to 10^4 CFU/mL). As expected, higher IS-6110 copy number provided lower C_T values. A rapid decline in bacilli, as indicated by increasing C_T values was observed over the first 4 hours with continued reduction in C_T values at 24 hours post treatment (Figure 1). At 24 hours, all mice exhibited low levels of MTB in blood, i.e., C_T values of 38.9 and 39.5 for IS-6110 and IS-1081, respectively (approximately 10^1 to 10^2 CFU/mL). Mice given MAB GG9 cleared MTB from the blood by 4 hours (C_T= 40 for IS-6110 and IS-1081 gene targets) at both 4 and 24 hours (Figure 1). PBS treated mice did not clear MTB at either 4 or 24 hours [IS-6110]; however, the less sensitive gene target IS-1081 exhibited a C_T value of 40 at 24 hours indicating no detection. MTB was also detected by qPCR in tissue homogenates, i.e., kidney, liver, spleen, and lung with lung tissue exhibiting the highest MTB concentration, i.e., lowest C_T values at both 4 and 24 hours *post* challenge (Figure 2). In contrast, kidney tissue exhibited the highest C_T value at 4 and 24 hours indicating few bacilli present at either time. Liver and spleen tissue homogenates exhibited decreasing C_T values at 4 and 24 hours is consistent with increased numbers of bacilli, and phagocytosis in these tissues.

Discussion

The presence of MTB in the blood was reported over 100 years ago [12]. However, with the convergence of HIV and TB in many parts of the world, MTB sepsis has become more common. Recent studies from Tanzania and Uganda have documented that bacteremic disseminated TB is rapidly fatal, especially with immunologically advanced HIV disease [3,5]. Since MTB is now a common cause of sepsis, rapid and sensitive detection of MTB in blood is urgently needed [5]. Data reported here using a novel mouse model suggest that MTB bacteremia in blood and tissues can be rapidly assessed in transported samples by qPCR. As such, this could be very helpful in: 1) early confirmatory diagnosis, and 2) monitoring therapeutic efficacy. Importantly, while qPCR is a rapid and sensitive diagnostic method not all facilities in low resource countries can perform such molecular testing. This multiplex assay described here uses not only the IS-6110 multi-copy target, but also the highly Conserved Is-1081 target. This could be critical for IS-6110 gene deleted strains [9,10]. Furthermore, data from Tanzania suggest that the yield in CFU from blood cultures are lower than the detection limit of Cepheid's GenXpert [13,14]. A recent study documented that GenXpert had a 21% sensitivity for diagnosing MTB bacteremia [15]. Furthermore, all individuals that were GenXpert positive died, likely indicating high level bacteremia in these patients [15]. It would be desirable to also rapidly identify people with low level bacteremia and begin appropriate treatment before the disease escalates to severe sepsis with a high mortality.

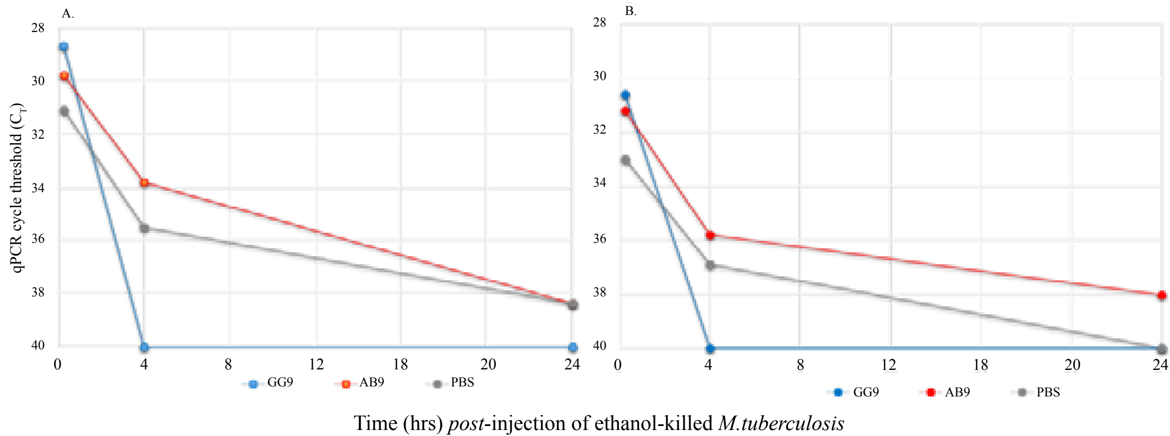

Figure 1: Detection of M. tuberculosis using qPCR targeting the IS-6110 (Frame A) and IS-1081 (Frame B) MTB genes. Total DNA was extracted from blood; treatment group MAB GG9 (blue), MAB AB9 (red), and phosphate buffered Saline (PBS) control (gray) before MTB challenge as described in the 'Materials and Methods'.

