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Research Article

Evaluation of Analytical-Sensitivity and -Specificity of a Commercially Available One-Step Real-Time Polymerase Chain Reaction Assay Kit for the Detection of *Burkholderia mallei*

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Abstract

Burkholderia mallei (B. mallei) is the etiological agent of glanders, a highly infectious and zoonotic disease of solipeds. Prompt and accurate detection of *B. mallei* and diagnosis of glanders is important for both, humans and animals. The present study was designed to evaluate the analytical sensitivity and specificity of a commercially available one-step real-time (RT) PCR assay kit (Genekam Biotechnology AG, Germany) for the detection of *B. mallei.* Furthermore, the comparative evaluation of the analytical sensitivity of an already published fliP RT-PCR protocol and this kit was made using a real-time PCR (RT-PCR) platform: the Mx3000P TM (Stratagene®, Canada). Diagnostic parameters were assessed with a panel of 20 B. mallei, 20 *B. pseudomallei,* 15 *B. mallei* related and 10 clinically relevant non-Burkholderia species. Using the one-step RT-PCR on the Mx3000PTM platform, the limit of detection (LOD) was determined as 10 fg. Applying a modified fliP RT-PCR using the Mx3000P platform resulted in a LOD of 100 fg. Authors conclude that the one-step RT-PCR kit is specific for DNA of *B. mallei* strains. The one-step RT-PCR assay kit is a robust, rapid, reliable, specific and sensitive tool with a fast turnaround time for the specific identification and detection of *B. mallei* from culture material.

Keywords: *Burkholderia mallei, Analytical specificity, Analytical sensitivity, One-step RT-PCR kit*

*Burkholderia mallei'*nin Tespitinde Ticari Tek-Basamaklı Gerçek-Zamanlı Polimeraz Zincir Reaksiyon Kitinin Analitik Özgünlüğü ve Özgüllüğünün Değerlendirilmesi

Özet

Burkholderia mallei (B. mallei) ruamın etiyolojik ajanı olup, hastalık tek tırnaklıların oldukça enfeksiyöz ve bulaşıcı bir hastalığıdır. *B. mallei*'nin hızlı ve doğru tespiti ve ruamın diagnozu hem insanlar hem de hayvanlar için önemlidir. Bu çalışma, *B. mallei*'nin tespitinde ticari olarak satılan tek-basamaklı gerçek-zamanlı (RT) PCR kitinin (Genekam Biotechnology AG, Almanya) analitik özgünlüğü ve özgüllüğünün değerlendirilmesi amacıyla yapılmıştır. Daha önceden yayınlanmış olan fliP RT-PCR protokolü ile mevcut test edilen kitin özgünlüğü ve özgüllüğünün karşılaştırılması gerçek-zamanlı PCR, Mx3000P TM (Stratagene®, Kanada) ile yapıldı. Diagnostik parametreler 20 B. mallei, 20 *B. pseudomallei*, 15 *B. mallei* alakalı ve 10 klinik olarak ilgili *Burkholderia* olmayan türler ile değerlendirilirdi. Mx3000PTM platformunda tek basamaklı RT-PCR uygulayarak tespit etme limiti (LOD) 10 fg olarak belirlendi. Mx3000PTM platformu kullanılarak uygulanan fliP RT-PCR ile LOD 100 fg olarak belirlendi. Tek basamaklı RT-PCR'ın *B. mallei* suşları için spesifik olduğu sonucuna varıldı. Tek basamaklı RT-PCR kiti, kültürden *B. mallei*'nin spesifik olarak identifiye edilmesi ve belirlenmesinde kullanılabilecek hızlı geri dönüş zamanına sahip güçlü, hızlı, güvenilir, spesifik ve hassas bir üründür.

