Specific *Bacillus anthracis* identification by a *plcR*-targeted restriction site insertion-PCR (RSI-PCR) assay

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**Keywords**

*Bacillus anthracis*; *plcR*; RSI-PCR; anthrax.

**Abstract**

A RSI-PCR assay was developed for the detection of a *Bacillus anthracis*-specific nonsense mutation in the *plcR* gene. The assay specificity was tested using 170 *Bacillus* spp. strains including 47 strains of *B. anthracis*. The *plcR* RSI-PCR distinguished *Bacillus cereus* group strains closely related to *B. anthracis* from the anthrax agent. The assay was found to be a robust, simple and cost effective tool for *B. anthracis* identification. In contrast to previously developed real time PCR-based methods, the RSI-PCR needs basic molecular biology equipment only, and thus may be easily introduced in developing countries, where anthrax is endemic.

**Introduction**

*Bacillus anthracis* causes anthrax, a zoonotic disease spread worldwide. It also causes acute and often lethal diseases in humans. Although the disease is not frequently observed in developed countries, it remains an important problem in developing countries where it is endemic (Turnbull *et al*., 1999; Koehler, 2002; Clegg, 2006). *Bacillus anthracis* identification in environmental samples is difficult and time consuming since this pathogen needs to be distinguished from other, closely related species of the *Bacillus cereus* group (Koehler, 2002). Therefore, among various molecular approaches for *B. anthracis* identification developed to date, only few have been considered as specific (for reference see Cherif *et al*., 2002; Zasada *et al*., 2006).

In *B. cereus* and *Bacillus thuringiensis*, the transcriptional activator PlcR promotes secretion of non-specific virulence factors, such as haemolysins (Slamti *et al*., 2004). It has been previously shown that a nonsense mutation in the *B. anthracis* gene *plcR* generates a termination codon (UAA) in place of glutamic acid (GAA) in position 209 of the amino acid sequence of the functional PlcR (Agaisse *et al*., 1999). The lack of haemolytic activity that is characteristic of *B. anthracis* was attributed to this nonsense mutation (Agaisse *et al*., 1999; Slamti *et al*., 2004). Noteworthy, the nonsense mutation was detected in *B. anthracis* strains of 89 different multiple locus variable number tandem repeats analysis (MLVA) genotypes but not in any of 29 *B. cereus* group strains considered the genetic near-neighbours of *B. anthracis* (Easterday *et al*., 2005a). This suggested that the nonsense mutation in the *plcR* gene may be a highly specific *B. anthracis* marker.

Recently, two approaches that detect the nonsense mutation in the *plcR* gene were developed for *B. anthracis* identification. The first one was a TaqMan-minor groove-binding allelic discrimination assay (Easterday *et al*., 2005a). The second is a highly sensitive TaqMan mismatch amplification mutation assay (Easterday *et al*., 2005b). Both the approaches require expensive real-time PCR platforms while most of the anthrax endemic areas are located in developing countries, where such equipment is limited. Taking into consideration the necessity for *B. anthracis* identification in samples isolated from humans and animals in endemic areas, as well as the importance of monitoring this microorganism in animal burial sites, which often are sources of outbreaks (Koehler, 2002; Nutsch & Spire, 2004), there is a need for reliable but simple and cost-effective methods for *B. anthracis* identification.

In this study, a restriction site insertion-PCR (RSI-PCR)-based assay was developed as a cost effective and simple alternative method for *B. anthracis* identification. The assay precisely detects the *B. anthracis*-specific nonsense mutation
in \( \text{plcR} \) by restriction digestion with the endonuclease SspI. The RSI-PCR was recently proposed for \( B. \) \( \text{cereus} \) and \( B. \) \( \text{thuringiensis} \) identification (Daffonchio et al., 2006) as well as for discrimination and typing of closely related strains (Daffonchio et al., 1999). A similar technique was used for detection of a single nucleotide mutation of \( \text{gyrA} \) in fluoroquinolone-resistant strains of \( \text{Campylobacter coli} \) (Alonso et al., 2004). The requirements of RSI-PCR are limited to standard PCR equipment and an agarose or a polyacrylamide gel electrophoresis (PAGE) apparatus that makes this assay available for most routine diagnostic laboratories world-wide.

