Virulence plasmid stability in environmentally occurring *Bacillus anthracis* from North East Turkey

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Abstract

The *Bacillus anthracis* virulence plasmid pXO2 which encodes for a polypeptide capsule, can be lost during long term laboratory storage. To determine if pXO2 is lost in nature we screened *B.anthracis* isolates obtained from *B.anthracis* spores contaminate animal burial site in Turkey for there ability to express a capsule upon primary culture. A total of 672 *B. anthracis* colonies were examined of which 10 produced a mixed mucoid (capsule +ve) /non-mucoid (capsule –ve) phenotype and a further one colony yielded non-mucoid colonies upon repeated culture. Screening by PCR using pXO2 specific primers revealed that 7 of these isolates had eliminated the plasmid. Of the 4 colonies which were positive by PCR, one regained the ability to express a capsule upon repeated culture suggesting that the defect was reversible. This is an important observation as

capsule expression is a principal marker of virulence and in the absence of PCR serves as a key diagnostic marker. The results of this preliminary study suggests that pXO2 is lost in nature and that further studies are need to determine the mechanisms by which this occurs.

Introduction

Bacillus anthracis is a Gram-positive spore forming bacteria that is primarily a pathogen of cattle and whose virulence is mediated by two plasmids (pXO1 and pXO2). The bacterium is released into the environment via contaminated fluids following the animals slaughter (Bellan et al. 2013). Once released it converts into its spore form in which it can persist for prolonged periods of time. There is limited information available on the retention of *B. anthracis* virulence plasmids in the environment. The current study sought to determine the stability of the *B. anthracis* virulence plasmids under "real world" conditions over the course of a year.

Materials and methods

Three sites in the North East of Turkey, contaminated with *B. anthracis* between 2006 and 2011 from the slaughter or disposal of infected cattle, were sampled throughout 2013 (May, August, October). Sites were selected from a total of 56 known animal burial sites in the Kars region that had been screened for the presence of *B. anthracis* (Buyuk et al, unpublished data). Due to extreme temperatures and high snowfall during the winter, samples could only be obtained during the summer months. As the result of a large civil engineering project, site A was destroyed during October 2013 rendering it inaccessible. All culture work was performed under appropriate safety conditions at the Veterinary Microbiology Faculty of Kafkas University (Kars, Turkey). About 100 g of soil was removed from the top 10 cm of up to 5 separate locations per site and stored at room temperature in clear plastic containers until processed. Samples were removed from the epicentre of the site and at a distance of 1 m at the major compass points (N, S, E, and W). Forty grams of soil was suspended in 120 mL of sterile distilled water, shaken vigorously by hand and allowed to settle for 30 minutes at room temperature. Five hundred µL aliquots were removed, serially diluted 1:10 in sterile distilled water and heated for 20 minutes at 60°C. Following heat shock, 150 µL was spread over the surface of 5% (v/v) sheep blood agar plates, allowed to air dry at room temperature for 5 minutes and incubated overnight at 37°C. Colonies which exhibited "classical" *B. anthracis* colony morphology (ground glass, non-haemolytic) were picked and stored at -20 °C in Brucella broth supplemented with 20% glycine until processed. Suspect colonies were phenotypically confirmed to be *B. anthracis* by streaking to a 5% (v/v) sheep blood agar plate and exposed to penicillin (10 units) and Gamma phage. Colonies were also screened for capsule expression (encoded by pXO2) by culture on 0.7% bicarbonate agar supplemented with 10% horse serum in the presence of carbon dioxide. Suspect colonies were also analysed by PCR for the presence of the virulence plasmids pXO1 and pXO2 as described by Buyuk et al. (2015) and Wang et al. (2011). While a single gene target (PA) was used to confirm the presence of pXO1,

multiple gene targets spanning the entire circumference of the pXO2 plasmid were employed (Wang et al. 2011). A fully virulent *B. anthracis* (pXO1+, pXO2+) isolate was included as a positive control while *Escherichia coli* was used as a negative control for both culture and PCR.

