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Development and validation of TaqMan real-time PCR for the detection of *Burkholderia pseudomallei* isolates from Malaysia

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Abstract. Rapid detection of *Burkholderia pseudomallei*, the etiologic agent of melioidosis, allows for timely initiation of appropriate treatment and better clinical outcomes. In the current gold standard, the culture method is time consuming and suffers from low sensitivity. Meanwhile, previously reported molecular assays are fast and sensitive, but their performance on isolates from Malaysia, an endemic region of melioidosis is under reported. This study designed oligonucleotides targeting *orf2* of Type III secretion system (TTSS) genes cluster for the detection of Malaysian *B. pseudomallei* isolates and evaluated the assay on 95 local *B. pseudomallei* strains, 58 other microorganisms and 71 clinical specimens from patients. The developed assay exclusively detected all tested *B. pseudomallei* isolates with a detection limit of 20 fg per reaction (equivalent to ~2.5 copies). Subsequent testing on clinical samples showed that the assay detected all confirmed specimens with the growth of *B. pseudomallei* (n = 10/10). None of the negative specimens had a detectable signal of our TTSS-*orf2* assay (n = 0/61). In conclusion, the present study provides crucial preliminary data for a subsequent study and should be considered as a potential alternative to current time-consuming culture method for the detection of *B. pseudomallei*.

INTRODUCTION

Melioidosis, caused by highly pathogenic Gram negative bacillus *Burkholderia pseudomallei* is an important tropical disease that infects between 68 000 and 412 000 people annually with more than 50% deaths (Limmathurotsakul *et al.*, 2016). The bacteria survive in robust environmental conditions such as low temperature, extreme pH, increased salinity, low nutrient and drought (Wang-Ngarm, Chareonsudjai & Chareonsudjai, 2014). Cases of melioidosis are commonly associated with some

climatic factors including increased sea surface temperatures, cloud cover, rainfall rate, groundwater and severe weather events (Kaestli *et al.*, 2016). Such incidence also occurred during drier occasions (Cheng *et al.*, 2008).

B. pseudomallei infects human through aerosol inhalation, aspiration, ingestion or skin inoculation (Lim, Peacock & Limmathurotsakul, 2016). The incubation period ranges from 2 to 21 days, but reactivation of latency may happen after more than 50 years (Ngauy *et al.*, 2005). Clinical manifestation includes pneumonia, localised abscess or sepsis with or without bacteraemia depending on the route and duration of infections and inoculum size (Ngauy *et al.*, 2005; Lim, Peacock & Limmathurotsakul, 2016).

Prompt detection of melioidosis plays important roles for timely diagnosis and better treatment outcomes. However, the culture method, as the current gold standard, takes more than 48 hours for isolation and identification of *B. pseudomallei* with inconsistent sensitivity (Lau *et al.*, 2015). As alternatives, several probe-based molecular assays have been developed for early detection of *B. pseudomallei*, targeting the *fliC*, *lpxO*, *rrs*, *mprA*, Yersinia-like fimbrial (YLF), *Burkholderia thailandensis*-like flagellum and chemotaxis (BTFC) gene clusters, single nucleotide polymorphisms (SNPs) and open reading frames (ORFs) of Type III secretion system (TTSS) (Thibault, Valade & Vidal, 2004; Tomaso *et al.*, 2005; Kaestli *et al.*, 2012; Lau *et al.*, 2015).

