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1 **The effect of prolonged storage on the virulence of isolates of *Bacillus anthracis* obtained**
2 **from environmental and animal sources in the Kars Region of Turkey**

3

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21

22 **Keywords:** *Bacillus anthracis*, storage, virulence, pXO1, pXO2, vaccine

23

24 **Running Title:** Impact of prolonged storage on *B. anthracis*

25

26 **Abstract**

27 The stability of the plasmid mediated virulence factors of *Bacillus anthracis*, a tripartite toxin
28 located on pXO1 and an anti-phagocytic capsule encoded by genes located on pXO2,
29 following long term storage was investigated. A collection of 159 isolates of *B. anthracis*
30 were collected from the Kars region of Turkey between 2000 and 2013 and stored at -20 °C in
31 Brucella broth supplemented with 20% glycerine. 142 isolates were recovered of which one
32 failed to express a capsule upon primary culture. A further 35 isolates yielded a mixture of
33 mucoid and non-mucoid colonies, the majority of which had lost the pXO2 plasmid as
34 determined by PCR analysis. Results would suggest that pXO2 is more unstable than pXO1
35 and that this instability increases with the length of storage. It is possible that the pXO2
36 deficient isolates of *B. anthracis* described here could be developed into a vaccine to treat at
37 risk animals in the Kars region as many animal vaccines are based upon pXO2 deficiency.

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48 **Introduction**

49 The storage and maintenance of well-defined bacterial isolates is one of the cornerstones of
50 microbiology providing reproducibility and confidence in results. Long term storage can be

51 achieved through several different methods (agar slants, lyophilisation etc.) and has be shown
52 to have an impact on properties of the resurrected cultures (Michel & Garcia, 2003). The
53 maintainance of virulence characteristics is of particular importance for those working in
54 infectious disease modelling or vaccine development. The influence of *in vitro* culture
55 conditions and storage conditions on virulence has been demonstrated in bacterial species
56 such as *Flavobacterium columnare* (Zhang *et al.*,2014) and human pathogens such as
57 *Eshcerichia coli* and *Salmonella* (Huang *et al.*, 2014; Yang *et al.*, 2014).

58 *Bacillus anthracis* is a well characterised spore forming bacterium that can establish
59 infections in both animals and humans and was one of the first bacterial species to have an
60 effective attenuated vaccine (Pasteur, 1881; Baillie, 2009). The virulence of *B. anthracis* is
61 mediated by the production of a tripartite toxin and the presence of an antiphagocytic capsule
62 (Green *et al.*, 1985). The genes responsible for these factors are encoded on two seperate
63 plasmids, pXO1 which encodes the toxins and pXO2 which carries the genes for capsule
64 production (Hugh-Jones & Blackburn, 2009).

65 These plasmids play a key role during infection, however, their contribution to survival
66 outside of an infected host is less clear. Isolates lacking pXO2 have been reported in both
67 environmental samples, as has the spontaneous loss of the plasmid during culture suggesting
68 that under certain conditions pXO2 may be lost (Turnbull *et al.*, 1992). The loss of pXO1
69 appears to be more infrequent than the loss of pXO2 (Turnbull *et al.*, 1992; Bowen & Quinn,
70 1999; Pavan & Cairo, 2007) and may account for the routine use of the Sterne strain of *B.*
71 *anthracis* as a live animal vaccine (Turnbull, 1991).

72 This current investigation screened the historic *B. anthracis* culture collection maintained
73 at Kafkas University since 2000 to determine the stability of pXO1 and pXO2 in Turkish
74 isolates. In addition to determing the efficacy of the in house storage conditions on strain

75 survival and plasmid stability we also sought to determine if any of the isolates yielded
76 attenuated variants that could potentially be developed into animal vaccines.

77

78 **Materials and Methods**

79 All culture work and identification of *B. anthracis* was performed in a Class II biosafety
80 cabinet in the Veterinary Faculty of Kafkas University (Kars, Turkey). All consumables and
81 reagents were obtained from either Fisher Scientific (Loughborough, UK) or Sigma Aldrich
82 (Dorset, UK) unless otherwise stated in the text.

