A Hexaplex PCR Assay Developed for Simultaneous Detection of *Bacillus anthracis* and *Yersinia pestis* and Distinguish Their Virulence Levels Tested on Vietnamese Samples

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Abstract

Bacillus anthracis and *Yersinia pestis* are at high risk of bioterrorism, but they have different strains of virulence that exist in the natural environment, making it difficult to diagnose the risk of disease. This study aimed to develop a novel hexaplex PCR assay to simultaneously detect *B. anthracis* and *Y. pestis* and quickly distinguish their virulence levels. The *vrnA*, *pagA*, and *capA* genes from *B. anthracis* and *ypj2088*, *pla*, and *cafI* genes from *Y. pestis* were specifically amplified to generate six PCR fragments of 222, 751, and 610 and 103, 438, and 271 bp, respectively. Furthermore, a recombinant competitive internal amplification control was designed in the assay, which coamplified with the *capA* gene primer pairs, producing a fragment of 1000 bp in length. Thirty-eight bacterial strains including 4 reference strains and 34 screening strains derived from human, herbivores, and environments in the North of Vietnam, as well as spiked soil samples, were used to evaluate the assay. The new hexaplex PCR assay was in accordance with the conventional culture methods to detect *B. anthracis* and *Y. pestis*. In addition, this assay can be quickly discriminated among pathogenic and non-pathogenic *B. anthracis* and *Y. pestis* strains, as well as rapidly differentiate levels of virulence among strains isolated from human and soil samples in some areas of Vietnam. The new hexaplex PCR is a useful tool in screening for both pathogenic and non-pathogenic *B. anthracis* and *Y. pestis*, necessary in use for biodefense purposes and disease prevention.

Keywords *Bacillus anthracis* · *Yersinia pestis* · Hexaplex PCR · Internal amplification control · Pathogenic

Son Thai Nguyen and Hang Thi Thu Dinh contributed equally to this work.

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Background

Bacillus anthracis and *Yersinia pestis* are the pathogenic agents of anthrax and bubonic plague, respectively. They are zoonotic pathogens causing life-threatening diseases in humans and animals and posing high risks for public health [1, 2]. The Centers for Disease Control and Prevention (CDC) classified these pathogens as bioterrorism agents in category A of the most dangerous group of biological agents [3]. One of the most typical examples of bioterrorism, the “Anthrax letters” case in September 2001 in New York, USA, has affected the population with a huge impact at a psychological and political level [4]. Rapid screening of suspected specimens, therefore, helps to control the spread of epidemics, to contain the causative agents and to ensure the accurate and specific treatment.

Currently, there are a number of diagnostic methods for identification of *B. anthracis* and *Y. pestis* based on their phenotypic features, including bacteriological isolation, serology, and immunoassays. These approaches are time-

consuming and required the use of skillful personnel and biosafety level 3 (BSL3) laboratory [3]. Moreover, serology and immune assays have limited sensitivity and specificity, particularly in case of immune assay-based rapid tests [5]. In recent years, a number of molecular approaches targeting genomic DNA have been developed for rapid and accurate identification of *B. anthracis* and *Y. pestis*, including conventional and simple multiplex polymerase chain reaction (mPCR), real-time PCR, and microarray assay applied for detection/discrimination of the pathogens in humans and environment [6–12]. Generally, in these assays, the detection has been targeted to virulence gene(s) on a symbiotic plasmid of each pathogen only; thus, identification and discrimination between pathogenic strains and non-pathogenic strains lacking either one or both virulence plasmids were limited. For *B. anthracis*, accurate diagnosis of pathogenic strains in nature is based on simultaneous detection of specific genes on their chromosomes and two virulent plasmids, pXO1 and pXO2. One of these plasmids may be lost in some strains, leading to a decrease in their pathogenicity [13]. Similarly, *Y. pestis* contains two virulence plasmids, pPCP1 and pMT1, with their respective genes encoding for virulence factors [14, 15]. In some cases, these plasmids are unstable and easy to be lost due to the environmental conditions or during serial subcultures [14, 16, 17]. Other factors that may inhibit successful PCR or generate false products regarding amplification of *B. anthracis* and *Y. pestis* from the environmental samples are the substances usually found in soil and water. Therefore, internal amplification control (IAC) in diagnostic PCR assays is necessary to be designed to detect false negative amplifications [18].

