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Original Article

Polyphasic characterization of *Bacillus* species from anthrax outbreaks in animals from South Africa and Lesotho

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Abstract

Introduction: *Bacillus anthracis* is the causative agent of anthrax, a disease endemic in regions of Northern Cape Province and Kruger National Park of South Africa. Accurate identification of virulent *B. anthracis* is essential but challenging due to its close relationship with other members of *B. cereus* group. This study characterized *B. anthracis* and *Bacillus* species that were recovered from animals and the environment where animals died of anthrax symptoms in southern Africa using a polyphasic approach.

Methodology: For this purpose, 3 *B. anthracis* and 10 *Bacillus* isolates were subjected to microbiology tests, BiologOmniLog identification system (Biolog), 16S ribosomal RNA (rRNA) sequence analysis, polymerase chain reaction (PCR) detection of protective antigen (*pag)* and capsule (*cap*) regions, and real-time PCR using hybridization probes targeting chromosomal, *pag*, and *cap*C genes.

Results: The *Bacillus* isolates were non-hemolytic, non-motile, and susceptible to penicillin, which is typical of *B. anthracis*, but resistant to gamma phage, unlike typical *B. anthracis*. The Biolog system and 16S rRNA gene sequence analysis identified most of the *Bacillus* isolates as *B. endophyticus* (7 of 10). Conventional PCR revealed that most of the *Bacillus* isolates contained *cap*BCA gene regions. This highlights the limitation of the specificity of conventional PCR and the fact that the real-time PCR is more specific and reliable for anthrax diagnosis.

Conclusions: Real-time PCR, 16S rRNA sequencing, and confirmatory microbiology tests including phage resistance distinguished *Bacillus* isolates from *B. anthracis* in this study. Identification of *B. anthracis* should be done using a polyphasic approach.

Key words: *Bacillus* species; *Bacillus anthracis*; anthrax; bacteriology; molecular techniques.

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Introduction

Bacillus cereus sensu lato group comprises six members, namely *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis*. The *B. cereus* group consists of three pathogenic species, namely *B. anthracis*, *B. cereus*, and *B. thuringiensis*, which share highly conserved chromosomes but differ in pathogenicity, which is mostly plasmid encoded. *Bacillus cereus* is a foodborne pathogen due to the production of an emetic toxin, enterotoxins, and degradative enzymes [1]; *B. thuringiensis* is widely used in agriculture as an insect pathogen with plasmid-encoded insecticidal crystal proteins [2], while *B. anthracis* is a pathogen due to the presence of toxin genes. Homologous recombination and horizontal transfer of genetic material within the *B. cereus sensu lato* group, including phage transmission, has been reported [3,4].

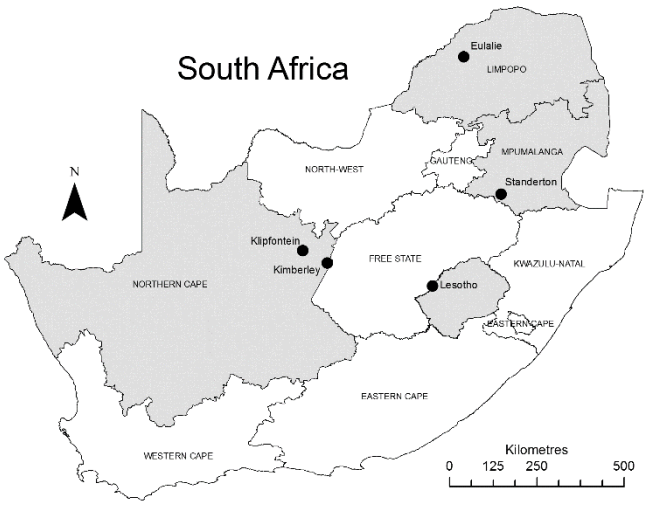
Bacillus anthracis is the causative agent of anthrax that primarily affects herbivorous animals. The plasmid-encoding toxin and capsule proteins are encoded by pXO1 and pXO2, which have been found in other members of *B. cereus sensu lato* and are not only restricted to *B. anthracis* as previously thought [5,6]. The pXO1 plasmid (182 kb) encodes a tripartite protein exotoxin complex and plasmid pXO2 (95 kb) encodes the polypeptide capsule genes. The toxin genes on pXO1 consist of the protective antigen (*pag*), lethal factor (*lef*), and edema factor (*cya*) [7]. The capsule encoded by pXO2 consists of a five-gene operon (*cap*BCADE) that synthesizes the poly-γ-D-glutamic acid capsule of *Bacillus* species, which enable host

immune system evasion by protecting the vegetative cells from being phagocytosed by macrophages [8].

It is paramount to provide rapid and accurate diagnosis of *B. anthracis* to curb the spread of this zoonotic pathogen. For this purpose, *B. anthracis* can be distinguished from closely related *B. cereus* members based on criteria that are recommended by the World Health Organization (WHO) and Centers for Disease Control (CDC). Based on these criteria, *B. anthracis* are non-motile, non-hemolytic, and they are sensitive to penicillin and gamma phage. Nevertheless, it is imperative to make use of DNA-based methods for consistent, accurate diagnosis of anthrax due to the challenge associated with inconsistent attributes of some isolates that resemble *B. anthracis*. The use of 16S ribosomal RNA sequence analysis for identification of *B. anthracis* revealed that the genes are homologous to the *B. cereus* group, and the group could be considered as a single taxon [9,10]. Confirmation of *B. anthracis* virulence genes and specific chromosomal regions can be done by polymerase chain reaction (PCR). However, PCR presents challenges for the discrimination of *B. anthracis* from closely related bacteria with similar capsule genes and *B. anthracis* virulence gene(s) [6,11].

The Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) in South Africa performs classical microbiological tests and PCR targeting pXO1 and pXO2 genes for diagnosis of suspected anthrax cases or outbreaks. Numerous samples from carcasses and the environment of animals showing symptoms that resembled anthrax were received at ARC-OVI for anthrax diagnosis (Table 1, Figure 1). In this study, the accuracy of diagnostic methods, including various microbiological assays, conventional PCR, and real-time PCR to identify *B. anthracis* were investigated using *B. anthracis* and *Bacillus* species isolated from suspected anthrax cases in South Africa and Lesotho.

Figure 1. South African map indicating the locations of *Bacillus anthracis* and *Bacillus* species used in this study.



Methodology

Bacillus species isolates

Soil and tissue samples were obtained during an anthrax outbreak in 2008–2009 at Klipfontein and Kimberly in the Northern Cape Province (NCP), as well as from cases in Limpopo and Mpumalanga provinces and Maseru in Lesotho (Figure 1, Table 1). The animals showed clinical symptoms such as sudden death and bleeding from natural orifices that resembled anthrax. The samples were processed at the ARC-OVI and identified. All isolates from the suspected anthrax cases were freeze-dried and stored in the bacteriology culture collection at ARC-OVI, South Africa (Table 1). *Bacillus anthracis* Sterne strain, *B. thuringiensis* (isolated from soil by ARC-OVI) and *B. cereus*

Table 1. *Bacillus* isolates from South Africa and Lesotho that where isolated from animals with anthrax clinical symptoms.

Cases	Strain number	Animal species	Isolate source	Date	Location[1]	*Bacillus* species
1	3617_3C	Kudu 1	Soil	May 2009	Klipfontein, NCP	*Bacillus* spp.
1	3617_2C	Kudu 1	Ear	May 2009	Klipfontein, NCP	*Bacillus* spp.
2	3618_C	Kudu 2	Ear	May 2009	Kimberly, NCP	*Bacillus* spp.
2	3618_2D	Kudu 2	Soil	May 2009	Klipfontein, NCP	*B. anthracis*
3	3566_3D	Kudu 3	Rib bone	Jan 2009	Klipfontein, NCP	*Bacillus* spp.
3	3566_1B	Kudu 3	Ear	Jan 2009	Klipfontein, NCP	*Bacillus* spp.
4	3631_6C	Kudu 4	Bone	May 2009	Kimberly, NCP	*Bacillus* spp.
4	3631_9D	Sheep 1	Ear	May 2009	Kimberly, NCP	*Bacillus* spp.
4	3631_10C	Sheep 2	Ear	May 2009	Kimberly, NCP	*Bacillus* spp.
4	3631_1C	Kudu 5	Ear	May 2009	Klipfontein, NCP	*B. anthracis*
5	8334	Giraffe 1	Soil	1995	Maseru, Lesotho	*Bacillus* spp.
6	7424	Buffalo 1	Lung	Nov 2011	Eulalie, Limpopo	*Bacillus* spp.
7	20SD	Sheep 3	Ear	2001	Standerton, MP	*B. anthracis*

[1] All isolates from South Africa except 8334; NCP: Northern Cape Province; MP: Mpumalanga Province.

(isolated by the bacteriology laboratory of Department of Veterinary Tropical Diseases, University of Pretoria) were included as controls in this study.

Microbiology tests

Bacillus isolates were grown on 5% sheep blood agar (Onderstepoort Biological Products, Pretoria, South Africa) and incubated at 37°C for 24 hours to determine hemolytic activity. The isolates were subjected to Gram stain, motility tests in thioglycollate, gamma phage, and penicillin sensitivity according to the Office International des Epizooties (OIE) [12]. Capsule visualization of the *Bacillus* isolates was conducted by transferring purified colonies on trypticase soy agar (Selecta-MEDIA, Johannesburg, South Africa) containing 0.8% sodium bicarbonate and incubating the colonies at 5% CO_2 at 37°C for 24 hours. The capsules were stained using India ink and visualized by light microscopy. Biochemical tests such as indole, citrate, oxidase, urease, and catalase were conducted using standard protocols as described by CDC/ASM/APHL [13]. *Bacillus anthracis* Sterne strain was used as a positive control and *B. cereus* and *B. thuringiensis* strains were included as negative controls.

API assay

The *Bacillus* isolates were subjected to a set of carbohydrate fermentation tests based on the API 50 CHB system. The tests were undertaken according to the manufacturer's instructions (BioMerieux, Marcy l'Etoile, France) and results were interpreted using the Analytical Profile Index (API) database (Apiweb software version 1.2.1; BioMerieux, Marcy l'Etoile, France).

Table 2. Summary of the primer and probe sequences, gene targets, and expected PCR product sizes[1].