83

84 **Bacterial strains and revival**

85 One hundred and fifty nine *B. anthracis* isolates from the historical collection at Kafkas
86 University were examined at part of this study. Isolates were obtained between 2000 and 2013
87 from animal and environmental sources using the same cultivation methods by trained
88 personnel. Prior to storage isolates were confirmed as *B. anthracis* by phenotypic assessment
89 (i.e. colony morphology, sensitivity to penicillin G and gamma phage). Isolates were stored at
90 -20°C in Brucella broth (Oxoid, Basingstoke, UK) supplemented with 20% glycerine.

91 Isolates were revived by the addition of 200 µL defrosted stock to 5 mL Brain Heart
92 infusion broth (BHI; Oxoid, Basingstoke, UK) and then incubated for 48 h at 37°C. After
93 incubation a 0.1 mL sample was spread over the surface of a pre-prepared 7% (v/v) sheep
94 blood agar plate and incubated at 37°C for 18-48 h.

95

96 **Phenotypic assessment**

97 Individual colonies were assessed using standard microbiological criteria i.e. classical *B.*
98 *anthracis* colony morphology (ground-glass appearance, flat, opaque, tenacious and grayish-
99 white), sensitivity to penicillin G (10 Units; Oxoid, Basingstoke, UK) and gamma phage

100 (~10⁹ PFU/mL). Once confirmed as *B. anthracis*, isolates were phenotypically assessed for
101 the presence of the pXO1 (toxin production) and pXO2 (capsule expression) virulence
102 plasmids.

103 The presence of pXO1 was determined using the XO media as described by Hoffmaster &
104 Koehler (1997). In brief, this media contained glucose (0.5% w/v), ferric chloride (40 µg/mL),
105 thiamine hydrochloride (10 µg/mL), glycine (200 µg/mL), L-methionine (40 µg/mL), L-
106 proline (40 µg/mL), L-serine (40 µg/mL), L-threonine (40 µg/mL), (NH₄)₂SO₄ (2 mg/mL),
107 KH₂PO₄ (6 mg/mL), K₂HPO₄ (14 mg/mL), sodium citrate (1 mg/mL), MgSO₄.7H₂O (0.005
108 mg/mL), MnSO₄.H₂O (0.00025 mg/mL), L-glutamic acid (2 mg/mL), and agar (1.5% w/v).
109 Plates were then incubated for 72 h at 37°C and assessed for the presence or absence of
110 growth (Fig. 1a).

111 The presence of pXO2 was determined on the basis of capsule production. In brief,
112 colonies were streaked to Nutrient Agar plates supplemented with 0.7% (w/v) Sodium
113 bicarbonate and 10% (v/v) Horse serum (Oxoid, Basingstoke, UK; Knisely, 1965). Plates
114 were then incubated for 48 h in candle jars at 37°C. At 24 and 48h post incubation, growth on
115 agar plates was assessed for the production of mucoid (capsule producing) and non-mucoid
116 (non-capsule producing) colony types (Fig. 1b). In order to confirm the results obtained using
117 candle jars, capsule production was also assessed by incubation with gradually increasing CO₂
118 concentration (between 5 and 20%) in a static incubator.

119 Capsule production was visually confirmed using the McFadyean stain (Owen *et al.*,
120 2013). In brief, individual colonies were suspended in 10 µL horse serum and smeared over
121 the surface of a microscope slide and allowed to air dry at room temperature. Slides were
122 immersed in absolute ethanol for 10 min and allowed to air dry. A single drop of methylene
123 blue was spread over the slide, left for 1 min and then gently rinsed with clean water and

124 examined under x1000 magnification using a CX21FS1 light microscope (Olympus
125 Corporation, USA) for the presence of capsule (Fig. 1c and d).