These two contagious pathogens have been detected and persisted in some areas in Vietnam. The sporadic cases of anthrax still occasionally appear in the North of Vietnam [19], and *Y. pestis* still persists in rodents in the Tay Nguyen area has the potential to cause epidemics [20]. An accurate and rapid identification assay to detect these pathogens is essential for early treatment and prevention of epidemic breaks. In the present study, we describe a rapid hexaplex PCR assay simultaneously targeting six genes developed for accurate identification of pathogenic *B. anthracis* and *Y. pestis* with the IAC included in the reaction to verify the positive and to avoid false negative results.

Materials and Methods

Experiments were carried out in the Division of Microbiology, the Institute of Biomedicine and Pharmacy, and the Department of Medical Microbiology, Military Hospital 103, Vietnam Military Medical University (VMMU).

Bacterial Strains and Growth Conditions

The strains of *Bacillus anthracis* 17JB, *Bacillus cereus* ATCC 6464, *Yersinia enterocolitica* ATCC 9610 and *Escherichia coli* ATCC 25922, are used as reference strains in this study. The screening bacterial strains from suspected various soil and aquatic environments (B1, B3–5, B8–13, B17–22, Y1), patients (B2, B6, B23, Y2–11), and livestock animals (B7, B14–16) in the north of Vietnam were collected by the research teams of Military Institute of Preventive Medicine, Vietnam Military Department of Medicine and VMMU, identified by conventional culture and PCR. Reference and screening bacterial strains were grown on broth of brain heart infusion (BHI) (Difco Laboratories, USA) and 5% (v/v) sheep blood agar plates (BioMérieux, Marcy l'Etoile, France) at 37 °C for 24 h for *B. anthracis*, and at 28 °C for 48 h for *Y. pestis*. Other reference strains were routinely cultivated on 5% (v/v) sheep blood agar overnight at 37 °C. Presumptive colonies studied the shape, the structure, and the colony characteristics; besides, Gram staining and biochemical tests of the isolates were carried out according to the manufacturer's instructions (API50CHB, API20NE, BioMérieux, Marcy l'Etoile, France, Vitek2 Compact).

Genomic DNA Extraction from Bacterial Culture

All bacterial culture samples were heat inactivated at 121 °C for 20 min, and follow-up experiments were carried out in a BSL2 facility [21]. Genomic DNA was extracted using the commercial QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Extracted DNA samples were prepared at a concentration of 10^5 copies/ μ l and stored at 4 °C until use.

Primer Design and Standard PCR Assays

Sequences of specific genes in GenBank for both pathogenic and non-pathogenic *B. anthracis* and *Y. pestis* were selected as references for primer design for multiplex PCR. The *vrnA* (chromosome), *pagA* (pXO1 virulent plasmid), and *capA* (pXO2 virulent plasmid) genes, in particular, were selected as markers for *B. anthracis*. For *Y. pestis*, the *ypo2088* (chromosome), *pla* (pPCP1 virulent plasmid), and *cafI* (pMT1 virulent plasmid) genes, similarly, were selected. All primer pairs for the selected genes were self-designed in silico by using Primer3 tool in the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/>) and BioEdit software. List of primers designed, target genes, and amplicon sizes are shown in Table 1. Standard PCR and multiplex PCR assays were performed in a Master proS Cyclor (Eppendorf, Hamburg, Germany) in the volume of 25 μ l containing $1\times$ PCR buffer (2 mM $MgCl_2$), 0.2 mM of each dNTP, 0.2 μ M of each primer, 1 U Dream Taq

Table 1 List of primer pairs developed for internal amplification control and multiplex PCR in this study

Organism	Primer	Gene target	Sequence (5' to 3')	Amplicon (bp)
Competitive IAC originated from <i>B. anthracis</i>	IC1F/ <i>Bam</i> HI	Lethal factor gene	GTGGATCCTATCTTGCCAGC ATCCGT	654
	IC1R/ <i>Nde</i> I		TCCATATGAAAGAGGCAGCA GAAAAGC	
<i>B. anthracis</i>	<i>vr</i> AF1* <i>vr</i> AR1*	Variable repeat region A	CTGTAAGCCCTGTCGTCGAA CCTCGCGCACTTCTTTTCT	222
	<i>pag</i> AF1* <i>pag</i> AR1*	Protective antigen gene	AAATGGAGCACGGCTTCTGA AGCCTGTATCCACCCTCACT	751
	<i>cap</i> AF1* <i>cap</i> AR1*	<i>CapA</i> gene, IAC plasmid	TGACGATGACGATGGTTGGT GCTTCCTGTCTAGGACTCGG	610, 1000
<i>Y. pestis</i>	<i>ypo</i> F1* <i>ypo</i> R1*	Ypo2088 putative methyltransferase gene	GGATGAGATAACGCGGGTGT AGAGAATCGTGATGCCGTCC	103
	<i>pla</i> F1* <i>pla</i> R1*	Plasminogen activator gene	CGGGATGCTGAGTGGAAAGT ATTACCCGCACTCCTTTCGG	438
	<i>caf</i> F1* <i>caf</i> R1*	F1 capsular antigen gene	CGCTTACTCTTGGCGGCTAT GCTGCAAGTTTACCGCCTTT	271