Anahtar sözcükler: *Burkholderia mallei, Analitik özgüllük, Analitik özgünlük, Tek basamaklı RT-PCR kiti*

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INTRODUCTION

Glanders is a highly infectious zoonotic disease of solipeds caused by Gram-negative pathogen *Burkholderia mallei (B. mallei)*. *B. mallei* is considered to be a clone of *B. pseudomallei* causes melioidosis both in animals and humans [1,2]. Main clinical signs of equine and human glanders are lymphangitis (farcy pipes), pneumonia and/or lymphadenitis (farcy buds) [3]. Glanders can be considered a re-emerging disease as the number of outbreaks in solipeds, zoo carnivores and dromedary is steadily increasing in the last two decades [2,4,5].

There is problem of accurate identification of *Burkholderia* spp. based on serology. False-positive and -negative serological results impose restrictions on the international trade of healthy animals and spread of glanders in disease free regions, respectively. However, combined use of serological and molecular methods increases the detection rate of glanders [2,6,7].

It is difficult to detect *B. mallei* directly from tissues and biological fluids of infected hosts as their low concentration. Cultivation and identification of *B. mallei* with conventional microbiological techniques is time-consuming and hazardous and should be performed under BSL (bio safety level)-3 conditions. However, isolation of *B. mallei* is the real proof of an infection.

Rapid and precise diagnosis of *B. mallei* is the mainstay for prompt treatment particularly in acute human glanders [6]. Similarly, rapid detection of equine glanders would be helpful for early countermeasures and quarantine time of suspicious cases of glanders can be shortened. Consequently, different polymerase chain reactions (PCR) and real-time PCRs (RT-PCR) with improved analytical-sensitivity and/or-specificity have been developed.

Many attempts have made to detect targets of the 16S rRNA, ISBma2transposase, 23S rRNA genes and the 16-23S rRNA spacer region for discrimination of *B. mallei* from *B. pseudomallei* [8-11]. In addition, RT-PCR assays have been developed that target the conserved type 3 secretion system (TTS1, *orf11* and *orf13)* encoded by both *B. mallei* and *B. pseudomallei* [12]. In a latter investigation, both *B. mallei* and *B. pseudomallei* were distinguished from *B. thailandensis* by amplification of *orf13* sequences, but the assay was not successful to differentiate *B. mallei* from *B. pseudomallei*. Recently, developed RT-PCR assay targeting the *B. pseudomallei* 16S rRNA, *fliC*, and the ribosomal subunit protein S21 *(rpsU)* genes could not differentiate *B. mallei* from *B. pseudomallei* [13]. Heterogenecity in the *fliC* gene from several *B. pseudomallei* isolates has been reported, which hindered the differentiation of *B. mallei* from *B. pseudomallei* [14]. It is demonstrated in another study that all *B. mallei* isolates contain specific *bimA* (intracellular motility A) gene with corresponding AT4 and AT5 primer pair would consider extremely useful for the identification of *B. mallei* and its differentiation from *B. pseudomallei* [15]. For rapid identification and screening of *B. mallei* and *B. pseudomallei*, a 5´ nuclease RT-PCR assay was developed and further improved for rapid, sensitive and specific identification and detection of *B. mallei* in clinical samples by targeting flagellin P (*fliP*) gene [16]. A Highly specific, rapid and validated RT-PCR assay "BurkDiff" which targets a single nucleotide polymorphism (SNP) and differentiates between *B. mallei* and *B. pseudomallei* has been developed [17]. The latest development is a commercially available one-step RT-PCR assay kit (Genekam Biotechnology AG, Germany) for the detection of *B. mallei*.

In the current study, analytical-sensitivity and -specificity of this kit for the diagnosis of glanders has been evaluated. The development of this kit is another step to reduce the turnaround time for the detection of *B. mallei*.

