Development of Real-Time PCR Assay for *Mycobacterium bovis* BCG Detection

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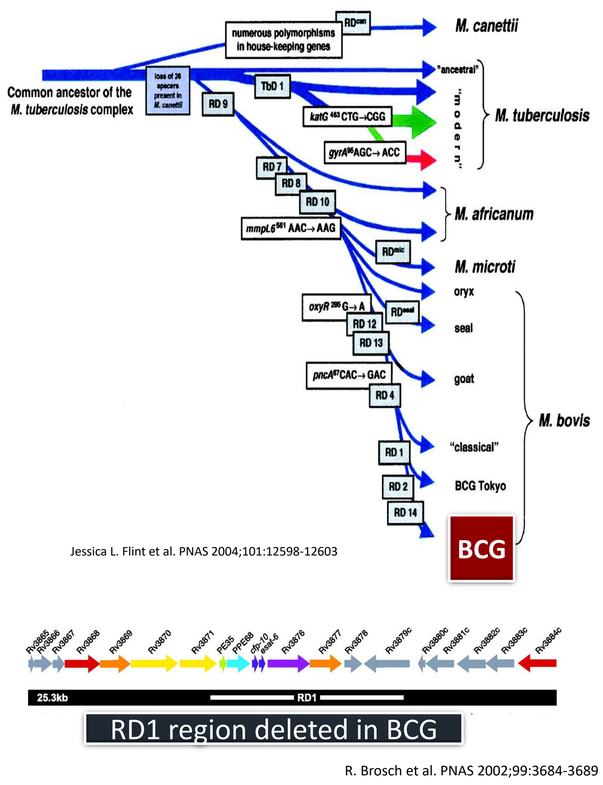
ABSTRACT

BCG vaccine is the only current available vaccine effectively against TB in children. However, complication after the vaccination have been reported. To timely identify the causative agent, this research aimed to develop a real time PCR assay for detection of *M. bovis* BCG in clinical specimens. This assay showed an analytical specificity of 100% for identification of *M. bovis* BCG among strains of *Mycobacterium* spp. and bacteria. Limit of detection was 10 copies per reaction with a good amplification efficiency at the coefficient 0.993 in clinical specimens. The assay provides as a rapid diagnostic tool of *M. bovis* BCG that could be useful for clinical samples from allow timely diagnosis and selection of appropriate treatment for pediatric patients.

INTRODUCTION

M. bovis Bacillus Calmette–Guérin (BCG) vaccine is live attenuated strain of a virulent *M. bovis*. BCG's attenuation is result of losing major virulent gene, region of difference 1 (RD1). A 9.5 kb RD1 DNA segment, encoded the most important virulence of *M. tuberculosis* complex (TBC), but the segment found to be permanently absent from BCG but present in all the other species of TBC. Despite the vaccine is highly effective to prevent military tuberculosis (TB) and TB meningitis, BCGosis complication has been reported.

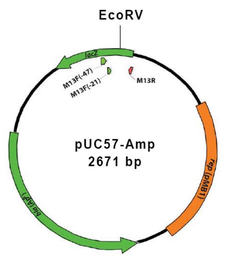


This research aimed to produce the effective real-time PCR with internal control for detection of *M. bovis* BCG in paediatric clinical samples.

MATERIALS AND METHODS

1. Primers and hydrolysis probes with 6FAM were designed at the region of difference I (RD1) which lacked in *M. bovis* BCG, but presented in all pathogenic TBC.

Name	Sequence (5' → 3')	Position (Rv3871 gene)
Forward primer	AACCTGAAGAAGCGGTTGCC	1,332
Reward primer	GTTTGGGGAGGGCAGTAGG	1,501
BCG-probe	6FAM-TGGCACATCCAGCCGCCCGG-BHQ1	1,431
IC-probe	Cy5-CTGCGTCCACTCTAACGACGG-BBQ	non



2. Internal control and positive control plasmids were designed and constructed by using pUC57-Amp plasmid DNA Vector. The positive control contained the synthetic sequence position 1332-1501 of *Rv3871* gene (complete genome position; 4318465-4319937) *M. bovis* BCG str. Tokyo 172.
3. The PCR condition was optimized using iTaq™ Universal Probes Supermix BioRad® and run on CFX96 ™ Real-Time System (BioRad®).
4. The analytical specificity was tested with DNA of 39 reference organisms which were 17 mycobacteria and 22 bacteria such as *M. bovis* BCG, *M. bovis* BCG Pasteur ,*M. tuberculosis* H37Rv ,*M. tuberculosis* H37Rv, *M. avium, M. intracellulare, M. kansasii , M. scrofulaceum , M. terrae , M. simiae , M. xenopi , M. gordonae , M. chelonae, M. fortuitum, M. smegmatis , M. abscessus, M. sherrisii*) (3 *K. pneumonia, Ps. Aeruginosa,* 3 *S. aureus, S. saprophyticus, S. epidermidis,* 3 *E. faecalis,* 6 *E. coli,* ETEC, EPEC, E. coli O157:H7.
5. The analytical sensitivity was assessed by using triplicate ten-fold serial dilution of positive control in TE buffer and in TBC negative specimen extracted DNA .