126

127 **PCR assessment of pXO1 and pXO2**

128 Individual colonies of interest (non-mucoid and mucoid phenotypes) taken from bicarbonate
129 agar plates were used to inoculate 1.5 mL of BHI broth and incubated overnight at 37°C.
130 Control organisms of *B. anthracis* Sterne (pXO1+, pXO2-), *B. anthracis* K-125 (pXO1+,
131 pXO2+) and *Escherichia coli* OP50 were also assessed. Following incubation, overnight
132 cultures were centrifuged at 14,000 x g for 10 min, the supernatant discarded and the pellet
133 resuspended in 1.5 mL molecular biology grade water (Eppendorf, Germany). Centrifugation
134 was repeated as before and the pellet resuspended in 100 µL DNase-Rnase free water
135 (Invitrogen, UK). Samples were boiled at 100°C for 10 min in a waterbath, cooled on ice and
136 centrifuged at 14, 000 x g for 10 s and stored at -20°C until use.

137 A summary of primers used in the current investigation can be found in Table 1. A 50 µL
138 reaction mixture (25µL Taq PCR Master Mix Kit (Qiagen, UK), 5µL primer, 5µL Template
139 DNA and 15µL dH₂O (supplied as part of the Taq PCR Master Mix Kit)) of each primer was
140 then run using the following PCR conditions;

141 For PA5/8, CAP6/103, CapBF/R, CapCF/R and pXO2-007F/R, initial denaturation step at
142 94°C for 5 min, followed by 30 cycles of 94°C for 40 s, 58°C for 40s and 72°C for 40s with a
143 final elongation step of 72°C for 5 min.

144 For CapAF/R, initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C
145 for 40s, 45°C for 40s and 72°C for 40s with a final elongation step of 72°C for 5 min.

146 Products were analysed on a 1% (w/v) agarose gel stained with SafeView nucleic acid
147 stain (NBS Biologicals, Huntingdon, UK).

148

149 **Statistical analysis**

150 The degree of association between plasmid profiles and storage time was measured using a
151 chi-square statistic (Preacher, 2001).

152

153 **Results**

154 A total of 151 (94.9%) isolates were successfully recovered from storage for which 142
155 (89.3%) were confirmed as *B. anthracis* on the basis of morphology, susceptibility to Gamma
156 phage, penicillin sensitivity and capsule expression.

157 Confirmed *B. anthracis* isolates originated from animal (n=130) and environmental samples
158 (n=12). The remaining 17 isolates (10.7%) were shown to be non-viable (n=8) or non *B.*
159 *anthracis* (n=9).

160 All isolates identified as *B. anthracis* also grew on XO media suggesting that they
161 contained the pXO1 plasmid (Fig. 1a; Table 2).

162 When cultured on bicarbonate agar in the presence of CO₂, only one isolate was non-
163 mucoid upon primary culture while a further 35 isolates yield a mixed phenotype of mucoid/
164 non- mucoid colonies when incubated using a candle jar. Reincubation of these non-mucoid
165 colonies in fixed 5% CO₂ atmosphere failed to restore capsule expression, while 5 of 35
166 mixed phenotypes regained the ability to express a capsule. Non-mucoid colonies from the
167 remaining 30 isolates failed to regain capsule expression suggesting either the complete loss
168 of pXO2 or the inactivation of genes essential for capsule expression.

169 PCR analysis was performed on the non-mucoid colonies obtained from all 36 isolates
170 using primers specific for one target on pXO1 (the pag gene) and 5 targets within the pXO2
171 plasmid, 3 of which are within the capsule synthesis operon. A total of 5 distinct PCR profiles
172 were observed (Fig. 2; Table 3).

173 Profile A (n=20) comprised of pXO1 primer positive, pXO2 primers negative isolates.
174 Repeated culture (n=3) of these non-mucoid isolates in a CO₂ incubator failed to restore
175 capsule expression suggesting that the pXO2 plasmid may have been lost from these isolates.

176 Profile B (n=10) comprised of pXO1 primer positive isolates which gave a positive
177 response with the Cap6/103 primers but failed to produce a product with the remaining pXO2
178 specific primers. Following repeated culture (three passages) only one isolate regained the
179 ability to express a capsule.