Primer or probe	Sequence (5'-3')	Target gene	PCR fragment size (bp)
PCR			
Pag1	CAAGTTGTACTGGACCGATTC	*pag*A	732
Pag4	TTGTA ATTGGAGCCGTCCC		
PA8	GAGGTAGAAGGATATACGGT	*pag*A	996
BAPA-R	CCGGTTTAGTCGTTTCTAATGGAT		
1234	CTGAGCCATTAATCGATATG	*cap*B/C	846
1301	TCCCACTTACGTAATCTGAG		
Cya2	GGATAAATCTCTAGATCCAGAG	*cya*	598
Cya3	TCCTTTCTCGACAGCTAATTG		
57	ACTCGTTTTTAATCAGCCCG	*cap*C	244
58	GGTAACCCTTGTCTTTGAAT		
MO1	GCTGATCTTGACTATGTGGGTG	*cap*A	287
MO2	GGCTTCCTGTCTAGGACTCGG		
Let4	TACAGGTGGATAGTAGTAATCC	*let*	647
Let5	CCCTAATGCTTTATTCCATTCC		
Melt-MAMA PCR			
PlcR derived	CGGGGCGGGGCGGGGCGGGCTTATTTGCATGACAAAGCGCATA	*plcR*	70–90 bp
PlcR ancestral	TTTGCATGACAAAGCGCCTC		
Reverse	AAAGCATTATACTTGGACAATCAATACG		
Real-time PCR			
BAPA-S	CGGATCAAGTATATGGGAATATAGCAA	*pag*	204
BAPA-R	CCGGTTTAGTCGTTTCTAATGGAT		
BAPA-FL(probe)	TGCGGTAACACTTCACTCCAGTTCGA-X		
BAPA-LC (probe)	CCTGTATCCACCCTCACTCTTCCATTTTC-P		
CapS	ACGTATGGTGTTTCAAGATTCATG	*cap*C	291
CapA	GATTGCAAATGTTGCACCACTTA		
CapC-FL (probe)	TATTGTTATCCTGTTATGCCATTTGAGATTTTT-X		
CapC-LC (probe)	AATTCCGTGGTATTGGAGTTATTGTTCC-P		
ANT-F	GCTAGTTATGGTACAGAGTTTGCGAC	*sasp*	102
ANT-Amt	CCATAACTGACATTTGTGCTTTGAAT		
ANT-FL (probe)	CAAGCAAACGCACAATCAGAAGCTAAG-X		
ANT-LC (probe)	GCGCAGCTTCTGGTGCTAGC-P		

[1] Primer sequences are based on Birdsell *et al*., [14]; Ramisse *et al*. [17]; OIE [12]; WHO [15]; Makino *et al*. [32]; Beyer *et al*. [33]; Patra *et al*. [34] and S. Klee (personal communication), where *cap* refers to capsule genes, *pag* refers to protective antigens, and SASP refer to small acid-soluble protein. Melt-MAMA refers to melt analysis of mismatch amplification mutation assays; [2] Indicates probe sequences; [3] Estimated between 70–90 bp, size depends on derived or ancestral PlcR region.

BiologOmniLog identification

Suspensions of *Bacillus* isolates were prepared according to the manufacturer's instructions (OmniLog ID System User Guide, Biolog, Hayward, USA). Briefly, the bacterial suspensions were inoculated into wells of microplates containing 95 different carbon sources. The plates were incubated in the OmniLog incubator at 30°C for 4 to 24 hours, depending on the growth requirements of the organisms. The microplates were evaluated using the Biolog's microbial identification system software (OmniLog Data Collection), which contains biochemical fingerprints of different species (Biolog, Hayward, USA).

Bacterial DNA extraction

All isolates (Table 1) were inoculated in 2 mL nutrient broth (Selecta-MEDIA, Johannesburg, South Africa) and incubated overnight at 37°C. The bacterial cells were then harvested by centrifugation at 7,500 rpm for 10 minutes. Genomic DNA was extracted from the harvested cells using the DNAeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was quantified using the Qubit fluorometric method (Life technologies, Carlsbad, Califolnia, USA) according to the manufacturer's instructions. In order to check for integrity, the DNA was electrophoresed in 0.8% ethidium bromide-stained agarose gel and visualized under UV light.

Conventional PCR

Different primer sets (Table 2) were used to amplify the pXO1, pXO2, and chromosomal gene regions. Primers *pag*1, *pag*4, *let*4, *let*5, *cya*2, and *cya*3 (Table 2), which target *B. anthracis*-specific virulence genes on pXO1, were obtained from S. Klee, Robert Koch Institute, Germany. The 25 µL PCR reaction contained 2x Dream Taq Green PCR Master Mix (Fermentas, Vilnius, Lithuania), 0.2 µM of each primer, and 4 ng target DNA. The PCR conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for '30 seconds, annealing for 30 seconds and extension at 72°C for 30 seconds, with a final extension for 5 minutes at 72°C. The PCR annealing temperature was 60°C for primer pairs *pag*1/4, *pag*8/BAPA-R, *let*4/5, *cya*2/3, 55°C for *cap*B/C, 58°C for *cap*C, and 65°C for *cap*A (Table 2). *Bacillus anthracis* Sterne strain was used as a positive control.

Agarose melt-mismatch amplification mutation assay (melt-MAMA) assay of the PlcR marker and the derived and ancestral controls were amplified as described by Birdsell *et al*. [14]. The 10 µL PCR reaction contained 1x MyTaq PCR Master Mix (Bioline, Taunton, USA), 2 mM $MgCl_2$, 0.2 µM of each primer (Table 2), and 2 ng target DNA. The PCR conditions consisted of initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The derived and ancestral controls were amplified using the same PCR reaction and conditions as described above, except that an annealing temperature of 58°C was used. Derived and ancestral PCR amplicons were used as control templates at 1/100,000 dilution. *Bacillus anthracis* Sterne, Ames, and Vollum strains were used as positive controls, and *B. cereus* and *B. thuringiensis* strains were included as negative controls. The PCR amplicons were electrophoresed at 100 V for 90 minutes on a 3% ethidium bromide-stained agarose gel, and then visualized under UV light.