MATERIAL and METHODS

One-step RT-PCR kit has been manufactured by Genekam Biotechnology AG, Germany to detect glanders. This kit works on the principle of fluorogenic probes: 6-caboxyfluorescein (FAM/reporter) at the 5' end and black hole Quencher-1 (BHQ1/quencher) at the 3' end of the probe. Fluorescence indicates the presence of PCR amplified product. Regarding the manufacturers' instructions, this kit needs DNA which can be isolated from blood, serum, nasal swabs, infected lymph nodes, respiratory tract, bacterial culture, body fluid and other tissues of glanderous animals or humans. The RT-PCR kit (tube A, B, D1 and D2) was used according to manufacturers' instructions. Briefly, after thawing, 8 µl from tube 'A' and 10 µl from tube 'B' mixed together in a nuclease free PCR-tube or alternatively in an optical 96-well microtiter plate (q PCR 96-well plates, Micro Amp TM, Applied Biosystem). This mixture is now referred as a ready-to-go "mastermix" (18 µl) for one test sample. As this whole procedure of mixing was performed in one-step that's why it is called one-step RT-PCR. After addition of 2 µl of isolated template DNA the reaction mix (20 µl) was ready-to-go in a thermocycler. Similarly, D1 (positive control) and D2 (negative control) were used in a separate mastermix (18 µl) in each run. No internal amplification control is included in the kit to detect any amplicon contamination and/or amplification failure. In the current study, the performance of the one-step RT-PCR kit was compared in RT-PCR platform: Mx3000P™ real-time PCR system (Stratagene© La Jolla, Canada) software version 2.0 was used for amplification and detection. The real-time PCR reaction was performed in duplicate in this platform. Results were interpreted according to one-step RT-PCR assay kit manufacturers' instructions.

Furthermore, for the comparative evaluation of analytical sensitivity of aforementioned thermocycler, a real-time hot-start *fliP* PCR with little modification [18] was performed in a Mx3000P. In Mx3000P, the 25 µl

Table 1. *List of bacterial strains analysed in current study*

Species	Strain	Source	Geographic Origin	Year	+/- in one-step RT-PCR kit
B. mallei	235	un	un		+
	237	un	un		+
	242	un	un		+
	32	un	un		+
	M2				+
	34	un	un		+
	ATCC 23344^T	Human	China	1942	+
	Bogor	Horse	Indonesia		+
	Dubai 7	Horse	UAE	2004	+
	M1	un	un		+
	Mukteswar	Horse	India		+
	NCTC 10230	Horse	Hungry	1949	+
	NCTC 10247	Horse	Turkey	1960	+
	NCTC 10260	Human	Turkey	1949	+
	NCTC120-Lister	un	London/Lister	1920	+
	PRL-1	Donkey	Pakistan	2002	+
	PRL-3	Horse	Pakistan	2005	+
	PRL-4	Horse	Pakistan	2005	+
	Rotz 7 (SVP)	un	un		+
	Zagreb	Horse	ex-Yugoslavia		+
B. pseudomallei	09RR8920	Ring trail	Germany	2009	–
	AB2056	Human	Kenya	1980	–
	ATCC 23343^T	Human	un	<1957	–
	D4899/303	Environment	Venezuela		–
	EF15660	un	un		–
	Hainan 106	Environment	China		–
	Heckeshorn	Human	Germany	1999	–
	Holland	un	Holland		–
	NCTC 1688	Rat	Malaysia	1923	–
	NCTC 4845	Monkey	Singapore	1945	–
	PITT 225A	Human	Thailand	1986	–
	PITT 521	Human	Pakistan	1988	–
	PITT 5691	un	un		–
	SID 2889	Human	Bangladesh	1923	–
	SID 3477	Human	Thailand	1999	–
	SID 3511	Human	Bangladesh		–
	SID 3783	Human	Malaysia	1999	–
	SID 4075	Human	N Thailand	1999	–
	Soil 1977	Environment	Madagascar	1977	–
	UE10	Human	NE Thailand		–
***B. mallei* related strains**					
B. cenocepacia	DSM 16553^T	Human	NE Thailand		–
B. ambifaria	DSM 16087^T	Human	NE Thailand		–
B. cepacia	DSM 7288^T	Human	NE Thailand		–