In contrast to *rrs* and *fliC* that are also present in other organisms, the ORFs of the TSSS are more exclusive to *B. pseudomallei* (Rainbow, Hart & Winstanley, 2002). This cluster of genes encodes for more than 30 proteins that are responsible for transporting virulence effector proteins and survival in phagocytes that determine the pathogenicity of *B. pseudomallei* (Gong *et al.*, 2015; Kang *et al.*, 2016). Currently, several TTSS putative genes, such as the *orf2* and *orf*11 have been utilised as biomarkers that indicate the presence of *B. pseudomallei* using PCR assays and have been tested on a large number of *B. pseudomallei* isolates (Thibault, Valade & Vidal, 2004; Novak *et al.*, 2006; Al-Marzooq and Mustafa, 2011; Zhang *et al.*, 2012). Most of the reported assays did not describe the origins of the tested *B. pseudomallei* isolates. Meanwhile, the BurkDiff assay was evaluated on only two clinical isolates from Malaysia (Novak *et al.*, 2006; Bowers *et al.*, 2010). Such validation analysis on local *B. pseudomallei* strains is necessary because *B. pseudomallei* genomes are large, diverse, complex with frequent horizontal gene transfer and usually clustered according to the location of isolation (Price, Currie & Sarovich, 2017). This is evident in a previous study done by our group that found high genotypic variability within Malaysian *B. pseudomallei* isolates (Zueter *et al.*, 2015). In a subsequent exploration, the same group reported 13 novel *B. pseudomallei* genotypes from Malaysia, such as ST1324, ST1325 and ST1326. It is also noticeable that other 19 reported genotypes can be found in other Southeast Asian, China and Indian sub-continents (Zueter *et al.*, 2018). As a continuation to these findings, we developed primers and probe targeting the *B. pseudomallei* TTSS-*orf2* to evaluate its performance on 95 Malaysian *B. pseudomallei* isolates that represent high genotypic diversities of this ubiquitous melioidosis agent.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A total of 95 *B. pseudomallei* strains, 58 other bacteria, *Plasmodium* and fungi isolated from human clinical samples, plus a number of ATCC strains were used in this study (Table 1). The isolates were provided by the Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia and Makmal Kesihatan Awam Kota Bharu. The bacteria were cultured aerobically in nutrient broth overnight at 37°C. Meanwhile, *Leptospira* strains were maintained in EMJH media, incubated at 30°C on a rotating platform at 40 rpm. Identifications of microbial species were previously carried out using the

standard identification method, VITEK-2 (bioMérieux, United States) and/or partial 16S rRNA gene sequencing.

Clinical specimen collection

A total of 71 clinical specimens (69 blood specimen, 2 blood culture fluid (BCF)), previously analysed using the conventional culture method, were collected from the Medical Microbiology and Parasitology Laboratory, Hospital Universiti Sains Malaysia between April 2016 and November 2017. Of that, 10 specimens had positive growth of *B. pseudomallei* and 11 specimens had growth of other organisms; *Staphylococcus* spp. (3), *Klebsiella pneumoniae* (2), *Escherichia coli* (1), Group A *Streptococcus* (1), *Campylobacter jejuni* (1), *Salmonella* Typhi (1), *Burkholderia cepacia* and *Acinetobacter baumanii* (1) and *Acinetobacter lwoffii* & *Pseudomonas stutzeri* (1). Meanwhile, the remaining 50 samples had no growth of any organism. Retrospective clinical data were obtained when necessary.

The inclusion criteria were patients aged more than 18 years with fever (38°C and more) and have proven or clinical suspicion of severe infection or sepsis. Patients with suspected or proven healthcare-associated pneumonia (HAP/HCAP) or ventilator-associated pneumonia (VAP) and/or dengue were excluded.

In addition to clinical specimens, a further 90 *B. pseudomallei* clinical isolates were spiked into sterile human blood specimens from healthy donors in accordance to a published study (Podnecky *et al.*, 2013) and used for specificity testing. Briefly, the overnight *B. pseudomallei* culture was suspended in phosphate-buffered saline (PBS) to obtain a McFarland standard between 1 and 2, which provided an estimated concentration of 4.5×10^8 colony forming units per millilitre. The suspended colonies were used for spiking in 90 whole bloods in EDTA tubes. One hundred microliter of the spiked specimen was plated on nutrient agar in triplicates and incubated at 37°C for 48 hours.

This study was approved by the Human Ethics Committee of Universiti Sains Malaysia (Protocol code: USM/JEPeM/16080260) and Medical Research and Ethics Committee (MREC) of the Ministry of Health Malaysia (Protocol code: NMRR-16-2117-33181).

Isolation of genomic DNA

DNA was extracted from pure bacterial culture using NucleoSpin® Tissue DNA Extraction kit (MACHEREY-NAGEL GmbH & Co. KG, Germany) according to the manufacturer instructions with a minor modification during the final elution step, in which the column was incubated at room temperature for 10 minutes prior to centrifugation at 11 000 × *g* for 1 minute. The total DNA was quantified using the Eppendorf BioPhotometer (Eppendorf Scientific, Inc., New York, United States), adjusted to 20 ng/µl, and stored at -20°C until use.

