Characterization of *Bacillus anthracis* spores isolates from soil by biochemical and multiplex PCR analysis

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التعرف على خصائص أبواغ العصوية الجمرية المستفرَدة من التـربة بالتحليل الكيميائي الحيوي وتفاعل البوليميراز السلسلي المتكرر

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الخلاصة: يتكرر التبليغ عن فاشيات العصوية الجمرية لدى الحيوانات في جمهورية إيران الإسلامية. وقد أجرى الباحثون في هذه الدراسة تحليلاً لعينات التـربة من المناطق الموطونة بالمرض في إيران، وجرى تحديد مستفردات العصوية الجمرية بالطرق البكتـريولوجية والكيميائية الحيوية المعتادة. كما استخدم تفاعل البوليميراز السلسلي المتعدد كبديل للتعرف على المستفرَدات، وقد أثبت هذا التفاعل أنه يمثِّل مقايسة تشخيصية سريعة وحساسة ونوعية. وأكدت النتائج وجود 25 عينة تحتوي على العصوية الجمرية، منها تسع عينات مُفَوَّعة للفئران والقُبَيْعة. وتثبت الدراسة إمكانية استخدام تفاعل البوليميراز السلسلي المتعدد كبديل يعوّل عليه في كشف أبواغ العصوية الجمرية، وفي الدراسات الوبائية، وفي تـرصُّد مرض الجمرة الخبيثة ولاسيَّما في المناطق الموبوءة.

ABSTRACT Outbreaks of *Bacillus anthracis* in animals are repeatedly reported in the Islamic Republic of Iran. In this study soil samples were analysed from endemic regions of the country, and *B. anthracis* isolates were identified by classical bacteriological and biochemical methods. A multiplex polymerase chain reaction (PCR) assay was also developed as an alternative for identification of isolates, and was shown to be a rapid, sensitive and specific diagnostic assay. The results confirmed that 25 samples contained *B. anthracis*, of which 9 were virulent for mice and guinea pigs. This study suggests that multiplex PCR can be used as a reliable alternative for the detection of *B. anthracis* spores.

Caractérisation d'isolats de *Bacillus anthracis* provenant du sol par analyse biochimique et PCR multiplex

RÉSUMÉ Des épidémies de *Bacillus anthracis* chez les animaux sont souvent signalées en République islamique d'Iran. Dans cette étude, des échantillons de sol venant de régions endémiques du pays ont été analysés et des isolats de *B. anthracis* ont été identifiés grâce aux méthodes bactériologiques et biochimiques classiques. Un test d'amplification en chaîne par polymérase (PCR) multiplex a également été mis au point pour identifier les isolats, et il s'est avéré que cette autre solution constituait un test diagnostique rapide, sensible et précis. Les résultats ont confirmé que 25 échantillons contenaient *B. anthracis*, et que 9 souches étaient virulentes pour les souris et les cobayes. Cette étude suggère que la PCR multiplex peut être utilisée comme solution de remplacement fiable aux fins de la détection de spores de *B. anthracis*.

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Introduction

Bacillus anthracis is a gram-positive, aerobic, spore-forming bacterium that causes anthrax in mammals [*1*]. *B. anthracis* spores are very resistant to various conditions. The spores can survive for many years in soil. This long survival is important in epidemiological studies and in planning for control and prevention of anthrax. The success of the attenuated Sterne veterinary vaccine in 1930 resulted in a global reduction of anthrax cases in livestock in response to national vaccination programmes.

Unfortunately there are still regions where anthrax is endemic. The Islamic Republic of Iran is an endemic region with many reported outbreaks in recent years. Outbreaks occur not only in wild animals [*2*] but also in domestic animals [*3*]. Animals become infected after coming into contact with soil-borne spores when grazing. Humans become infected after contact with infected animals or their contaminated products [*4*]. Surveillance and monitoring of anthrax is therefore a focus in public health.

B. anthracis is closely related to several species, including *B. cereus* and *B. thurengiensis* [*5,6*]. The virulent strains of *B. anthracis* harbour 2 virulent plasmids, toxin-encoding plasmid pX01 (181.7 kb) [*7,8*] and plasmid pX02 (96.2 kb) which codes for the capsule [*9*]. The current routine laboratory diagnostic method for *B. anthracis* is microbiological analysis [*4,10*]. Avirulent *B. anthracis* strains lack pX01 and pX02 and cannot be distinguished from other related species with these time-consuming microbiological analyses [*11,12*]. Therefore, development of a specific and rapid method for detection of *B. anthracis* is required.

In this study soil samples were collected from regions of the Islamic Republic of Iran where there are repeated reports of outbreaks of *B. anthracis*. These samples were analysed for the presence of *B. anthracis* using routine assay and a multiplex polymerase chain reaction (PCR) assay that was established in-house (at the Razi Vaccine and Serum Research Institute, Mashhad, Islamic Republic of Iran).