*Primers used in multiplex PCR

polymerase (Thermo Scientific, Wilmington, DE, USA), and 5 µl genomic DNA (approximately 10 ng). Thermocycling parameters were as follows: an initial denaturation at 94 °C for 4 min, followed by 30 cycles consisting of 94 °C for 1 min, 56 °C for 45 s, and 72 °C for 50 s, and a final extension at 72 °C for 7 min.

Optimization of Hexaplex PCR Assay

A hexaplex PCR was carried out by simultaneous addition of six primer pairs for *vr*rA, *pag*A, *cap*A, *ypo*2088, *pla*, and *caf*I genes (Table 1) in the same reaction mixture. Initially, equimolar primer concentration of 0.2 µM each was used in the multiplex PCR and adjustment of the proportion of each primer pair from 0.1 to 0.6 µM in the reactions to obtain optimal amplification of targeted genes. Secondly, a gradient of PCR annealing temperature was performed from 54 to 58 °C to find the optimal temperature allowing simultaneous and specific amplification of six different amplicons. Additionally, to increase the product yield of hexaplex PCR assay, other PCR components were also evaluated including MgCl₂ 0.2–2.0 mM, dNTPs 0.25–0.8 mM, buffer 1–1.5×, and number of PCR cycles from 30 to 40 cycles. Adjuvants such as BSA (bovine serum albumin), betaine (trimethylglycine) and DMSO (dimethyl sulfoxide) to be considered additional if necessary. After each amplification assay, PCR fragments were analyzed on 1.5% agarose gel electrophoresis in 0.5× TBE buffer at 110 V for 50 min and 0.5 µg/ml of ethidium bromide (Bio Basic, Markham, Ontario, Canada) stained and visualized with a BioDoc-it Imaging System (UVP, Upland, CA, USA).

Sensitivity and Specificity of the Hexaplex PCR Assay

Genomic DNA samples from *B. anthracis* and *Y. pestis* were serially diluted from approximately 20 ng/µl to 20 fg/µl with 5 µl from each dilution used for hexaplex PCR to identify the analytical sensitivity, corresponding to a range from 2×10^7 to 20 genome copies/reaction, respectively. Specificity of primer pairs in the developed hexaplex PCR was assessed using genomic DNA samples from *B. cereus*, *Y. enterocolitica*, and *E. coli*. The time stability of developed PCR assay which was stored at –20 °C was tested monthly in the same standard concentration sample of *B. anthracis* and *Y. pestis*.

Hexaplex PCR Assay for the Detection of Soil Pseudo-Sample Contaminated Bacteria

In order to validate the multiplex PCR with the environment sample, we spiked the inactivated *B. anthracis* and *Y. pestis* culture samples into the soil. The final concentration of pseudo-samples was from 10^0 to 10^7 colony forming units/milliliter (CFU/ml). One milliliter of each sample was added to aliquots of soil (1 g) and incubated the contaminated soil for 24 h [22]. The suspension that was created from the soil by adding 2 ml of distilled water and carefully vortex was used to extract the DNA using the Soil DNA isolation kit (Norgen Biotek Corp., Canada) according to the manufacturer's instruction.