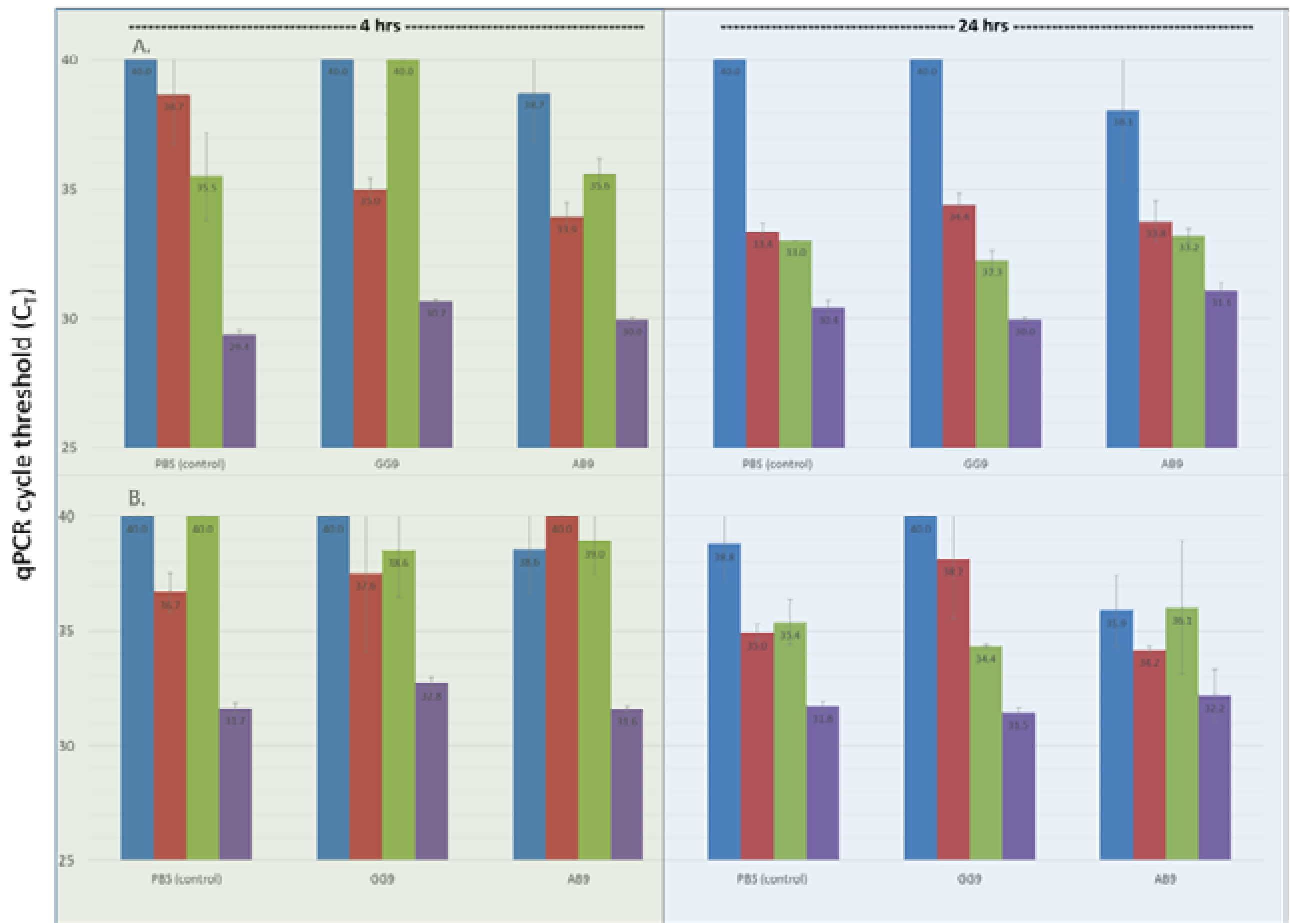

Figure 2: qPCR detection of M. tuberculosis from murine kidney (blue), liver (red), spleen (green), and lung (purple) DNA targeting IS- 6110 (Frame A) and IS-1081 (Frame B) according to treatment and time after MTB challenge. qPCR was performed for each tissue in duplicate with average mean and standard error bars shown. A qPCR cycle threshold (CT) value of 40 represents no detection.

The collection, transport, extraction and qPCR multiplex MTB assay used in this study has been previously validated for sensitive detection of MTB in sputum [16-19]. Here we demonstrate that mouse blood and tissue specimens can be transported at ambient temperature to a distant facility for qPCR analysis, i.e., within one day of specimen arrival.

Although a mouse bacteremia model was used in the study, this approach could significantly impact the diagnosis and treatment of MTB bacteremia and sepsis in patients in many low resource areas where hospitals lack treatment and molecular detection infrastructure. With certainty, clinical studies are needed in high risk patients in Africa using the collection to detection approach described here.

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