180 Pattern C (n=3) comprised isolates which yielded PCR products of the expected size from
181 all primers. All isolates regained the ability to expression a capsule upon repeated culture in
182 the presence of CO₂.

183 Pattern D (n=1) gave the expected size PCR products with the pXO1 specific and
184 Cap6/103, CapB and Cap C PCR primers but did not regain the ability to express a capsule
185 upon repeated culture (three passages).

186 The final profile, E (n=1) failed to produce a PCR product with the pXO1 specific primers
187 but produced expected PCR products with all pXO2 specific primers.

188 The majority (80%) of the strains which produced a mixed capsule morphology were
189 isolated from cattle while the remaining animal isolates came from sheep (20%). The majority
190 (76.6%) of conversions from mucoid to non-mucoid were from strains isolated between 2000
191 and 2003 while the remainder were from 2005 (3.3%), 2007 (3.3%), 2008 (3.3%), 2011
192 (3.3%) and 2013 (10%) (Table 2). No significant difference ($P > 0.05$) was observed between
193 the production of a non-mucoid phenotype and sample type. However, conversion to a non-
194 mucoid phenotype was found to increase significantly with increasing length of storage ($P <$
195 0.05).

196

197 **Discussion**

198 Although it is well established that *B. anthracis* spores can survive and persist in the
199 environment for decades and still maintain the ability to establish an active infection once a
200 suitable host arises (Wilson & Russell, 1964; De Vos, 1990), there are limited studies which
201 investigate the effect of long term storage on viability and virulence. In this current
202 investigation, 142 (89.3%) isolates of *B. anthracis* were recovered from long term storage.
203 This recovery rate compares favourably to that of Marston and colleagues who were only able
204 to recover 53.8% of isolates following long term storage (Marston *et al.*,2005). The
205 preservation method employed in this study, a liquid medium supplemented with glycerin and
206 the storage at -20°C may account for the greater recovery rate.

207 The ability of *B. anthracis* to cause anthrax is primarily attributed to the presence of two
208 virulence plasmids; pXO1 which carries the genes which encode the tripartite anthrax toxin
209 (*pagA*, *lef*, and *cya*) and pXO2 which carries the *capBCADE* gene cluster which is responsible
210 for the expression of the poly-D-glutamic acid capsule (Makino *et al.*, 1989; Okinaka *et al.*,
211 1999ab; Pannucci *et al.*, 2002; Candela & Fouet, 2005). While either plasmid can be lost from
212 the bacteria, it is suggested that pXO2 is more susceptible to loss than pXO1 (Turnbull *et al.*,
213 1992; Marston *et al.*, 2005).

214 While the mechanisms responsible for the loss of these plasmids are unclear, factors such
215 as genetic damage, sporulation inadequacies as a result of poor nutrient availability,
216 temperature induced stress and the presence of antibiotics such as novobiocin have all be
217 linked to the phenomenon in the laboratory (Ezzell, 1988; Marston *et al.*, 2005).

218 A single isolate (PCR profile E) failed to generate a PCR product of the expected size with
219 our pXO1 specific primers it was able to grow on XO media suggesting the presence of at
220 least some or all of the plasmid while all other isolates produced positive results for both PCR
221 and culture assessment. Further studies are required to determine if this failure in PCR

222 assessment was due to the lost of the target DNA or a mutation in the primer recognition
223 sequence.

224 A single isolate was non-mucoid upon primary isolation and failed to regain capsule
225 expression following repeated culture in a CO₂ incubator. PCR screening with pXO2 specific
226 primers failed to generate any products (profile A) suggesting a complete loss of the plasmid.

227 A further 35 isolates yielded a mixed phenotype of mucoid/non-mucoid colonies upon
228 primary culture. Of these non-mucoid colonies 20 failed to regain capsule expression
229 following repeated culture on bicarbonate agar in a CO₂ rich atmosphere and yielded negative
230 results with all pXO2 specific primers (profile A) suggesting that the plasmid had been lost.

231 This study has yielded 21 isolates from the historic collection with the potential to be
232 developed as a live spore animal vaccine due to the non-function or absence of pXO2.