Results and discussion

During the course of the study, *B. anthracis* spore levels varied between 35 and 74400 spores/g of soil and no correlation between sampling time (time of year), site and spore content was observed (Table 1). Given the small number of sites sampled the results can at best be considered preliminary. To determine the validity of these finds a much larger study is in progress involving over 50 contaminated sites spread across the Kars region. Of a total of 672 phenotypically confirmed *B. anthracis* colonies, only 10 exhibited a mixed mucoid (capsule expression)/non-mucoid phenotype when grown on sodium bicarbonate agar while a single colony exhibited a pure non-mucoid phenotype (Table 1). All colonies yielded a PCR product of the expected size for pXO1 confirming the presence of the plasmid (Fig. 1). With regards to pXO2, all mucoid colony variants tested produced PCR products of the expected size while the non-mucoid variants yielded two distinct PCR profiles: the first showed no PCR products (n=7) for any of the pXO2 targets, while the second showed products of the expected size (n=4) for each of the pXO2 primers tested suggesting a defect in the genes regulating capsule expression (Fig. 1). The four isolates which were initially non-mucoid on primary culture but which generated positive pXO2 PCR reactions were sequentially sub-cultured on bicarbonate agar to assess the stability of the non-mucoid phenotype. Of these 4 isolates, only one regained the ability to produce a capsule suggesting that for at least one variant the phenotype is reversible.

B. anthracis is well known to be environmentally persistent with two contradictory theories attempting to explain the prolonged survival of *B. anthracis* in the environment: the dormant spore (Hugh-Jones and Blackburn 2009) and actively replicating lifecycle (Saile and Koehler 2006; Dey et al. 2012). The persistence of spores in soil and their ability to infect susceptible animals has been linked to a range of factors including the presence of organic material, calcium ions, a pH > 6.0, ambient temperature above 15.5° C and cycles of local flooding during which spores are transported to the surface via ground water saturation (Dragon and Rennie 1995; Smith et al. 2000; Hugh-Jones and Blackburn 2009). The area of Turkey used in the current investigation possesses some of these factors: the soil is rich in calcium, the pH >6.0 and there are cycles of local flooding

when the winter snow melts. These environmental conditions suggest spores could leach into the surrounding soil from contaminated carcasses and could be transported to surface as the water table rises.

However, there is limited information on the retention of the virulence plasmids under environmental conditions, as most studies have focused on laboratory storage (Turnbull et al. 1992; Bowen and Quinn 1999; Marston et al. 2005; Buyuk et al. 2015). When compared to these reports, our data suggests a lower frequency of pXO2 loss (1.75% compared to ~20%) suggesting that long term storage has a detrimental effect on plasmid stability (Marston et al. 2005; Buyuk et al. 2015). While the underlying mechanisms responsible for the loss of pXO2 during long term laboratory storage have yet to be determined the results highlight the importance of using fresh isolates to determine plasmid stability in the field.

The loss of capsule expression was not only due to the complete loss of the pXO2 plasmid as four non-mucoid isolates gave positive signals when probed by PCR suggesting a loss of gene function. Of these isolates, one regained the ability to produce a capsule suggesting that the phenotype. This is an important observation as capsule expression is a key phenotypic marker of virulence and in the absence of PCR serves as a key diagnostic marker. The ability of PCR to detect target genes is no doubt a powerful diagnostic tool, however, it is also limited by an inability to provide information on gene functionality. Thus care needs to be taken when basing diagnostic decisions on the presence or absence of a single marker and highlights the need to include a phenotypic screen in addition to PCR to confirm the potential for capsule expression.

To our knowledge this pilot study represents the first attempt to characterise plasmid stability in isolates of *B. anthracis* taken directly from burial sites and hence the potential virulence of *B. anthracis* isolates in Turkey. Further studies are required to determine the mechanisms of plasmid loss and loss of function and how this affects the ability of the bacterium to infect domestic animals.