### Materials and methods

Altogether, 170 strains of \( \text{Bacillus} \) spp. were tested (Table 1). In order to detect thymidine (T), a key nucleotide in the \( \text{plcR} \) nonsense mutation of \( B. \) \( \text{anthracis} \), an artificial SspI-specific cleavage site was introduced into a PCR product generated using the 60 bp-long AplR primer (5'-ATGTCA TACTATTAATTTGACACGATAGTTCAATAGCTTTATTTG CATGACAAAGCGAAT-3'), modified at the 3' end to specifically introduce the SspI cleavage site in the amplified \( \text{plcR} \) fragment. At positions 58 and 59 of the AplR primer, two adenine (A) bases were incorporated to generate an artificial cleavage site (AATTT) for SspI in the expected PCR product of the \( \text{plcR} \) gene of \( B. \) \( \text{anthracis} \). These two adenines replaced cytosine (C) and thymine (T), which complement G and A at positions 1147 and 1146 (positions referred to sequence accession number AF132086), respectively, of the truncated \( \text{plcR} \) of \( B. \) \( \text{anthracis} \) strain 9131 (Agaisse et al., 1999). Primer AplF (5'-GCTCAATCAA CAATTGGCAGG-3') was used in combination with primer AplR for the amplification of a 278 bp fragment of the \( \text{plcR} \) gene. Localization of the primers, the nonsense mutation and the artificial SspI site is shown in Fig. 1.

The PCR was carried out in 25 \( \mu \)L reaction mixture, containing 1 U of Taq DNA polymerase (Polgen, Warsaw, Poland) and 1 × PCR buffer (10 mM Tris-HCl pH 8.9, 2 mM MgCl2, 50 mM KCl) supplied with the polymerase, 10 pmol of each the aforementioned primer, 0.2 mM of each deoxynucleotide and 2.5 \( \mu \)L of the template DNA solution (about 5–10 ng of genomic DNA) prepared as described previously (Gierczyński et al., 2004). Briefly, bacterial pellet from 1 mL of culture in tryptic soy broth was mixed with 40 \( \mu \)L of 10 mg mL\(^{-1}\) lysosome and incubated for 30 min at 37 °C. Then 160 \( \mu \)L of lysis buffer (10 mM Tris-HCl pH 8.0; 10 mM EDTA; 1% Triton X100 and 2 mg mL\(^{-1}\) proteinase-K) was added and tubes were placed at 52 °C until the suspension became transparent. Finally, 0.8 \( \mu \)L of 10 mM Tris-HCl, pH 8.0 was added and tubes were heated at 95 °C for 30 min.

An initial denaturation step at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s and a final extension at 72 °C for 5 min were performed in a Mastercycler 5333 thermal cycler (Eppendorf, Hamburg, Germany). Enzyme digestion was performed in a 20 \( \mu \)L mixture containing 6 \( \mu \)L of PCR product and 7 \( \mu \)L of the enzyme SspI (Fermentas, Vilnius, Lithuania) using thermal conditions and reaction buffer recommended by the manufacturer. At the beginning of this study, digestion products were separated in 4% nondenaturing polyacrylamide gel (Applichem, Germany) was applied. Separation was

### Table 1. Number of tested strains and results of RSI-PCR assay

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of tested strains</th>
<th>No. of ( \text{plcR} ) PCR-positive strains</th>
<th>No. of RSI-PCR-positive strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>( B. ) \text{anthracis}</td>
<td>47</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>( B. ) \text{thuringiensis}</td>
<td>55</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>( B. ) \text{cereus}</td>
<td>36</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>( B. ) \text{weihenstephanensis}</td>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>( B. ) \text{mycoides}</td>
<td>12</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>( B. ) \text{pseudo}mycoides</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>( B. ) \text{subtilis}</td>
<td>4</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>( B. ) \text{circulans}</td>
<td>3</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>( B. ) \text{brevis}</td>
<td>2</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>( B. ) \text{licheniformis}</td>
<td>1</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Total</td>
<td>170</td>
<td>92</td>
<td>47</td>
</tr>
</tbody>
</table>

*\( \text{Bacillus} \) \text{anthracis}-specific SspI pattern is with bands of about 60 and 220 bp (pattern indistinguishable from \( B. \) \text{anthracis} Sterne 34F2).