Real-time PCR

Real-time PCR was conducted for the detection of *pag* and *cap*C regions on both virulence plasmids and a specific *B. anthracis* chromosomal target (small acid soluble protein, SASP) [15] using the LightCycler Nano instrument (Roche Applied Science, Mannheim, Germany). The 20 µL PCR mixture consisted of 4 mM $MgCl_2$, 1/10 volume of FastStart Master Mix (Roche Applied Science, Mannheim, Germany), 0.5 µM of each primer, 0.2 µM of each probe, and 5 ng DNA (Table 2). *Bacillus anthracis* Sterne, Ames, and Vollum DNA were used as positive controls and *B. cereus* and *B. thuringiensis* strains were included as negative controls. The LightCycler experimental protocol was used as indicated by OIE experimental protocol [12], but the cutoff was after 35 cycles. The LightCycler Nano software was used to analyze the amplification of the genes. The PCR products were run on 2% agarose gels in order to confirm the amplicon sizes.

16S rRNA PCR

The 16S rRNA genes were amplified using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') [16]. The 25 µL reaction contained 1x Dream Taq Green PCR Master Mix (Fermentas, Vilnius, Lithuania), 0.2 mM of each primer and 50 ng of template DNA. The PCR conditions used to amplify the 16S rRNA gene consisted of an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and

extension at 72°C for 1 minute, with a final elongation at 72°C for 7 minutes. The PCR products were electrophoresed in 1.5% ethidium bromide-stained agarose gels, followed by visualization under UV light.

Sequencing

The 16S rRNA amplicons were purified using a High Pure PCR product purification kit (Roche, Basel, Switzerland) and sequenced in both directions at Inqaba Biotechnologies (Pretoria, South Africa). All the sequences were inspected and corrected using the CLC-Bio 6.0 Workbench software. In order to obtain preliminary identifications of the *Bacillus* spp. isolates, the basic local alignment search tool for nucleotide sequences (BLASTN) was used to compare the sequences to those in the database of nucleotides in the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). The preliminary identifications were refined through phylogenetic analyses. For this purpose, the 16S rRNA sequence alignments were generated with MEGA version 5.05 software using ClustalW multiple sequence alignment. The alignments also incorporated nucleotide sequences of other relevant *Bacillus* species that were obtained from GenBank. The 16S rRNA phylogenetic relationships based on maximum likelihood were inferred with MEGA version 5.05 software. Non-parametric bootstrap analysis was used to estimate branch support, and it was based on 1,000 replicates.

The *cap*C region sequences were obtained from draft whole genome sequences of *B. anthracis* 20SD, *B. endophyticus* 3618_1C and 3631_9D (unpublished data), and the whole genome sequence of *B. anthracis* Ames (GenBank accession number AE017335). The *cap*C sequence alignment was generated with CLC Genomic workbench software using classical sequence alignment analysis.

Results

Phenotypic identification of the Bacillus species

All *Bacillus* isolates formed white, non-hemolytic colonies on blood agar except for *Bacillus* isolate 8334, which was white-grey in color. These colonies appeared circular, rough, and dry. The colonies of *B. anthracis* isolates (20SD, 3631_1C, and 3618_2D) were distinct from other *Bacillus* isolates as they were characterized by a "medusa head", which appeared as curl-like projections. The colonies of *Bacillus* isolates were typically Gram-positive rods showing slightly variable thickness, non-motile, sensitive to penicillin like classic *B. anthracis*, but in contrast to the classic anthrax bacteria, the *Bacillus* bacteria were not lysed by gamma phage. All isolates, including *B. cereus* and *B. thuringiensis* controls, were catalase positive, oxidase

Figure 2. Micrographs of Gram stains showing morphological characteristics of *Bacillus anthracis* and *Bacillus endophyticus* isolates. Morphology of (A) *B. anthracis* Sterne, and (B) *B. anthracis* 3631_1C compared to (C) *B. endophyticus* 3618_1C, and (D) *B. endophyticus* strains 3617_2C.

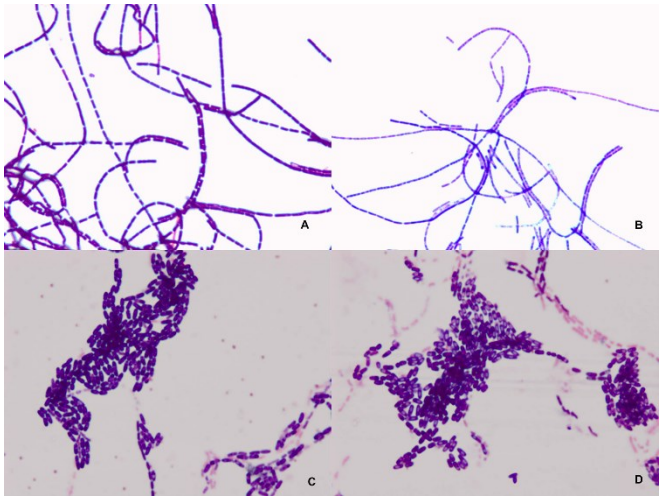


Table 3. Identification of the *Bacillus* isolates by means of API 50 CHB biochemical test, BiologOmniLog system, and 16S ribosomal RNA sequencing.

	API 50 CHB		BiologOmnilog	16S ribosomal RNA
Isolate ID	**Taxon assigned to**	**Similarity[1] (%)**		
3618_1C	*Bacillus megaterium*	58.3	*Bacillus endophyticus*	*Bacillus endophyticus*
3631_9D	*Bacillus subtilis/amyloliquefaciens*	98.3	*Bacillus pumilus*	*Bacillus endophyticus*
8334	*Bacillus circulans*	69.0	No identification	*Bacillus thuringiensis*
3631_6C	Could not identify		*Bacillus endophyticus*	*Bacillus endophyticus*
3631_10C	*Bacillus subtilis/amyloliquefaciens*	84.9	*Bacillus endophyticus*	*Bacillus endophyticus*
3566_1B	*Brevibacillus* non-reactive	73.7	*Bacillus endophyticus*	*Bacillus endophyticus*
3566_3D	*Geobacillus thermoglucosidasius*	70.5	*Bacillus endophyticus*	*Brevibacterium frigoritolerans*
3617_3C	*Bacillus subtilis/amyloliquefaciens*	92.8	*Bacillus endophyticus*	*Bacillus endophyticus*
3617_2C	*Bacillus pumilus*	95.8	*Bacillus endophyticus*	*Bacillus endophyticus*
7424	*Bacillus megaterium*	58.4	*Bacillus amyloliquefaciens*	*Bacillus endophyticus*