Internal Amplification Control Designing

A competitive IAC used to verify the diagnosis results was a chimeric plasmid, and its construction is briefly described. A 654 bp fragment of *lef* gene of *B. anthracis* was amplified

using IC1F/*Bam*HI and IC1R/*Nde*I primer pairs (Table 1). This PCR product was cloned into a pJET1.2-capA plasmid consisting of the sequence of pJET1.2-blunt vector (Thermo Fisher Scientific Inc., Waltham, USA) and the *capA* gene of *B. anthracis* after it was linearized by *Bam*HI and *Nde*I restriction enzyme. The ligation product was conventionally transformed into *E. coli* DH5 α (Invitrogen Corp., Carlsbad, USA) [18]. The recombinant IAC named pJET1.2-capA-IC1 was sequenced to confirm its identity. Determination of the lowest reproducible IAC concentration during genomic DNA extraction was evaluated from 10^9 to 10^0 copies/sample from IAC dilutions.

Evaluation of Developed Hexaplex PCR with IAC

Evaluation of hexaplex PCR assay with pJET1.2-capA-IC1 was performed with a total of 38 samples, including *B. anthracis* ($n = 11$), *B. cereus* ($n = 14$), *Y. pestis* ($n = 1$), *Y. enterocolitica* ($n = 1$), and *E. coli* ($n = 11$), and compared with conventional culture methods. Additionally, the strains of *B. anthracis* and *Y. pestis* specifically determined by the hexaplex PCR assay were exactly confirmed by gel extraction of target bands and direct Sanger sequencing.

All the experiments in the present study were performed in triplicate and controls included.

Results

Selection and Primer Pair Design of Three Target Genes for *B. anthracis* and Three Target Genes for *Y. pestis*

In *B. anthracis*, to distinguish between pathogenic strains and non-pathogenic strains, we selected both the *pagA* gene (751 bp amplicon) and *capA* gene (610 bp amplicon) from pXO1 and pXO2, respectively, and a *B. anthracis*-specific target of the variable repeat region A—the *vrnA* gene (222 bp amplicon) as a stable chromosomal marker for a *B. anthracis* detection triplex PCR assay.

Similarly, the *pla* and *cafI* are common target genes which are used to identify *Y. pestis* in molecular methods. Particularly, the *pla* gene (amplicon size 473 bp) encodes plasminogen activator located on the virulent plasmid pPCP1. Whereas, the *cafI*/R1 primer pairs targeted the *cafI* gene (amplicon size 271 bp) located on the virulent pMT1 plasmid which encoded the F1 fraction. Furthermore, the *ypa2088* gene (amplicon size 103 bp) is a specific chromosomal marker of *Y. pestis*. Uniplex PCR amplicons of different genes of *B. anthracis* as well as *Y. pestis* yielded the products of expected sizes on agarose gel electrophoresis. The specificity of the primer pairs was checked in pooled two DNA templates of *B. anthracis* and *Y. pestis* by tri-primer

PCR assays of each agent, to ensure that there was no cross-amplification (Fig. 1, Table 1).

Optimization of Hexaplex PCR Assay

The optimization of a hexaplex PCR assay for high amplification efficiency of the specific PCR products has been conducted as follows. Firstly, the concentration of individual primer pairs was evaluated, and the optimized concentrations of three primer pairs for *B. anthracis* and three primer pairs for *Y. pestis* were 0.6 and 0.1 μ M, respectively. Secondly, an optimal annealing temperature of 56 $^{\circ}$ C was found. Next, $MgCl_2$ was obtained for 0.6 mM as the best concentration. After the concentration of $MgCl_2$ was determined as of 0.6 mM, the dNTP concentration from 0.25 to 0.8 mM was tested and 0.25 μ M was found the most optimal concentration. Finally, other components found for the hexaplex PCR were 2.0 U of Dream *Taq* polymerase, 1.2 $\times$ buffer, and 32 amplification cycles. The optimal mPCR assay revealed the presence of six amplicons clearly separated on agarose gel by the electrophoresis (Fig. 2).

Evaluation of the Detection Capability for Multiplex PCR Assay

The limit of detection of hexaplex PCR by using serious dilutions of bacterial genomic DNA as templates, for the individual detection of *B. anthracis* and *Y. pestis*, was 0.5 pg/reaction, equivalent to approximately 100 bacterial cells. The sensitivity of hexaplex PCR with spiking soil study for *B. anthracis* and *Y. pestis* was about 10^4 CFU/ml, and there were no