DNA from clinical blood specimens and spiked blood specimens were extracted using the NucleoSpin® Blood QuickPure (MACHEREY-NAGEL GmbH & Co. KG, Germany) DNA extraction kit. The DNA extraction procedure was performed according to the manufacturer's instruction with minor modifications to increase its yield by eluting the DNA in 50 µl pre-warmed TE buffer. The column was incubated at room temperature for 10 minutes prior to the final centrifugation at 11 000 × *g* for 1 minute.

DNAs from blood culture fluid (BCF) were extracted according to a published protocol via the M5 method (Villumsen *et al.*, 2010). Briefly, 100 µl BCF was added to 100 µl lysis buffer (5 M guanidine hydrochloride in 100mM Tris-HCl, pH 8.0) and 10 µl proteinase K (20 mg/ml) and incubated at room temperature for 10 minutes. Next, 600 µl water and 800 µl benzyl alcohol were added to the mixture. Samples were then centrifuged at 20 000 × g for 5 minutes at room temperature. Subsequently, 200 µl supernatant were collected and mixed with 200 µl absolute ethanol and 200 µl of BQ1

buffer. The mixture was then processed according to procedures for the blood specimens using the NucleoSpin® Blood QuickPure kit with minor modifications as described previously.

Fungal genomic DNA used for specificity evaluation was supplied by Ms. Nor Suhada Anuar from the Central Research Laboratory, Universiti Sains Malaysia.

Design of TaqMan probes and PCR primers

The primers and probes were designed using the IDT DNA PrimerQuest® online tool (https://sg.idtdna.com/Primerquest/Home/Index) based on *B. pseudomallei orf2* of Type III secretion system (TTSS) associated genes (GenBank accession no.: AF074878). The *in silico* specificity of the primers and probes was assessed using the Primer-BLAST programme from the National Centre for Biotechnology Information (NCBI).

Real-time PCR parameters

The PCR reaction for the amplification of TTSS- *orf2* was prepared in a total volume of 20 uL, containing 10 µL 2 × SsoAdvanced™ Universal Probes Supermix (Bio-Rad, California, United States), 1 uL PCR grade distilled water (Gibco®, Massachusetts, United States), 200 nM primers (F-CCTGG GAGAGCGAGATGTT, R-GCTGGATGAGAA GAAAGTCC) (Integrated DNA Technologies, Singapore), 100 nM probe (TexasRed-CCACGCACGGCGGAGATTCT-IAbRQ) (Integrated DNA Technologies, Singapore) and 8 uL DNA template. The real-time PCR amplification was conducted using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, California, United States) with the following thermal cycling conditions; an initial denaturation at 95°C for 5 minutes, followed by 50 cycles of 95°C for 30 seconds and 61.3°C for 30 seconds. The baseline threshold for the post-amplification analysis was set at 50. Any Cq value ≤40 was considered positive. All the amplifications in this study were carried out in triplicates, unless specified otherwise.

Sensitivity and specificity analyses

The analytical sensitivity of the assay was carried out in triplicates using 10-fold dilutions of the extracted *B. pseudomallei* DNA ranging from 10 ng/uL to 1 fg/uL. Two microliter of each dilution was used in each reaction. Concentration of bacteria in colony forming unit (CFU) per reaction was assumed to be equal to the gene copies/reaction, as the target gene presents a single copy. Copy number was calculated based on a formula previously described by Aghamollaei *et al.* (Aghamollaei *et al.*, 2015). The genome size of *B. pseudomallei* is 7.24 Mb (Holden *et al.*, 2004). Meanwhile, analytical specificity of the assay was determined using the extracted non-*B. pseudomallei* DNAs (Table 1).