positive, and indole negative (Supplementary Table 1S). Most *Bacillus* isolates (8334, 3631_6C, 3631_10C, 3566_1B, 93566_3D, 3617_2C, 3618_1C, and 7424) utilized citrate as a sole source of carbon except for isolates 3631_9D, 3617_3C, *B. anthracis* Sterne, and reported *B. anthracis* isolates (20SD, 3631_1C, and 3618_2D) in the study (Supplementary Table 1S). The isolates hydrolyzed urea into ammonia and carbon dioxide except for 8334, *B. anthracis* 3618_2D, 3631_1C, and *B. anthracis* Sterne isolates.

The microscopic appearances of *Bacillus* isolates from this study are summarized in Figure 2. The *B. anthracis* Sterne strain (Figure 2A) and other *B. anthracis* isolates (20SD, 3618_2D, and 3631_1C (Figure 2B) had long square (box)-ended rods in long chains. *Bacillus* isolate 3618_1C (Figure 2C) and 3617_2C (Figure 2D) had small broad rods in short chains that clustered together.

The results of the API 50 CH and BiologOmniLog identification system are summarized in Table 3. Identification at a species level is assigned as an acceptable profile with a percentage greater than 75%. The BiologOmniLog system assigned most of the *Bacillus* isolates to *B. endophyticus*, except isolates 3631_9D and 7424, which were identified as *B. pumilus* and *B. amyloliquefaciens*, respectively. The *Bacillus* isolate 8334 from Lesotho could not be identified using the BiologOmnilog system.

Conventional PCR and sequencing analysis

The PCR assays confirmed the presence or absence of the pXO1 and pXO2 virulence genes. The *Bacillus* species isolates did not contain the *pag*A, *let*, and *cya* regions on the pXO1 plasmid, while *B. anthracis* isolates (3618_2D, 3631_1C and 20SD) amplified these regions (Table 4). However, non-specific binding band was observed on isolate 3631_9D on the *pag*A marker using primer pair pag1/4 (data not shown). The *cap*C gene was present in the *Bacillus* isolates (8334, 3618_2D, 36319D, 3631_6C, 3631_10C, 3566_1B, 3566_3D, 3617_3C, 3617_2C, 3618_1C, and 7424) and *B. anthracis* (20SD and 3618_2D), but not in *B. anthracis* isolates 3631_1C and Sterne (Table 4). The *Bacillus* isolates produced varying results with the different regions of the capsule genes, namely *cap*A, *cap*B/C, and *capC* (Table 4). *Bacillus anthracis* isolates 3618_2D and 20SD amplified all the capsule regions (*cap*A, *cap*B/C and *capC*), but *B. anthracis* Sterne and 3631_1C lacked these regions (Table 4).

The PlcR melt-MAMA marker did not amplify the *Bacillus* isolates (7424, 3618_1C, 8334, 3631_9D, 3566_3D, 3566_1B, 3631_6C, 3617_2C, 3617_3C) as well as *B. cereus* and *B. thuringiensis* (Supplementary Figure 1S). The PlcR-positive *B. anthracis* control strains (Vollum, Ames, and Sterne) amplified the same PCR size product as the positive derived control as indicated by Birdsell *et al*. [14].

Real-time PCR

The real-time PCR assay was used for the detection of regions specific to *B. anthracis* in *pag* (pXO1), *cap*C (pXO2), and chromosomal SASP regions. None of the *B. anthracis*-specific probes hybridized with the *Bacillus* isolates. The probes hybridized with the *B. anthracis* strains (Sterne, 3618_2D, 3631_1C, and

Table 4. Summary of the PCR results of *Bacillus anthracis* and *Bacillus* isolates for pXO1, pXO2 plasmids, and/or chromosomal genes[1].

	Conventional PCR							Real-time PCR		
Isolates	***pagA*[2]**	***cap*B/C**	***capA***	***capC***	***let***	***cya***	***PlcR***	***pag***	***capC***	***SASP*[3]**
Sterne[4]	+	-	-	-	+	+	+	+	-	+
3618_2D[4]	+	+	+	+	+	+	+	+	+	+
20SD[4]	+	+	+	+	+	+	+	+	+	+
3631_1C[4]	+	-	-	-	+	+	+	+	-	+
3618_1C	-	+	+	+	-	-	-	-	-	-
3631_9D	-	-	+	+	-	-	-	-	-	-
8334	-	-	+	+	-	-	-	-	-	-
3631_6C	-	+	-	+	-	-	-	-	-	-
3631_10C	-	+	+	+	-	-	-	-	-	-
3566_1B	-	-	-	+	-	-	-	-	-	-
3566_3D	-	-	+	+	-	-	-	-	-	-
3617_3C	-	-	-	+	-	-	-	-	-	-
3617_2C	-	-	-	+	-	-	-	-	-	-
7424	-	-	-	+	-	-	-	-	-	-

[1] See Table 2 for primers used to amplify the target regions and probes for hybridization, (+) indicates amplification of the gene and (-) indicates no amplification; [2] Amplification with primer pairs *pag*1 and *pag*4 as well as PA8 and BAPA-R; [3] SASP: small acid-soluble proteins in *B. anthracis* chromosome; [4] Identified as *B. anthracis* based on conventional microbiology methods.