Methods

Soil sampling

Soil samples were randomly collected from anthrax-endemic regions of Isfahan province in the central part of the Islamic Republic of Iran. A total of 60 specimens were collected. In each collection, approximately 500 g of the topsoil to a maximum depth of 20 cm were included and transferred to labelled bags.

Spore extraction and bacteria identification

An overnight incubated soil suspension in sterile phosphate-buffered saline (PBS) was passed through a 0.45 μm filter and the deposit was suspended in sterile PBS. The aliquot was heated at 65 °C to destroy vegetative cells and activate the spores. Then the suspension was centrifuged and the resuspended pellet was streaked onto duplicate plates of PLET agar (a selective media for *B. anthracis*) and blood agar media. One set of cultured plates was incubated at 37 °C and the other at 40 °C, both aerobically. Colonies emerging were examined for morphological and cultural features of *B. anthracis* at the end of 24–48 hours of incubation.

Biochemical and biological analysis

The colonies identified as *B. anthracis* were selected and further biochemical tests were

Table 1 **Primers used for *Bacillus anthracis* gene amplification**

Primer	Sequence (5–3)	Expected size	Target gene
Bac F	AAT GAT AGC TCC TAC ATT TGG AG	150 bp	Chromosome
Bac R	TTA ATT CAC TTG CAA CTG ATG GG		
PA F	CGA AAA GGT TAC AGG ACG G	330 bp	pX01
PA R	CAA GTT CTT TCC CCT GCT A		
Cap F	GTA CCT GGT TAT TTA GCA CTC	209 bp	pX02
Cap R	ATC TCA AAT GGC ATA ACA GG		

Bac = B. anthracis *chromosome; PA = protective antigen; Cap = capsule.*

conducted according to classical bacteriological methods [*13*]. The saline suspension containing different numbers of spores was injected subcutaneously to mice and guinea pigs to evaluate the lethality of the isolates [*14,15*].

Total DNA extraction

Bacterial isolates were cultured on blood agar plates and then 1 colony was picked and resuspended in normal saline. All of the bacterial suspensions were inactivated in boiling water for 15 minutes. A simple and rapid method was used for total DNA extraction. Briefly, 200 µL of inactivated bacterial cultures were vortexed vigorously and then frozen in liquid nitrogen. The samples were thawed and vortexed again. These steps were repeated 3 times and then the samples were centrifuged at 12 000 × g for 1 minute. Finally, the obtained supernatants were used as DNA source directly.

Primers

Three pairs of primers were designed according to *B. anthracis* sequences deposited in the GenBank® database. The primers were synthesised by TIB MOLBIOL (Berlin, Germany). The primers that were used for specific amplification of *B. anthracis* chromosome (Bac) were designed on the basis of the previously published sequences [*16*]. Protective antigen (PA) and capsule (Cap) primers were used to confirm the presence of plasmids pX01 and pX02 respectively. These primers were confirmed to be specific for targets based on the previous studies and after comparison with *B. anthracis* sequences from the database of the National Centre for Biotechnology Information using the BLAST network server [*17–20*]. Sequences of these oligonucleotide primers are shown in Table 1.

Multiplex PCR

In order to optimize the PCR reaction, we first set each primer at a concentration of 5 pmol in separate amplification reactions. Subsequently we optimized the multiplex PCR reactions.

The final optimized PCR mixtures (25 µL) consisted of: 200 µM each of dATP, dCTP, dGTP and dTTP, 4 mM $MgCl_2$, 5 pmol of each primer, and 2.5 µL of 10 × reaction buffer (100 mM $(NH_4)2SO_4$, 200 mM Tris–HCl (pH 8.8), 1% Triton X-100, 100 mM KCl, 1 mg/mL BSA) and 0.05 pg of DNA extraction in 3 µL doubled-distilled water. PCR analysis was performed under the following conditions using the Techne 1600 (Techne, Cambridge, United Kingdom): 1× (94 °C for 5 min), 35 × (94 °C for 50 s followed by 58 °C for 50 s and 72 °C for 50 s), 1 × (72 °C for 1 min), cool to 25 °C.

Serial dilutions of extracted DNA were used as the template for multiplex PCR. The specificity of optimized multiplex PCR was tested using archived samples of the *Bacillus* genus: *B. subtilis*, *B. cereus*, *B. thuringiensis* and *B. mycoides*.

Analysis and detection of amplified DNA (PCR products)

Finally, 5 µL of each amplification reaction was analysed on 2% w/v agarose in Tris-acetate-EDTA buffer containing 0.5 µg/mL ethidium bromide. A 50 bp marker (Fermentas, Lithuania) was also included on every gel. The gel was analysed under ultraviolet illumination.