Fig. 1 Mono- and tri-primer PCR assay for the detection of *B. anthracis* and *Y. pestis*. Lane M, 100 bp DNA ladder (Thermo Scientific); NC, negative control (no DNA template). For the detection of *B. anthracis*: lane 1, mono-primer PCR assay consisting of *pagA*F1/R1 (virulent pXO1 plasmid); lane 2, *capA*F1/R1 (virulent pXO2 plasmid); lane 3, *vrnA*F1/R1 (chromosome). For the detection of *Y. pestis*: lane 4, mono-primer PCR assay consisting of *pla*A1/R1 (virulent pPCP1 plasmid); lane 5, *cafI*F1/R1 (virulent pMT1 plasmid); lane 6, *ypaF*1/R1 (chromosome). For the detection of pathogenic *Y. pestis* and *B. anthracis* with pooled two DNA templates: lane 7, tri-primer PCR assay including three primer pairs (*ypaF*1/R1, *pla*A1/R1, *cafI*F1/R1); lane 8, tri-primer PCR assay including three primer pairs (*vrnA*F1/R1, *pagA*F1/R1, *capA*F1/R1)

Fig. 2 Optimal hexaplex PCR assay with pooled two DNA templates of *B. anthracis* and *Y. pestis*. Lane M, 100 bp DNA ladder (Thermo Scientific); lane 1, hexaplex PCR assay; lane 2, negative control

notable differences in sensitivity for *B. anthracis* and for *Y. pestis* observed. However, some weak unspecific products appeared in the no-spiked and spiked soil samples (Fig. 3). The results proofed the overabundance of other microorganisms on soil samples in the study. Sequencing of six specific bands extracted from the gel confirmed the correspondent designed chromosomal and virulent targets of *B. anthracis* and *Y. pestis* (data not shown).

Optimization and Evaluation of the Multiplex PCR Assay Including Internal Amplification Control

Plasmid pJET1.2-capA-IC1 was designed to contain the primer sequence of capAF1/R1 of *B. anthracis* which was amplified simultaneously in the hexaplex PCR, for IAC product with a size of 1000 bp different from the six diagnosis amplicons of the hexaplex PCR in terms of size. The IAC concentration of 5×10^4 copies per sample was determined, and the components of the hexaplex PCR assay including IAC were adjusted to 0.64 μ M concentration of capAF1/R1 primer set (Fig. 4). The IAC was added to the samples to control the DNA extraction procedure and verify PCR negative cases.

The developed hexaplex PCR assay was validated on 38 bacterial samples including the reference strains and screening isolates. Sensitivity and specificity were calculated for each target gene individually for the hexaplex PCR results of *B. anthracis* and *Y. pestis*. Out of 38 genomic DNA samples, 24 samples (*B. anthracis* 17JB, B1-B23) showed a PCR product of the *vrroA* gene amplification (222 bp) for the “*B. cereus*” group, and four samples (*B. anthracis* 17JB, B2, B3, B23) showed two PCR products of both 751 and 610 bp of the *pagA* gene and *capA* gene, respectively, which are specific

Fig. 3 Multiplex PCR products from soil samples inoculated with *B. anthracis* and *Y. pestis*. Lane M, 100 bp DNA ladder (Thermo Scientific); lanes 1, 4, and 7, bacterial culture of *B. anthracis*, *Y. pestis*, and *B. anthracis* and *Y. pestis*; lanes 2, 5, and 8, soil not inoculated with *B. anthracis*, *Y. pestis*, and *B. anthracis* and *Y. pestis*; lanes 3, 6, and 9, soil inoculated with 10^6 CFU/ml of *B. anthracis*, *Y. pestis*, and *B. anthracis* and *Y. pestis*

to pathogenic *B. anthracis*. One sample (B5) showed a single product of 751 bp of the *pagA* gene present in the plasmid pXO1 in *B. anthracis* strain only and no amplification for the *capA* gene due to the absence of plasmid pXO2, indicating this is of a not fully virulent strain. One sample showed three bands of 103, 275, and 438 bp, which are specific to pathogenic *Y. pestis* (Y1). The rest samples did not show any band except IAC product in all examined samples (illustrated in Fig. 4). The hexaplex PCR assay had, thus, 100% specificity for *Y. pestis*.

Discussion

Rapid, reliable, and simultaneous identification of CDC category A bioterrorism agent plays a crucial role in disease prevention. In this study, we have developed and evaluated a novel hexaplex PCR assay for simultaneous detection of pathogenic *B. anthracis* and *Y. pestis* as well as discrimination with non-pathogenic strains in a single PCR tube. A multiplex PCR assay comprising a total of six primer pairs including *vrroA*/R1, *pagA*/R1, *capA*/R1, *ypof*/R1, *pla*/R1, and *caf*/R1 for *B. anthracis* and *Y. pestis* detection, respectively, was developed. Especially, the recombinant IAC, namely pJET1.2-capA-IC1, was designed with primer capAF1/R1 and was successfully co-amplified in the hexaplex PCR assay.