20SD), but the *cap*C probe did not hybridize with Sterne and 3631_1C (results not shown).

Sequencing and phylogenetic analysis

The *B. anthracis* Sterne and *B. anthracis* isolates (20SD, 3631_1C, 3618_2D) that were confirmed with conventional microbiology tests had identical 16S rRNA sequences and clustered with the *B. anthracis* Sterne strain (Figure 3). The 16S rRNA sequence of isolate 8334 from Lesotho clustered with the *B. cereus* group and was closely related to *B. thuringiensis* IAM 12077 in this cluster. The 16S rRNA sequence of isolate 3566_3D was closely related to *Brevibacterium frigoritolerans* and all the other *Bacillus* isolates clustered with *B. endophyticus* (n = 8) (Figure 3).

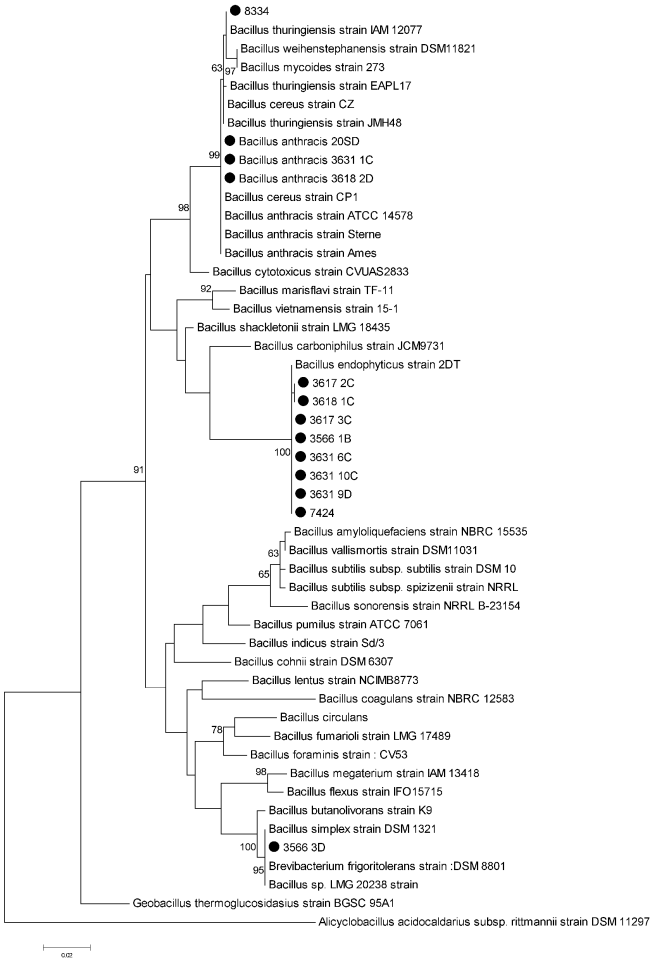
The *cap*C region of *B. anthracis* (Ames and 20SD) and possible *B. endophyticus* sequences (3618_1C and 3631_9D) were aligned and showed various nucleotide substitutions with no insertion or deletion (Supplementary Figure 2S). The primers 57 and 58 designed to amplify the *B. anthracis cap*C region [17] are present in both *B. anthracis* and *B. endophyticus* and have five to seven nucleotide differences in the primer sequences (Supplementary Figure 2S).

Discussion

In this study, *B. anthracis* and *Bacillus* isolates that were isolated from diagnostic samples from suspected anthrax cases send to ARC-OVI were identified using a polyphasic approach to investigate the accuracy of these diagnostic methods. These isolates were obtained either from animals that showed symptoms reminiscent of anthrax or from soil that was beneath animals that died of anthrax symptoms. We therefore investigated the isolates using a combination of classical microbiological techniques, including API CH50B, BiologOmniLog, conventional and real-time PCR targeting various virulence genes, and 16S rRNA gene sequencing. We determined that *B. anthracis* can be differentiated from other similar *Bacillus* species using a combination of classical microbiological techniques, real-time PCR, and sequencing. The Omnilog and 16 S rRNA sequencing identified most of the *Bacillus* isolates as *B. endophyticus.* The polyphasic approach indicated that these possible *B. endophyticus* isolates had similar test results with *B. anthracis* when using selected microbiology and molecular methods. Conventional PCR revealed the presence of the capsular or polyglutamate genes in *Bacillus* isolates using *B. anthracis* primers (Table 4, Supplementary Figure 2S). This study highlights the need to determine the significance of ruling out *B. endophyticus* isolates from *B. anthracis* anthrax outbreaks for diagnostic purposes. However, it is important to note that this study did not show evidence that the *Bacillus* spp. were the causative agents of anthrax, as microbiological and molecular tests results indicated that these isolates did not contain toxin genes of anthrax.

The classical microbiological tests indicated that *Bacillus* isolates from this study differed from the classical *B. anthracis*, as they were resistant to gamma phage [12,18]. In this study, 20SD and 3631_1C were *B. anthracis*, and they were sensitive to both penicillin and gamma phage. Currently, classical *B. anthracis* diagnosis is based on the observation of capsule, non-motile, non-hemolytic isolates that are sensitive to penicillin and gamma phage [18]. However, the resistance to gamma phage was observed in 15% of *B.*

Figure 3. Maximum likelihood (ML) phylogeny for the southern African *Bacillus anthracis* and *Bacillus* species isolates based on 16S rRNA sequences with other *Bacillus* group obtained from GenBank. Bootstrap values > 60% are indicated at the internodes. *Geobacillus thermoglucosidasius* and *Alicyclobacillus acidocaldarius* were used as outgroups to root the tree. Scale bar indicates nucleotide substitutions per site.



anthracis in previous studies [19], and in South Africa, a survey indicated that up to 16% of *B. anthracis* isolates from soil and carcasses were resistant to gamma phage [20]. Although it is a known fact that a single diagnostic attribute may not necessarily compromise the correct identification of classical *B. anthracis* [5,21], the combination of various tools used in study has shown that the non-conformance of one diagnostic trait may exclude *B. anthracis*. All the other identifying methods (API CH50B, BiologOmnilog, and 16SrRNA sequencing) identified isolates as *B. endophyticus*, *B. thuringiensis*, and *Brevibacterium frigotoleransis* despite the fact that resistance to gamma phage was the only differential microbiological criteria that excluded these isolates from being *B. anthracis*.