1 Restriction pattern different from that of \( B. \) \text{anthracis} was observed with bands of about 120 and 160 bp.

NA, not applicable.
conducted for 3 h at a constant voltage of 6 V cm\(^{-1}\) using a conventional chamber for vertical electrophoresis (Mini-Protean II, Bio-Rad). DNA was visualized in the polyacrylamide gels by ethidium bromide staining or silver staining using a SilverStain kit (Kucharczyk, Warsaw, Poland).

**Results and discussion**

Amplification product of about 280 bp was obtained for all the 47 *B. anthracis* strains tested and for 58.2%, 25.0%, 28.6%, 16.7% and 0.0%, respectively, of the *B. thuringiensis*, *B. cereus*, *Bacillus weihenstephanensis*, *Bacillus mycoides* and *Bacillus pseudomycoides* strains tested (Table 1). Strains of species not belonging to the *B. cereus* group did not give any PCR product. To check the template DNA quality of strains, which were *plcR*-PCR negative, a control fragment of the 23S rRNA gene (Radnedge *et al.*, 2003) or the 16S–23S rRNA gene intergenic transcribed spacers (Daffonchio *et al.*, 2003) was amplified as described in the appropriate reference. The template DNA samples from all the *plcR*-PCR-negative strains yielded the control amplicon and were found PCR-inhibitors free.

Among strains that yielded the expected *plcR* fragment, all *B. anthracis* strains gave the same SspI restriction profile (Table 1) with two fragments of about 60 and 220 bp (Fig. 2) that were in agreement with the expected sizes of 60 and...
218 bp. Strains of B. anthracis were tested that were isolated from four different countries, France, Georgia, Poland and Russia, including strains used as vaccine strains (data not shown). Among them, there were strains belonging to the markedly different MLVA genotypes (Gierczyński et al., 2004; Merabishvili et al., 2006). The presence of the plcR nonsense mutation in all the strains confirmed the highly monomorphic genetic nature of the species, and the canonical nature (Keim et al., 2004; Easterday et al., 2005a) of this single nucleotide polymorphism. Of all the other species analyzed, only seven B. thuringiensis strains gave plcR fragments that were digested by SspI. However, the fragments sizes were different from those of B. anthracis strains, being of about 120 and 160 bp (Table 1). DNA sequence analysis of plcR fragments from these strains, by a previously described method (Gierczyński et al., 2004), showed a native SspI restriction site that splits PCR product into fragments of 120 and 158 bp. The evident size differences of the SspI restriction profile between B. anthracis and the aforementioned B. thuringiensis strains confirmed the test specificity for B. anthracis.

Taken together, the results are in agreement with previous findings that the nonsense mutation in the plcR gene is the highly specific B. anthracis marker useful for anthrax agent identification (Radnedge et al., 2003; Easterday et al., 2005a, b). Furthermore, this study shows the usefulness of the plcR-targeted RSI-PCR assay for B. anthracis identification. The assay distinguished from the genuine anthrax isolates, strains B. cereus strains DSMZ 318 and 336, which were recently shown to be among the closest neighbors to B. anthracis described to date (Daffonchio et al., 2006). Bacillus cereus strains AH812, AH817, AH818 and AH831 were also analyzed that were previously associated with periodontitis in humans (Daffonchio et al., 2006) and were shown to be genetically related to B. anthracis on the basis of multi-locus enzyme electrophoresis (Helgason et al., 2000; Daffonchio et al., 2006) and multi locus sequence typing (Helgason et al., 2004). The plcR PCR product was obtained only with strain AH818, but it was not cut by SspI indicating that this strain, even though related to B. anthracis, does not share the same nonsense mutation in plcR. These findings support a hypothesis that the nonsense mutation in the plcR gene plays an important role in anthrax pathogenicity and is strongly conserved by the selective pressure (Mignot et al., 2001).

The RSI-PCR assay can be conducted using agarose or PAGE. The first method was sufficient for screening of multiple samples while the latter method appeared to be more suitable for reference analyses, especially when the gels were silver stained. This method of DNA staining supported effective detection of short DNA fragments. The developed assay was found to be specific, robust and easy to introduce in a routine laboratory equipped with a conventional PCR apparatus. Thus, it may serve as an alternative method for plcR nonsense mutation detection in laboratories lacking expensive equipment, like real-time PCR platforms.

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References


Easterday WR, Van Ert MN, Simson TS, Wagner DM, Kenefic LJ, Allender CJ & Keim P (2005a) Use of single nucleotide...