RESULTS

The developed real time PCR assay with dual fluorescents probes with internal control for false negative detection has ability to detect *M. bovis* BCG in clinical specimen and isolates.

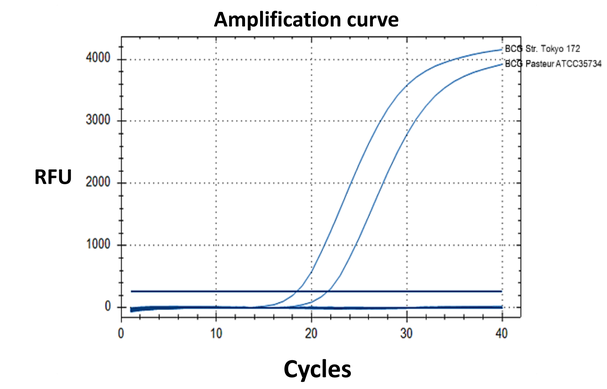


Fig 1. This assay success to 100 % specificity for the identification of *M. bovis* BCG among 39 strains of *Mycobacterium* spp. and bacteria.

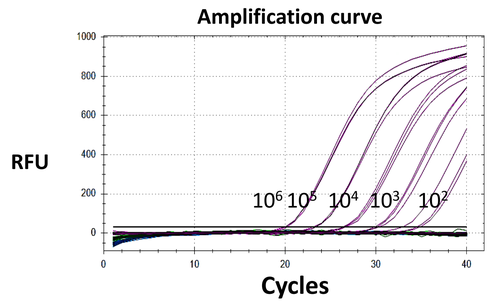
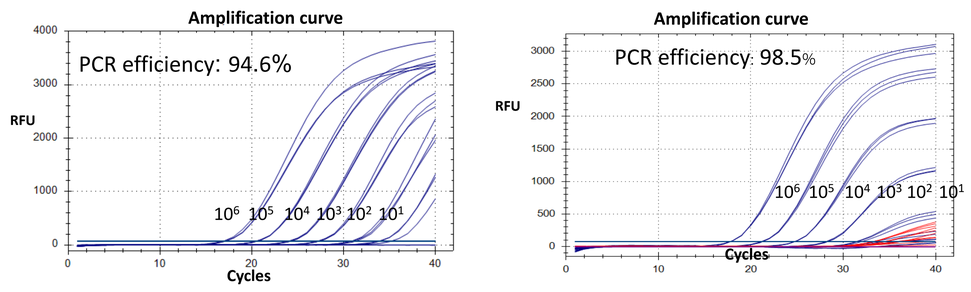


Fig 2. 10-fold internal control (IC) serial dilution show the limit of detection (LOD) for IC at 10 copies/µL. Therefore, 2 Log10 of the LOD, 10^3, was selected to add in the assay as a false negative internal control.



(A) (B)

Fig 3. The assay ability of detection all positive control DNA dilution 10^1–10^6 in TE buffer (A) similar to in negative specimen extracted with IC (B) which show the limit of detection for *M. bovis* BCG at 10 copies/µL.

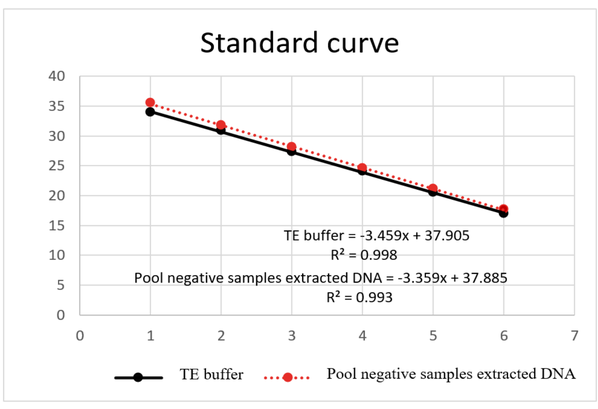


Fig 4. Inverse linear relationship between copy number of BCG positive control in TE buffer and in negative TBC extracted DNA with number of Ct, show the good of line at the coefficient (R2) $\geq$ 0.99.

DISCUSSION &CONCLUSION

Future studies will assess another part of validation that compost of repeatability and reproducibility which are precision and accuracy of the test. Additionally, evaluation will be estimated with clinical specimens collected from BCG vaccinated children aging less than 3 years old.

This assay provides as a rapid diagnostic tool of *M. bovis BCG* that could be useful for clinical samples allowing timely diagnosis and selection of appropriate treatment for paediatric patients. This assay could be implemented in clinical mycobacteriology laboratories and also public health programs and epidemiological studies.

Acknowledgement

This project was supported by the National Vaccine development (Public Organization) and Faculty of Graduate studies, Mahidol University