Table 1. *List of bacterial strains analysed in current study (Continue)*

Species	Strain	Source	Geographic Origin	Year	+/- in one-step RT-PCR kit
B. dolosa	DSM 16088^T	Human	NE Thailand		–
B. fungorum	DSM 17061^T	Human	NE Thailand		–
B. gladioli	DSM 11318	Human	NE Thailand		–
B. glathei	DSM 50014^T	Human	NE Thailand		–
B. multivorans	DSM 13243^T	Human	NE Thailand		–
B. stabilis	DSM 16586^T	Human	NE Thailand		–
B. thailandensis	DSM 13276^T	Human	NE Thailand		–
B. vietnamiensis	DSM 11319^T	Human	NE Thailand		–
P. aeruginosa	ATCC 9027				–
P. alcaligenes	ATCC 14909				–
P. fluorescens	ATCC 13525				–
P. putida	ATCC 12633				–
Non-*Burkholderia* strains					
E. coli	DSMZ 30083				–
P. multocida	DSM 5281				–
R. equi	DSM 20307				–
Str. agalactiae	DSM 6784				–
Str. equi subsp. equi	ATCC 9528				–
Str. equi subsp. zooepidemicus	ATCC 700400				–
Str. equinus	DSM 20558				–
Str. parauberis	DSM 6631				–
T. equigenitalis	DSM 10668				–
Y. enterocolitica ssp. enterocolitica	ATCC 9610				–

B Burkholderia; E Escherichia; P Pasturella; Ps Pseudomonas; R Rhodococcus; Str Streptococcus; T Taylorella; un unknown history; Y Yersinia; + Positive; – Negative

reaction mixtures consisted of 9.75 µl of HPLC, 12.5 µl of 2x TaqMan™ Universal MasterMix (Applied Biosystem, Germany), 0.25 µl of each primer (Jena Bioscience, Germany) (10 µM/µl), 0.25 µl of the probes (Jena Bioscience, Germany) (10 µM/µl), and 2 µl of template DNA. Thermal profile conditions were as follows: 1 cycle at 50°C for 2 min (decontamination) followed by 95°C for 10 min (hotstart), initial amplification at 95°C for 25 s, followed by 50 cycles at 63°C for 1 min.

To determine analytical specificity (exclusivity and inclusivity) of one-step RT-PCR kit, a representative panel of genomic DNA from 20 *B. mallei*, 20 *B. pseudomallei,* 15 *B. mallei* related and 10 clinically relevant non-*Burkholderia* species *(Table 1)* were obtained from the Institute of Microbiology, Federal Armed Forces (Munich, Germany), the Department of Clinical Medicine and Surgery (University of Agriculture, Faisalabad, Pakistan), the German Collection of Microorganisms and Cultures (DSMZ, Braunschweig, Germany), and the strain collection of the National Reference Laboratory of Glanders at the Federal Research Institute for Animal Health (Friedrich-Loeffler-Institute, Jena, Germany). *B. mallei* detection limit of one-step RT-PCR kit was determined with little modification [19]. Briefly, analysis was made of a dilution series of purified DNA of *B. mallei* ATCC 23344^T in PCR HPLC from 1 ng to 100 ag. This linear quantification was used to prepare the standard curve *(Fig. 1)*. The concentration of DNA preparations and purity were determined by using NanoDrop ND-1000 UV Vis spectrophotometer (NanoDrop Technologies, Wilmington, Germany). Dilution series and RT-PCR reactions were performed in duplicate separately in Mx3000P using one-step RT-PCR kit and modified *fliP* RT PCR protocol. For the assessment of analytical specificity, all DNA *(Table 1)* was used in a concentration of 100 pg/µl in HPLC in a volume of 2 µl per assay.

RESULTS

The linear range of the one step real-time PCR for *B. mallei* covered concentrations from 1 ng to 100 ag of bacterial DNA/µl *(Fig. 1, Fig. 2)*. During evaluation of