233 The next biggest group (profile B) consisted of 10 isolates of which 9 failed to regain the
234 ability to expression a capsule upon repeated culture in a CO₂ incubator. The fact that one
235 non-mucoid colony producing isolate regained the ability to express a capsule suggests that
236 the conditions within the candle jar may have been non-optimal. It is known that capsule
237 expression is regulated in part by the level of CO₂ in the environment where it is thought to
238 serve as a signal of the mammalian host environment and act as cue for the induction of the
239 virulence factors (Uchida *et al.*, 1993; Drysdale *et al.*, 2005). The constant level of CO₂
240 achieved by the incubator compared to the candle jar may explain the reactivation of capsule
241 expression in this and other isolates (Fouet, 1996; Green *et al.*, 1985).

242 PCR analysis of all 10 isolates with pXO2 specific primers, a positive response was
243 obtained with the Cap6/103 primers but failed to yield products with any of the remaining
244 primers. Further studies are required to determine the reasons for this discrepancy and raises
245 the possibility that Cap6/103 may not be specific for pXO2 as first thought.

246 Isolates that belonged to profile C (n=3) produced mixed mucoid/ non- mucoid colonies
247 upon primary culture in a candle jar but regained capsule expression when cultured in a CO₂
248 incubator. PCR analysis generated products of the expected size for all of the pXO2 primers
249 suggesting that failure to express a capsule may be due to inefficient regulation of gene
250 expression.

251 Profile D comprised one non-mucoid isolate which failed to regain the ability to express a
252 capsule when cultured in a CO₂ incubator. PCR primers (Cap6/103, CapB, CapC) specific for
253 some but not all of the pXO2 located targets gave the expected gene products suggesting that
254 the plasmid was present but had been subjected to major mutational events.

255 The vast majority of isolates which yielded a mixed colony phenotype were intially
256 isolated between 2000 and 2003 suggesting that the length of storage has a significant
257 impact on the stability of pXO2. The results from this study suggest that the storage of *B.*
258 *anthracis* at -20°C in Brucella broth supplemented with 20% glycerine may not be the
259 optimum approach with which to ensure the maintainence of the original properties of the
260 isolate.

261

262 **Conclusions**

263 The results from this study suggests that the pXO2 plasmid is more susceptible to loss or
264 modification when stored in Brucella broth supplemented with 20% glycerine at -20°C than
265 pXO1 and that this instability increased with the storage duration. While the reasons for this
266 instability have yet to be determined, a number of isolates suggest a complete loss of the
267 pXO2 plasmid. The failure of a small number of isolates to express a capsule was linked to
268 the use of a candle jar suggesting that this is an unreliable method with which to identify
269 capsule producing bacteria. The reasons for the differences in pXO2 primer specificity is

270 currently unclear. Twenty one pXO2 deficient isolates which have the potential to be
271 developed as animal vaccines have been recovered and characterised.

272

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276

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- 334

335 **Table 1.** PCR primers used for the confirmation of *B. anthracis* virulence plasmids

Primer	Sequence	Plasmid Target	Location	Product Size (bp)	Reference
PA 8	GAGGTAGAAGGATATACGGT	pXO1	2452-2471	596	Beyer <i>et al.</i> (1995)
PA 5	TCCTAACACTAACGAAGTCG		3048-3029		
CAP 6	TACTGACGAGGAGCAACCGA	pXO2	506-525	1035	Beyer <i>et al.</i> (1995)
CAP 103	GGCTCAGTGTAACCTCCTAAT		1541-1522		
CAPA-F	CGATGACGATGGGTGAC	pXO2	54942-54931	676	Wang <i>et al.</i> (2011)
CAPA-R	AGATTGAAGTACATGCGGATG		54266-54287		
CAPB-F	GACGAGGAGCAACCGATTAAG	pXO2	56564-56544	550	Wang <i>et al.</i> (2011)
CAPB-R	AAGAACGCAGGCTTAGATTGG		56014-56034		
CAPC-F	GTATTAGGAGTTACTGAGCC	pXO2	55554-55533	345	Wang <i>et al.</i> (2011)
CAPC-R	GGTAACCCTTGTCTTTGAATTG		55208-55229		
pXO2-007F	GCGATGGTGGAACAGGAATG	pXO2	4497-4478	688	Wang <i>et al.</i> (2011)
pXO2-007R	TGCGTTGCTGCCGATATTG		3809-3727		