Results

The presence of *B. anthracis* in collected soil samples was determined and analysed morphologically and biochemically. A total of 25 samples were positive: 11 of these positive samples displayed rods with capsules by Giemsa staining, 9 of them were lethal to mice and guinea pigs and 8 of the samples did not produce toxin or capsule. Table 2 summarizes the results.

Our optimized multiplex PCR assay was able to successfully amplify 3 fragments of the expected sizes from extracted DNA of positive controls. Three fragments of 152 bp (Bac), 209 bp (Cap) and 330 bp (PA) reflected the presence of *B. anthracis* chromosome, capsule and toxin respectively. The sensitivity of this assay was tested by serial dilution of genomic DNA from the *B. anthracis* positive control. A minimum of 0.05 pg of total DNA was sufficient to be used as a template in the PCR. The multiplex PCR proved to be very specific for *B. anthracis* and did not result in false positives with any other bacteria. Therefore, the specificity for this protocol was calculated at 100%. The presence of *B. anthracis* was confirmed in all 25 samples by amplification of the 152 bp Bac DNA fragment. Cap and PA fragments were amplified in 12 and 14 samples respectively (Figures 1, 2 and 3).

Discussion

Anthrax is still one of the most serious infectious diseases in animals and man because of its highly resistant spores and wide distribution. Approximately 95% of anthrax cases in humans result from exposure to infected soil or animals, through skin lesions. Therefore, continuous surveillance for anthrax is essential to prevent this threat, especially in the Islamic Republic of Iran, where there are a number of endemic regions. During a 1945 outbreak in the Islamic Republic of Iran, 1 million sheep died, one of the largest anthrax epizootics in herbivores reported [*5*]. In recent years controlling the disease through vaccination has reduced anthrax cases in the country.

The survival of anthrax spores in soil is an important factor, especially in animal outbreaks. A classic case is the experience at Gruinard Island, Scotland, during the Second World War, where spores persisted and remained viable for 36 years. Decontamination of the island was completed in stages, beginning in 1979 and ending in 1987, when the island was finally declared fully decontaminated. There have been similar experiences in northern Canada and south Sudan [*18,19*]. Specific soil parameters, such as alkaline pH, adequate nitrogen, calcium and organic material, are required in conjunction with extreme weather changes to undergo a vegetative cycle and cause disease in grazing animals, producing the occasional outbreaks [*20*].

In our study 25 isolates of *B. anthracis* from infected regions of the Islamic Republic of Iran were obtained, of which 9 were virulent in guinea pigs and mice. *Bacillus* bacteria are notable for their

Table 2 **Results of screening of isolated *Bacillus anthracis* strains: success in detection by in-house polymerase chain reaction (PCR) assay or traditional methods**

Test number	Presence of *B. anthracis* chromosome by PCR	Capsule presence		Toxin production	
		By PCR	By Giemsa staining	By PCR	Lethality in mice or guinea pigs
1	+	+	+	–	–
2	+	–	–	–	–
3	+	+	+	+	+
4	+	–	–	–	–
5	+	+	+	+	+
6	+	–	–	+	–
7	+	+	+	+	+
8	+	–	–	–	–
9	+	+	+	+	+
10	+	+	+	+	+
11	+	–	–	+	–
12	+	–	–	+	–
13	+	–	–	+	–
14	+	+	+	+	+
15	+	+	+	+	+
16	+	–	–	–	–
17	+	+	+	+	+
18	+	–	–	+	–
19	+	–	–	–	–
20	+	+	–	–	–
21	+	+	+	–	–
22	+	+	+	+	+
23	+	–	–	–	–
24	+	–	–	–	–
25	+	–	–	–	–

phenotypic similarities. The species can be distinguished on the basis of time-consuming biochemical and microbiological analysis [*6*]. The main feature used to distinguish *B. anthracis* from other closely related *Bacillus* spp. is the presence of 2 virulent plasmids pX01 and pX02. The use of PCR as a highly sensitive, specific and rapid test for identification has been reported. Virulent genes on these plasmids have been used as markers to detect *B. anthracis* using PCR assay [*21,22*]. Avirulent strains

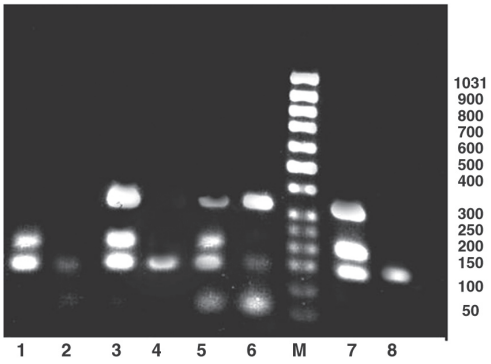

Figure 1 **Agarose gel electrophoresis of multiplex polymerase chain reaction products of *Bacillus anthracis* isolates (Bac = *B. anthracis* chromosome; PA = protective antigen; Cap = capsule)**