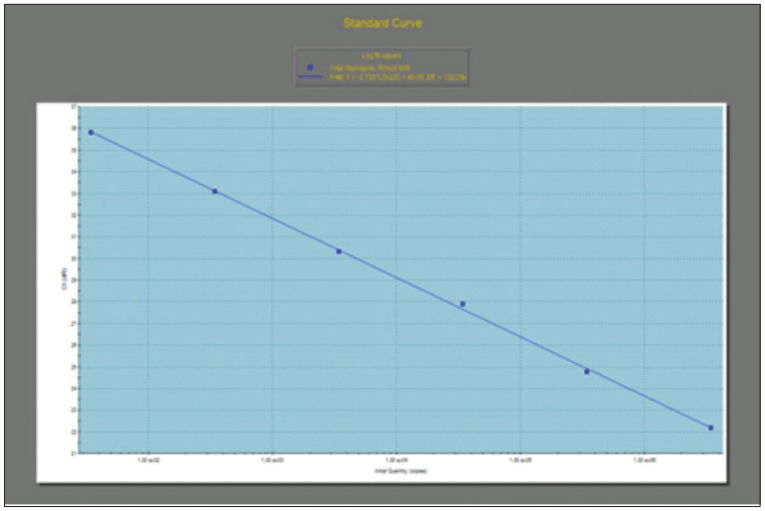
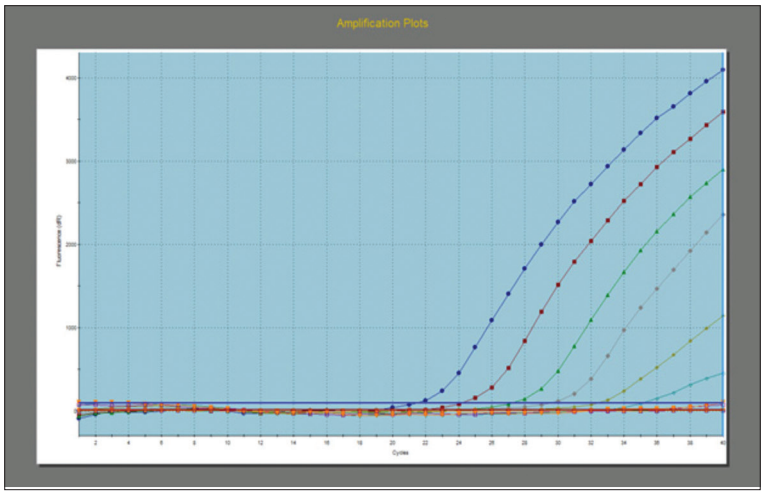


Fig 1. *Standard curve of dilution series containing 1 ng-100 ag/ µl DNA*

Fig 2. *Comparative evaluation of different RT-PCR assays at Mx3000P platform*



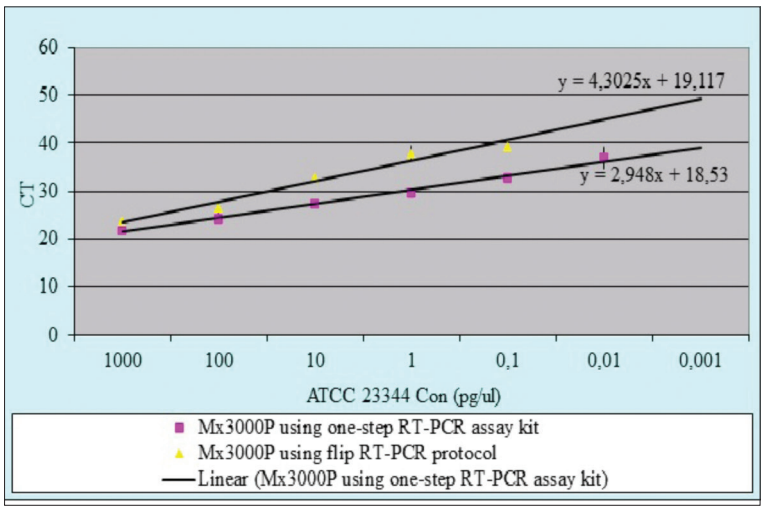


Fig 3. *Comparative evaluation of standard curve of different RT-PCR assays at Mx3000P platform*

analytical sensitivity of one-step RT-PCR with Mx3000P, lower limit of detection (LOD) for *B. mallei* ATCC 23344^T purified DNA was determined to be 10 fg, corresponding to 2 genome equivalents. However, *fliP* RT-PCR using Mx3000P platform determined LOD of 100 fg, 10 genome equivalents) *(Fig. 3)*.