The API 50 CHB identification system for *Bacillus* species results lacked correlation with 16S rRNA sequences and BiologOmnilog. The latter techniques identified isolates 3617_2C, 3618_1C, 2617 3C, 3566_1B, 3631_10C, and 3631_6C (n = 6) as *B. endophyticus* (Table 3, Figure 3). Indeed, the API 50 CHB system was shown to be inaccurate for diagnosis of *B. anthracis* and *Bacillus* isolates in this study. This was further evidenced by the low probabilities of identities obtained (Table 3) and the inability for identification of *Bacillus* isolates in the study.

Bacillus cereus strains were reported from chimpanzees that died of anthrax symptoms in Tai National Park, Cote d'Ivoire (CI) and a gorilla that died in Cameroon (CA) as a result of *B. cereus* biovar *anthracis* [5,6]. The *B. anthracis* virulence genes pXO1 and pXO2 were present in the *B. cereus* biovar *anthracis* CI and CA strains [5,6]. The plasmids pXO1 and/or pXO2 have also been reported in other *B. cereus* strains [11,22]. Hoffmaster *et al*. [23] also identified anthrax toxin genes in *B. cereus* G9241 that are capable of causing a severe inhalation anthrax-like disease. The genome sequence of *B. cereus* G9241 revealed the plasmid pBXO1, which is 99.6% similar to the pXO1 plasmid. The plasmid pXO1 genes of *B. anthracis* have been found to be significantly similar to the chromosomal encoded genes from the other members of the *B. cereus* group, *B. subtilis*, *B. hakodurans*, *Listeria* spp., and *Staphylococcus* spp. [24]. The *Bacillus* isolates did not amplify the lethal, edema, and protective antigen regions, and therefore these bacteria did not contain the pXO1 plasmid. The capsule region amplification of *Bacillus* species in this study indicated the consistent presence of *cap*C region. Whole genome sequencing would determine the presence of *B. anthracis* plasmid(s) in the *Bacillus* species as PCR only indicated the presence of similar capsule-like regions.

The *Bacillus* isolates tested positive for the three genes (*cap*A, *cap*B/C, and *cap*C) using conventional PCR. This suggests the presence of either poly-glutamic acid or polyglutamate (PGA) or capsular genes (Table 4). The sequence alignment of the capsular gene *cap*C shows that the possible *B. endophyticus* isolates 3631_9D and 3618_1C have single nucleotide variations with no insertions or deletions with *B. anthracis* Ames and 20SD in the *cap*C region (Supplementary Figure 2S). This indicates the presence of the *pgs*/*cap*C gene in these possible *B. endophyticus* strains. The capsular genes *cap*BCADE play a role in the synthesis of the PGA capsule of *B. anthracis*, and these are located on the pXO2 plasmid. PGA confers the ability to evade the host immune system by protecting the vegetative cells from phagocytotic killing by macrophages [25,26]. However, PGA is synthesized by many *Bacillus* species [27] and other organisms [25]. Three *B. anthracis* genes, *cap*B, *cap*C, and *cap*A, were shown to be necessary to drive the production of PGA weakly in *Escherichia coli* but could not promote PGA synthesis in *B. subtilis* [5]. Candela *et al*. [25] investigated the five *cap* genes and found *cap*A, *cap*B, *cap*C, and *cap*E genes all necessary for sufficient PGA synthesis by *B. anthracis*. The *cap*D gene encodes a ϒ-glutamyl transpeptidase or PGA depolymerase, which is important for the covalent anchoring of the PGA to the peptidoglycan in *B. anthracis* [26]. Therefore, isolates that lack *cap*D gene produce a loose slime layer of the PGA instead of a covalently linked capsule [25]. *Bacillus thuringiensis*, *B. subtilis* 168 IFO3336, *B. licheniformis* ATCC 14580, and *Staphylococcus epidermidis* ATCC 12228 also contain the *cap*B, *cap*C, *cap*A, and *cap*E genes similar to *B. anthracis*. Annotations of these genes are named differently among the species, but they synthesize the PGA [25]. In *B. subtilis* and *B. licheniformis*, they are named pgs (polyglutamate synthase) when PGA is released, which might help the bacterium to survive in high salt concentrations or confer resistance to adverse environment [26]. In a study characterizing the *cap*B, *cap*C, *cap*A, and *cap*D of *B. megaterium* and other *Bacillus* species that produced PGA, the *B. anthracis*-specific primers could not amplify capsule regions from these isolates using PCR [27]. These authors indicated that PGA-producing *Bacillus* species contain divergent capsule regions that cannot all be amplified using *B. anthracis* PCR-specific primers [27]. Therefore, the *Bacillus* isolates from South Africa and Lesotho contained polyglutamate genes (Table 4), which may

have contributed either to virulence or environmental survival.