336

337 **Table 2.** Summary of phenotypic screening of confirmed *B. anthracis* isolates

Isolate Type	N	Phenotypic characterisation					
		Penicillin	γ Phage	pXO1	pXO2 (CO ₂ incubator / Candle jar)		
					Mucoid	Mixed	Non-mucoid
Cattle	114	114	114	114	89 / 85	24 / 28	1 / 1
Sheep	15	15	15	15	9 / 9	6 / 6	-
Dog	1	1	1	1	1 / -	- / 1	-
Soil	10	10	10	10	10 / 10	-	-
Other	2	2	2	2	2 / 2	-	-

338

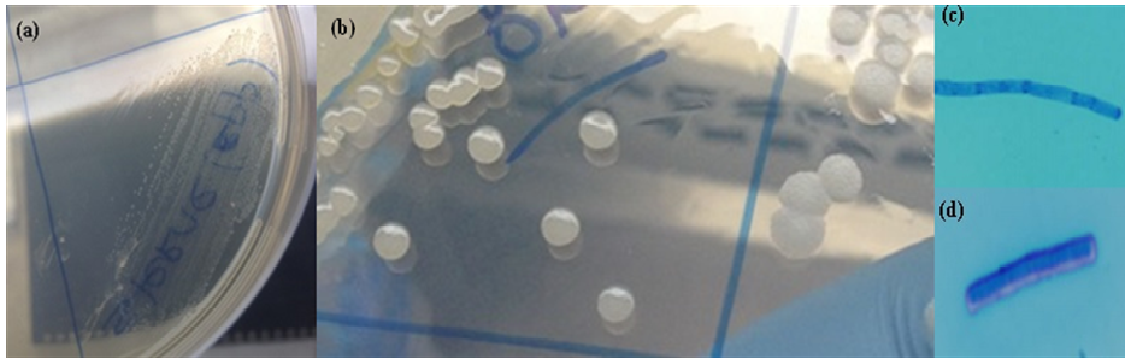
339 **Table 3.** Virulence plasmid profiles of *B. anthracis* strains

Sample Type	Strains ^a	Growth on XO medium	Capsule status	PCR results					
				PA	Cap6/103	CapA	CapB	CapC	pXO2-007
Control	K125 (pXO1 ⁺ , pXO2 ⁺)	+	Mucoid	+	+	+	+	+	+
	Sterne (pXO1 ⁺ , pXO2 ⁻)	+	Non-mucoid	+	-	-	-	-	-
	<i>E. coli</i> OP50 (Neg.cont.)	-	Non-mucoid	-	-	-	-	-	-
Mixed culture- Mucoid	30 (35)	+	Mucoid	+	+	+	+	+	+
Mixed culture- Non-mucoid	Pattern A 20 (20)	+	Non-mucoid	+	-	-	-	-	-
	Pattern B 9 (10)	+	Non-mucoid	+	+	-	-	-	-
	Pattern C - (3)	+	Non-mucoid	+	+	+	+	+	+
	Pattern D 1 (1)	+	Non-mucoid	+	+	-	+	+	-
	Pattern E - (1)	+	Non-Mucoid	-	+	+	+	+	+
Pure culture- Non-mucoid	Pattern A 1 (1)	+	Non-Mucoid	+	-	-	-	-	-

340 ^aPattern data in brackets represents samples cultured in candle jars.

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344 **Fig. 1.** Phenotypic screening of confirmed *B. anthracis* cultures. (a) *B. anthracis* isolate on
345 XO media after 72 h growth. (b) *B. anthracis* isolates on sodium bicarbonate agar after 48 h
346 growth (left side is mucoid and right is non-mucoid colony morphology). (c) no capsule
347 production and (d) capsule production with McFadyean staining. .

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