DISCUSSION

It is difficult to rule out erroneous serodiagnostic results because use of crude *B. mallei* spot antigen [2]. Recently, ELISA has been developed based on purified *rBimA* (recombinant *Burkholderia* intracellular motility A) protein

as spot antigen. This protein showed no cross-reaction with sera from melioidosis patients or healthy humans [20]. This serodiagnostic test still needs to be validated and it yet has not been commercialised. Rapid and accurate diagnosis of *B. mallei* infection is still a major challenge for veterinary clinician. Glanders is often falsely diagnosed as melioidosis, strangles, equine influenza and epizootic lymphangitis. Glanders is still a major problem in many developing countries where BSL-3 laboratory facilities do not exist for the cultivation of *B. mallei* which itself is time taking process. Among direct methods, development of PCR assays and real-time PCR assays for specific identification and detection of *B. mallei* would reduce the time turn around for interpretation of final results but also overcome aforementioned shortcomings. Potential use of *B. mallei* as a bio-weapon stresses its prompt detection to start immediate prophylactic treatment. The main objective of current study is to evaluate analytical-sensitivity and -specificity of a commercially available one-step RT-PCR kit for the identification and detection of *B. mallei*.

A RT-PCR assay was developed using LightCycler 4.0 software to detect *B. mallei* and found LOD 1 pg or 424 genome equivalents [15]. However, another RT-PCR assay was developed targeting the *fliP* gene of *B. mallei* and used internal amplification control and found the detection limit of 60 fg of *B. mallei* DNA [21]. The aforementioned difference of LOD might be due to use pure genomic DNA *(Table 1)* in current study while in previous studies [16,21], RT-PCR assays used natural outbreak clinical samples and/ or *B. mallei* ATCC 23344^T challenged animal tissue samples for the specific identification and isolation of *B. mallei* due to which chance of extraneous analyte contamination cannot be ignored.

There are many factors including instrumentation, operator error, reagent choice, calibration, accuracy and acceptance limits of assay controls, water quality, pH, ionicity of buffers and diluents, durations, and error introduced by detection of closely related analytes that can influence on the analytical performance of an assay. One-step RT-PCR kit might have already overcome these shortcomings during its optimisation process, on the other hand its read-to-use availability for clinical laboratories minimise the chances of humans inherent errors.

While establishing exclusivity and inclusivity of one-step RT-PCR assay kit, all 20 *B. mallei* species DNA were accurately identified positive and 20 *B. pseudomallei*, 15 *B. mallei* related species and 10 clinically relevant non-*Burkholderia* species DNA tested negative *(Table 1)*. No cross-reactivity was detected with these 45 species DNA. Results of analytical specificity correlate with recent studies for the development of RT-PCR for the specific identification and detection of *B. mallei* [15,16,21].

The slop of a standard curve is used to estimate the PCR efficiency of a RT-PCR reaction. A RT-PCR standard curve is graphically represented as a semi-log regression line plot of C$_T$ value versus input nucleic acid (pg) *(Fig. 2)*. A standard curve slope of 3.32 indicates a PCR reaction with 100% efficiency. One can draw the conclusion that Mx3000P using one-step RT-PCR kit relatively better in its amplification efficiency than Mx3000P using *fliP* based RT-PCR protocol for the detection of *B. mallei* DNA.

Commercially available one-step RT-PCR assay kit is robust, rapid, reliable, specific and sensitive tool with obvious less turnaround time for specific identification and detection of *B. mallei* within culture material. There is also need to validate one-step RT-PCR assay kit by screening a large panel of *B. mallei, B. pseudomallei* and *B. mallei* related and non-*Burkholderia* isolates. The application for clinical sample materials also needs to be evaluated. Generally, a positive PCR result in clinical samples is a confirming result in the diagnosis of glanders but a negative PCR result in any clinical sample cannot be used to exclude the disease.

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