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Sample taken	Site	*B. anthracis* content (spores g^{-1})	Phenotypic analysis			pXO2 PCR profile[3]	
			No. Colonies picked for analysis	Confirmed *B. anthracis* (% positive)[1]	Non-mucoid *B. anthracis* colonies (%)[2]	Positive	Negative
May	A	2330	112	101 (90)	0	-	-
	S	133	24	8 (30)	0	-	-
	D	266	108	60 (50)	0	-	-
August	A	199	119	117 (98)	2 (2)[a]	1	1
	S	133	118	116 (98)	0	-	-
	D	133	105	67 (63)	0	-	-
October	A	Site Destroyed					
	S	35	108	103 (95)	9 (9)[b]	3	6
	D	74 400	104	100 (96)	0	-	-

Table 1 Phenotypic screening of isolates of *B. anthracis* from individual burial sites. All percentages have been rounded to the nearest whole percent. [1] Confirmation was based on Gamma phage and Penicillin G susceptibility.[2] Production of a mucoid colonies on 0.7% bicarbonate agar supplemented with 10% horse serum in the presence of carbon dioxide.[3] Based on non-mucoid phenotype only. [a] Colonies were 1 pure non-mucoid phenotype and 1 mixed mucoid/non-mucoid phenotype. [b] All colonies were of mixed mucoid/non-mucoid phenotype.

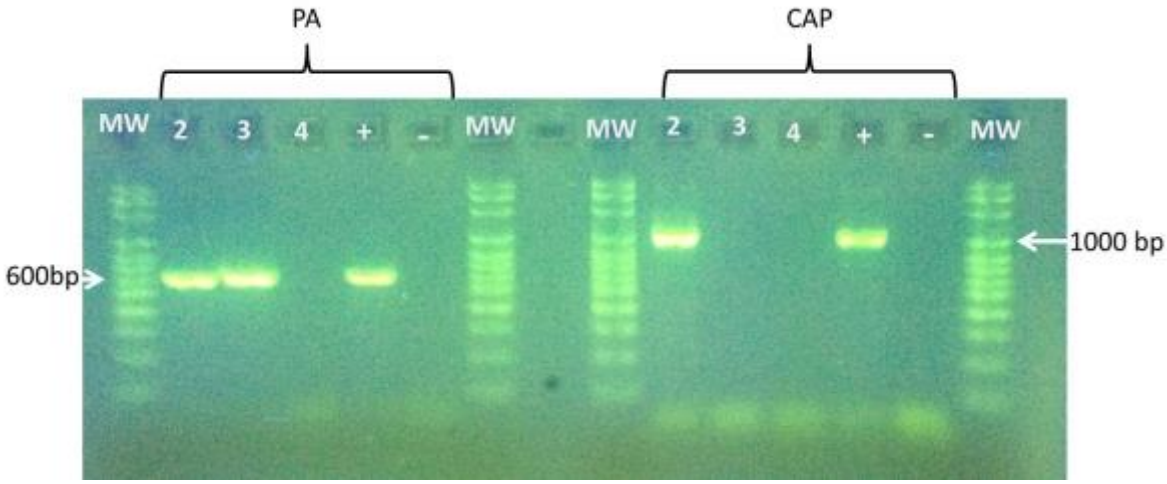

Fig. 1 PCR analyses of mucoid and non-mucoid variants of *B. anthracis* field isolate A38 using PA and CapB specific primers. PA specific primers PA5/PA8 produce a product of 596bp. CAPB specific primers CAP6/CAP103 produce a 1035bp product. MW- molecular weight markers; Lane 2: A38M (mucoid), Lane 3: A38NM (non-mucoid), Lane 4: *E. coli* control, Lane +: wild type *B. anthracis* (pXO1+, pXO2+), Lane -: *B. anthracis* Sterne (pXO1+, pXO2-).