In *B. anthracis*, the *pagA* gene which encodes the protective antigen belonging to the virulent plasmid pXO1 is recommended by the World Health Organization (WHO) [19]. Besides, the second virulent plasmid of *B. anthracis*, pXO2, contains the *capA*, *capB*, and *capC* genes encoding poly-D-glutamic capsule, in which the *capA* gene was widely used for diagnosis of *B. anthracis* [7, 23, 24]. In *Y. pestis*, both the *pla*

Fig. 4 Evaluation of the detectability of the optimal multiplex PCR including IAC from genomic DNA from screening isolates. M, 100 bp DNA ladder (Thermo Scientific); NC, negative control (*B. cereus* + *E. coli*); PC, positive control (*B. anthracis* and/or *Y. pestis* DNA); lane 1,

Y. pestis Y1 and *B. anthracis* B2; lane 2, *B. anthracis* B4; lane 3, *Y. pestis* Y1; lane 4, *B. anthracis* B3; lane 5, *B. anthracis* B5; lane 6, *B. cereus* B9; lane 7, *Y. enterocolitica*

and *cafI* genes were commonly used as the targets because of high estimated copy numbers (3 and 4 in a cell), respectively, which improved the sensitivity of PCR [12, 25, 26]. Additionally, we have used the *ypo2088* gene encoding for a putative methyltransferase to distinguish *Y. pestis* from other *Enterobacteriaceae* [15, 27].

In the developed hexaplex PCR assay, the recombinant IAC was also designed to control both DNA extraction and PCR processing, allowing the evaluation of the true negative to false negative results. In our study, we used no additional adjuvant supplementary materials, which were recommended by various authors to improve amplification efficiency and specificity of the multiplex PCR [28, 29]. Our results revealed that the simultaneous hexaplex PCR assay with IAC was effectively optimized in this study.

However, the study has some limitations that will be the focus of our future work. Firstly, due to the lack of a clinical sample for both *B. anthracis* and *Y. pestis*, we could not evaluate the hexaplex PCR assay for diagnostics in clinical and environmental samples. Instead, we had to validate the mPCR assay in soil pseudo-samples contaminated with the cultured bacteria. Secondly, for the 6 non-pathogenic *B. anthracis* isolates (B1, B4, B6–8, B10), which do not harbor both the virulent plasmids, pXO1 and pXO2, which harbored the *vrrA* gene as this gene is present in all species of “*B. cereus*” group including *B. anthracis*, *B. cereus*, and *B. thuringiensis* [5], these isolates, therefore, were indistinguished from *B. cereus* used as reference strains. Thus, the use of both plasmids pXO1 and pXO2 in combination to accurately identify pathogenic *B. anthracis* was recommended [13, 30, 31]. Here, the three pathogenic *B. anthracis* were explored by the hexaplex PCR assay which was to the best of our knowledge the first report for cases of anthrax during two past decades in Vietnam. Altogether, the above multiplex PCR assay has been proven the ability of accurate and simultaneous identification of

pathogenic *B. anthracis* and *Y. pestis* as well as discrimination with non-pathogenic strains in nature.

Conclusions

The hexaplex PCR assay was developed for both pathogenic *B. anthracis* and *Y. pestis* to allow rapid and accurate detection of six target genes simultaneously including two genes on chromosomes and four genes on virulence plasmids of both bacteria, help distinguish strains that cause disease with non-pathogenic strains of *B. anthracis* as well as *Y. pestis*. Furthermore, we successfully developed the recombinant IAC for concurrent PCR amplification, making it as reliable proof for avoiding false negative results. The hexaplex PCR can, therefore, be a useful tool in screening for both pathogenic and non-pathogenic *B. anthracis* and *Y. pestis*, necessary in use for biodefense purposes and disease prevention.

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Compliance with Ethical Standards

Ethics Approval and Consent to Participate Not applicable. The study was based on laboratory work performed with reference bacterial and screening strains isolated from environments, human, and herbivores. No data from human patients were used.

Consent for Publication Not applicable.

Conflict of Interest The authors declare that they have no conflict of interests.

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