Data analysis

The sequences of oligonucleotides used in this study were compared against Bacterial Genome Database in the National Centre for Biotechnology Information (NCBI) using the BLAST online web tool, available at (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Initial *in silico* specificity of the real-time PCR by Basic Local Alignment Search Tool (BLAST) showed that the TTSS oligonucleotides have specific homology with the expected *B. pseudomallei* TTSS region. The BLAST analysis also revealed that the developed oligonucleotides were 100% complementary to all the *B. pseudomallei* complete genomes available in the NCBI. No significant cross-reaction was detected with other microorganisms, especially with the genetically close relatives of *B. pseudomallei*, including *B. cepacia*, *B. mallei*, *B. thailandensis*, *Pandoraea* spp. and *Ralstonia* spp.

During empirical specificity evaluation using genomic DNA of 95 *B. pseudomallei* clinical strains (90 strains were spiked into blood specimens, 5 isolates were directly used), the newly developed qPCR assay was

Table 1. List of organisms used in the developed assay

Organism	No. tested (*n*)	Source / strain	qPCR result *n*, positive signal (%)
Positive control isolates			
B. pseudomallei	5	Clinical isolates	5 (100)
B. pseudomallei (spiked in bloods)	90	Clinical isolates	90 (100)
Negative control isolates			
Aspergillus fumigatus	1	Clinical isolate	0 (0)
Bacillus subtilis	1	Clinical isolate	0 (0)
B. cepacia	6	Clinical isolates	0 (0)
B. thailandensis	1	Clinical isolate	0 (0)
Candida albicans	1	Clinical isolate	0 (0)
Citrobacter freundii	1	Clinical isolate	0 (0)
Campylobacter jejuni	1	Clinical isolate	0 (0)
Entamoeba histolytica	1	Clinical isolate	0 (0)
Enterococcus faecalis	1	Clinical isolate	0 (0)
Klebsiella pneumoniae	1	Clinical isolate	0 (0)
L. biflexa serovar Patoc	1	UPM, Malaysia	0 (0)
L. borgpetersenii serovar Ballum	1	UPM, Malaysia	0 (0)
L. borgpetersenii serovar Celledoni	1	UPM, Malaysia	0 (0)
L. fainei serovar Hurtsbridge	1	UPM, Malaysia	0 (0)
L. inadai	1	USM, Malaysia	0 (0)
L. interrogans serovar Australis	1	UPM, Malaysia	0 (0)
L. interrogans serovar Autumnalis	1	UPM, Malaysia	0 (0)
L. interrogans serovar Bataviae	1	UPM, Malaysia	0 (0)
L. interrogans serovar Canicola	1	UPM, Malaysia	0 (0)
L. interrogans serovar Copenhageni	1	UPM, Malaysia	0 (0)
L. interrogans serovar Hebdomadis	1	UPM, Malaysia	0 (0)
L. interrogans serovar Icterohaemorrhagiae RGA	1	UPM, Malaysia	0 (0)
L. interrogans serovar Javanica	1	UPM, Malaysia	0 (0)
L. interrogans serovar Pomona	1	UPM, Malaysia	0 (0)
L. interrogans serovar Pyrogenes	1	UPM, Malaysia	0 (0)
L. interrogans serovar Tarassovi	1	UPM, Malaysia	0 (0)
L. kmetyi	4	USM, Malaysia	0 (0)
L. licerasiae serovar Varillal	1	UPM, Malaysia	0 (0)
L. meyeri	3	USM, Malaysia	0 (0)
L. wolffii	3	USM, Malaysia	0 (0)
Plasmodium falciparum	1	ATCC PRA-405D	0 (0)
P. knowlensi	1	Clinical isolate	0 (0)
P. vivax	1	Clinical isolate	0 (0)
Proteus mirabilis	1	Clinical isolate	0 (0)
Proteus vulgaris	1	Clinical isolate	0 (0)
Salmonella Typhi	1	ATCC 7251	0 (0)
Salmonella Paratyphi A	1	ATCC 9150	0 (0)
Salmonella Paratyphi B	1	ATCC BAA 1250	0 (0)
Salmonella Paratyphi C	1	ATCC 9068	0 (0)
Salmonella Enteritidis	1	Clinical isolate	0 (0)
Salmonella Typhimurium	1	Clinical isolate	0 (0)
Salmonella Heidelberg	1	Clinical isolate	0 (0)
Salmonella Weltevreden	1	Clinical isolate	0 (0)
Shigella dysenteriae	1	Clinical isolate	0 (0)
Staphylococcus aureus	1	Clinical isolate	0 (0)
S. saprophyticus	1	Clinical isolate	0 (0)

able to amplify all tested strains (Table 1). No cross-amplification signal was detected with the other microorganisms, including the closely related *B. cepacia* and *B. thailandensis*. The list of the tested organisms is shown in Table 1.