Lanes: (1) Bac+ Cap+ PA– (2) Bac+ Cap– PA– (3) Bac+ Cap+ PA+ (4) Bac+ Cap– PA– (5) Bac+ Cap+ PA+ (6) Bac+ Cap– PA+ (7) Bac+ Cap+ PA+ (8) Bac+ Cap– PA– (M) 50 bp DNA marker

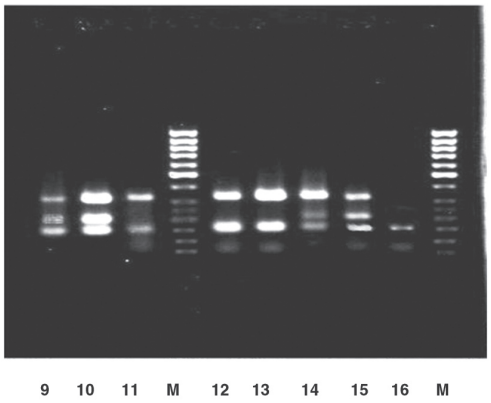

Figure 2 **Agarose gel electrophoresis of multiplex polymerase chain reaction products of *Bacillus anthracis* isolates (Bac = *B. anthracis* chromosome; PA = protective antigen; Cap = capsule)**

Lanes: (9) Bac+ Cap+ PA+ (10) Bac+ Cap+ PA+ (11) Bac+ Cap– PA+ (12) Bac+ Cap– PA+ (13) Bac+ Cap– PA+ (14) Bac+ Cap+ PA+ (15) Bac+ Cap+ PA+ (16) Bac+ Cap– PA– (M) 50 bp DNA marker

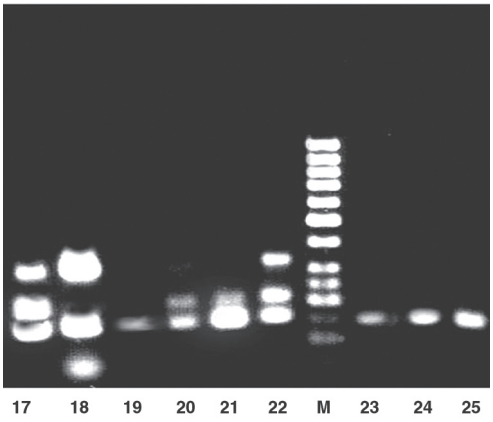

Figure 3 **Agarose gel electrophoresis of multiplex polymerase chain reaction products of *Bacillus anthracis* isolates (Bac = *B. anthracis* chromosome; PA = protective antigen; Cap = capsule)**

Lanes: (17) Bac+ Cap+ PA+ (18) Bac+ Cap– PA+ (19) Bac+ Cap– PA– (20) Bac+ Cap+ PA– (21) Bac+ Cap+ PA– (22) Bac+ Cap+ PA+ (23) Bac+ Cap– PA– (24) Bac+ Cap– PA– (25) Bac+ Cap– PA– (M) 50 bp DNA marker

that lack both of these plasmids cannot be distinguished from other *B. cereus* group bacteria with classical analysis [*5*].

In this study, a reliable and rapid method for detection and characterization of *B. anthracis* was developed. With multiplex PCR, simultaneous amplification of specific genes on bacterial chromosome and 2 plasmids was performed. This assay is suitable for general identification of *B. anthracis*, because a strain lacking both pX01 and pX02 cannot be distinguished from other related species by microbiological analysis. This sensitive and rapid assay is a reliable test for confirmation and characterization of *B. anthracis* in laboratories.

It is important to keep in mind that studies of the epidemiology of *B. anthracis* are an important component in planning control

and surveillance programmes for anthrax. We suggest that the multiplex PCR assay can be used for accurate diagnosis and analysis of toxic factors encoded by *B. anthracis* plasmids pX01 and pX02. This method reduces the time required for *B. anthracis* detection by about 3 hours in comparison with time-consuming classical microbiological and biochemical methods.

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Anthrax in humans and animals

This fourth edition of the anthrax guidelines encompasses a systematic review of the extensive new scientific literature and relevant publications up to end 2007, including all the new information that emerged in the 3-4 years after the anthrax letter events.

This updated edition provides information on the disease and its importance, its etiology and ecology, and offers guidance on the detection, diagnostic, epidemiology, disinfection and decontamination, treatment and prophylaxis procedures, as well as control and surveillance processes for anthrax in humans and animals.

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