The members of the *B. cereus* group are under the control of PlcR and its regulatory peptide PapR, which transcribes genes encoding collagenases, hemolysins, phospholipases, and enterotoxins [28]. The PlcR gene product of *B. cereus* and *B. thuringiensis* is known to upregulate the production of numerous extracellular enzymes, while *B. anthracis* has a nonsense mutation that is responsible for non-motile and non-hemolytic phenotype [28]. The PlcR melt-MAMA marker can be used as a specific marker for *B. anthracis*, because it differentiated *B. anthracis* strains from *Bacillus* isolates (Supplementary Figure 1S).

The SASPs found in *B. anthracis* spores were used as *B. anthracis*-specific protein markers [29]. This marker has been found to be insufficient to discriminate amongst closely related organisms [30]; however, real-time PCR consists of confirmation of three targets, namely the SASP, and plasmid targets, which improves the specificity of the test. In this study, real-time PCR, 16S rRNA sequencing, and conventional microbiology tests accurately discriminated *B. anthracis* from closely related *Bacillus* species. However, closely related *Bacillus* species isolates with virulence genes that are similar to those of *B. anthracis* should continuously be investigated, as they might contribute towards anthrax-associated disease in animals. It is therefore paramount not to use only one test for diagnosis or identification of *Bacillus* species, as this may lead to false-positive results. Microbiological tests should be coupled with molecular tests for accurate diagnosis of anthrax (this study; [5,21]). The significant role of *B. endophyticus* in anthrax outbreaks is not clearly understood; however, for every anthrax case in the NCP, these species were isolated together with *B. anthracis*, which needs to be investigated further, as *B. endophyticus* has been isolated from vegetation as plant-endophytic bacteria [31]. Whole genome sequencing will be subsequently used to obtain broader understanding of the genes that are shared between *B. endophyticus* strains and *B. anthracis*. This will further enhance the understanding of the *B. endophyticus* strains isolated in South Africa.

Conclusions

The BiologOmnilog system and 16S rRNA gene sequencing could identify most of the isolates as *B. endophyticus.* These techniques can identify *Bacillus* to species level (therefore differentiate *B. anthracis* from *Bacillus* species). However, the BiologOmnilog system may not be sufficient for diagnosis of *Bacillus* species isolates on its own, and neither of these methods would detect the virulence genes. The presence of virulence genes could be detected using PCR. However, PCR analysis provides limited information about the genetic basis or virulence of isolates, as it relies on previous known genetic sequences. Therefore, whole genome sequencing is needed to resolve the variability within species and sub-species groups that are closely related amongst the *B. cereus*/*subtilis* group. This is important for comparative analysis of the virulence genes that might be associated with anthrax symptoms.

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Authors' contributions

KEL contributed to the laboratory experiments and writing up the manuscript. AH participated in some of the experiments, whereas FC participated in the design of the study and in drafting the manuscript. JM and JR assisted in drafting the manuscript. EM and HvH participated in the design of the study, drafting the manuscript, revising it critically, and providing funding.

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Annex – Supplementary Items

Supplementary Table 1S. Microbiological features with biochemical tests of *Bacillus* isolates after incubation at 37°C for 48 hours.

Isolate	Motility (M)1	Hemolysis (H)[2]	Penicillin[3]	Gamma (γ)-phage[3]	Biochemical tests[4]				
					Citrate	Urease	Indole	Catalase	Oxidase
B. anthracis Sterne	N-M[5]	N-H	S	S	-	-	-	+	+
B. cereus	M	H	R	R	-	W+	-	+	+
B. thuringiensis	M	H	R	R	-	+	-	+	+
B. anthracis 3618_2D	N-M[5]	N-H	S	R	-	-	-	+	+
B. anthracis 3631_1C	N-M[5]	N-H	S	S	-	-	-	+	+
B. anthracis 20SD	N-M[5]	N-H	S	S	-	-	-	+	+
3631_9D	N-M	N-H	S	R	-	W +	-	+	+
8334	N-M[6]	N-H	S	R	+	-	-	+	+
3631_6C	N-M	N-H	S	R	+	W +	-	+	+
3631_10C	N-M	N-H	S	R	+	W +	-	+	+
3566_1B	N-M	N-H	S	R	+	W +	-	+	+
3566_3D	N-M	N-H	S	R	+	+	-	+	+
3617_3C	N-M	N-H	S	R	-	W +	-	+	+
3617_2C	N-M	N-H	S	R	+	W +	-	+	+
3618_1C	N-M	N-H	S	R	+	W +	-	+	+
7424	N-M	N-H	S	R	+	+	-	+	+

[1] M indicates motile; N-M indicates non-motile; [2] H indicates hemolysis; N-H indicates non-hemolysis; [3] S: sensitive; R: resistant; [4] W: weakly, + positive, - negative; [5] Inverted fir-tree; [6] Growth along the step line.

Supplementary Figure 1S. Conventional melt-MAMA PCR products using the pleiotropic transcriptional regulator (PlcR) marker for the *Bacillus* species on agarose. The lanes represent: Marker with 100 bp plus generuler ladder; water showing negative control; *Bacillus anthracis* Vollum, Ames; and Sterne positive control PCR products as according to Birdsell *et al*. [14]. Lanes labelled as *B. thuringiensis* and *B. cereus* consist of negative controls. *Bacillus* isolates (7424, 3618_1C, 8334, 3631_9D, 3566_3D, 3566_1B, 3631_6C, 3617_2C, 3617_3C, 3631_10C) did not amplify the PlcR marker region.

Supplementary Figure 2S. *Bacillus anthracis* 20SD and Ames as well as *B. endophyticus* 3618_1C and 3631_9D *cap*C region alignment with nucleotide differences highlighted in red. Primers 57 and 58 that amplify *cap*C region [17] are indicated by black boxes.