Next, the developed assay was validated for its amplification efficiency, linearity and sensitivity. We found that the Cq value decreased proportionally to the DNA concentration, as shown in Table 2. The qPCR assay has an amplification efficiency of 95.88%, correlation regression coefficient (R^2) of 0.9984 and detection limit of 20 fg per reaction, equivalent to ~2.5 copies (Figure 1).

Subsequent testing on the clinical samples found that the assay was able to amplify the *B. pseudomallei* DNA from previously confirmed clinical specimens (n = 10/10), as shown in Table 3. None of the clinical samples with negative growth or with organisms other than *B. pseudomallei* yielded a positive signal by our assay (n = 0/61).

Table 2. Cq values of 10-fold serial dilution of *B. pseudomallei* genomic DNA in 20 µl reaction

B. pseudomallei genomic DNA	Copies number	Mean Cq	SD
20 000 pg	2 560 000	18.62	0.45
2 00 pg	256 000	22.07	0.07
200 pg	25 600	25.39	0.07
20 pg	2 560	28.91	0.52
2 pg	256	32.28	0.16
0.2 pg	25.6	35.14	0.48
0.02 pg	2.56	39.58	–
0.002 pg	0.256	–	–
No template control	–	–	–

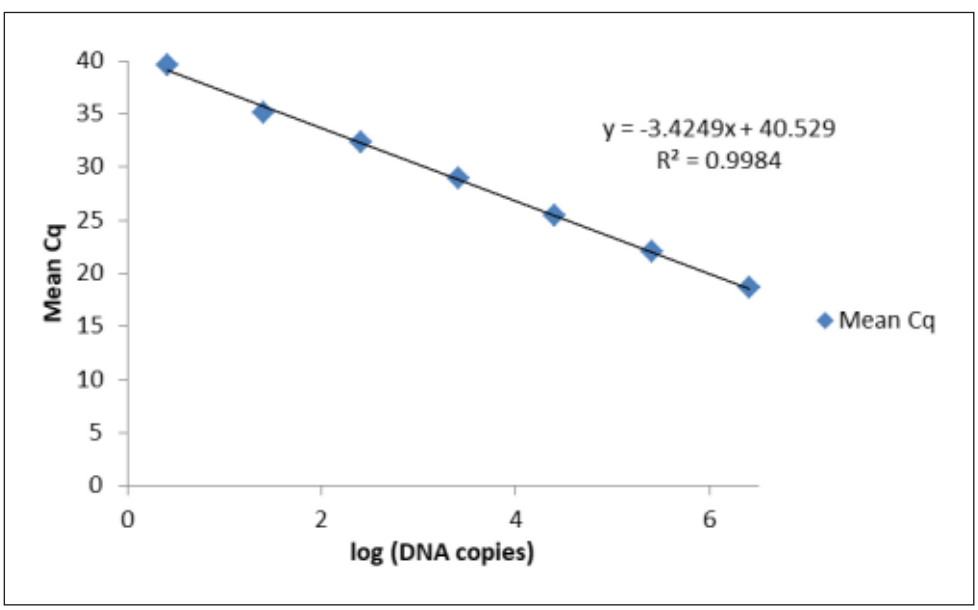

Figure 1. Standard curves of the developed TaqMan probe based qPCR assays showing amplification of *B. pseudomallei* genomic DNA at different concentrations.

Table 3. Evaluation of TTSS-orf2 Taqman qPCR on clinical specimens

Gold standard (*B. pseudomallei* culture)	Taqman qPCR		Total samples (n = 71)
	Positive	Negative	
Positive	10	–	10
Negative[a]	–	61	61

[a] Consisted of 11 samples with growth of non-*B. pseudomallei* and 50 samples of negative growth of any organism.

DISCUSSION

Early detection of melioidosis enables early initiation of a definitive treatment regime and significantly reduces the risk of mortality. Current laboratory diagnosis of melioidosis depends on conventional culture method and biochemical tests for bacterial identification, which often take two to seven days (Lau *et al.*, 2015). Such methods also suffer from low sensitivity (~60%) (Limmathurotsakul *et al.*, 2010). In the era of molecular technique advancement, PCR-based assay has a great potential to be utilised at healthcare facilities as it enables immediate results with good sensitivity and specificity (Cheng & Currie, 2005; Lau *et al.*, 2015). To improve the diagnostic capacity of our laboratory, we developed a probe-based qPCR assay to detect *B. pseudomallei* from clinical specimens. In comparison to previously reported probe-based molecular assays that can detect 5 – 10 copies of *B. pseudomallei* DNA per reaction, our assay was able to amplify approximately 2.5 DNA copies per reaction (Novak *et al.*, 2006; Al-Marzooq & Mustafa, 2011; Kaestli *et al.*, 2012; Zhang *et al.*, 2012).

In terms of specificity, the assay correctly identified all 95 *B. pseudomallei* strains used in our study. Validation of assays using local isolates is necessary as *B. pseudomallei* genomes are diverse and lateral gene transfers occur regularly (Holden *et al.*, 2004). Molecular epidemiological study of Malaysian *B. pseudomallei* isolates found a high genetic variation in comparison to neighbouring countries, the Indian subcontinents, China and Australia (Radua *et al.*, 2000; Zueter *et al.*, 2015). However, certain genotypes can also be present in other Asian countries such as Cambodia and Thailand (Zueter *et al.*, 2018). Phenotypically, Malaysian *B. pseudomallei* isolates are different to isolates from the other regions in terms of sensitivity to gentamicin due to non-synonymous mutation within the *amrB* gene (Podin *et al.*, 2014). This will affect the detection of *B. pseudomallei* when using selective media containing gentamicin. Besides, different performances can be observed on commercially-available biochemical identification kits tested with different strains of *B. pseudomallei* which further emphasises the need for validation of assay on local isolates (Podin *et al.*, 2013).

Potential cross amplification may occur with *B. pseudomallei* genetic relatives including *Burkholderia mallei*, *B. thailandensis*, *Burkholderia oklahomensis* and others of the *B. cepacia* complex (BCC). The *in silico* BLAST analysis showed low similarities of our primers and probe to these organisms. Even though *in vivo* validation remains crucial in order to confirm the analytical specificity of the assay, our study was only able to test a limited number of *Burkholderia* spp. due to unavailability or limited availability of the strains in our facility. Further study should emphasise on the evaluation of this assay on larger strains of *Burkholderia* spp.

Propriety of target genes for qPCR amplification also affects the assay efficacy. Molecular assays that utilised more common genes such as *rrs* and *fliC* may produce false positive results due to possible cross-amplification with other bacteria (Kunakorn *et al.*, 2000). Our assay utilised the *orf2* of Type III secretion system (TTSS) cluster

genes that are unique to *B. pseudomallei* (Rainbow, Hart & Winstanley, 2002). Although there have been some reported assays which targeted the TTSS-*orf2*, majority of the oligonucleotides used were not exclusively located on the target gene (Novak *et al.*, 2006; Al-Marzooq & Mustafa, 2011; Zhang *et al.*, 2012). For example, only the forward primer, as described by Winstanley and Hart (2000), is located within the *orf2*-TTSS (Data not shown) (Winstanley & Hart, 2000).

Clinical evaluation provides essential data on the performance of diagnostic assay in detecting *B. pseudomallei* directly from the specimens. Previously reported TaqMan assays have reported on clinical sensitivities and specificities between 70% and 100% (Meumann *et al.*, 2006; Mustafa *et al.*, 2011; Kaestli *et al.*, 2012). Even though our developed assay is shown to be able to amplify all melioidosis-confirmed specimens, it was tested on a limited number of clinical samples. Further clinical evaluation should be carried out on a larger cohort, using the developed molecular assay and other reported tests.

CONCLUSION

In conclusion, this study suggests that the developed assay is sensitive and specific for the detection of *B. pseudomallei* in clinical specimens. Even though the number of tested clinical samples is limited, it provides crucial preliminary data for a subsequent larger scale study in Malaysia.

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