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Full Length Research Paper

Numerous locus variable-number pair rehash examination of Bacillus anthracis disengaged from a human-animal Bacillus anthracis episode in the Luangwa valley of Zambia

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The incidence of anthrax, caused by *Bacillus anthracis*, in human and animal population of Zambia has increased recently. In this study, 34 strains of *Bacillus anthracis* from soil, hippopotamuses and humans, isolated in the 2011 outbreak were analyzed using the multiple-locus variable-number tandem repeat analysis. The analysis revealed that a single anthrax clone may have been involved in the epidemic. Considering the cyclical nature of *B. anthracis*, a link could be established with anthrax spores in soil getting ingested by hippopotamuses, followed by human contact resulting into an animal-human epidemic. These data confirm the importance of molecular typing methods for in-depth epidemiological analyses of anthrax epidemics.

Key words: *Bacillus anthracis,* hippopotamuses, variable number tandem repeat (VNTRs), epidemic, human.

INTRODUCTION

Bacillus anthracis is a zoonotic disease-causing agent for anthrax. It is a spore forming bacterium that causes disease in wild and domestic animals (Koehler, 2002; Habrun et al., 2011). Animals especially herbivores are mostly infected by oral ingestion of soil contaminated with anthrax spores, while humans are infected through contact with contaminated animal products (Spencer, 2003). In humans, the bacterium causes three major types of anthrax namely inhalational, gastrointestinal and cutaneous (Baykam et al., 2009).

B. anthracis has a worldwide dissemination as it is easy to propagate and has a history of being used as an agent of bioterrorism (Gierczynski et al., 2009). With this background, it is therefore important that the bacteria be identified and typed accurately in order to determine microbial forensics associated with disease and act appropriately in controlling the infection (Koehler, 2002). To enhance such an understanding, the phenotypic and

genetic properties of isolates from one source to the other must be determined. *B. anthracis* is highly mono-morphic in nature and differentiation of isolates from different regions has proved to be difficult (Brodzik and Francoeur, 2011). However attempts have been made to develop techniques that can resolve the minor genetic differences that can be found in *B. anthracis.* Genetic differentiation among very closely related individuals such as *B. anthracis* requires the use of molecular markers that exhibit very high diversity (Keim et al., 2004). These techniques include; multiple-locus variable-number tandem repeat analysis (MLVA), amplified fragment length poly-morphism (AFLP), single nucleotide repeats (SNR) and single nucleotide polymorphism (SNP) (Vos et al., 1995; Okinaka et al., 2008). SNP detection is facilitated by whole genome discovery approaches which are useful for identifying long branches or key phylogenetic positions (Keim et al., 2004) while AFLP has good resolution ana-lysis that require the use of MLVA. The MLVA is the most suitable technique to differentiate and subtype *B. anthracis* strains (Lindstedt, 2005). One powerful feature of the MLVA system is the ability to simultaneously employ multiple variable number tandem repeat (VNTRs) markers that exhibit varying levels of diversity and, there-fore, high resolving power. In this study, we analyzed and compared isolates of *B. anthracis* from wildlife (hippo-potamus), soil and humans reported in a human-animal anthrax outbreak in Zambia (Hang'ombe et al., 2012). Anthrax outbreaks have been recorded in Zambia, since 1989 (Turnbull et al., 1991; Turnbull, 1998; Tuchili et al., 1993; Siamudaala et al., 2006) without any report of direct molecular epidemiological linkage of *B. anthracis* strains from an epidemic scenario in Zambia.

MATERIALS AND METHODS

In this study, we analyzed 34 *B. anthracis* strains associated with a single anthrax epidemic in wildlife and human populations. A vaccine Sterne strain was used as a positive control and was compared with the epidemic isolates. Each *B. anthracis* strain was streaked onto 5% sheep blood agar plates and then incubated at 37°C for 24 h. Genotyping was done as previously described (Keim et al., 2004). The cultured isolates were inactivated by heating at 95°C for 30 min after which DNA was extracted using a DNA extraction kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Following DNA extraction, a panel of 21 VNTR markers was used in this analysis (Table 1). These were variable repeat region (*vrr*) markers; *vrrA, vrrB1, vrrB2*, *vrrC1*, *vrrC2*, CG3, pXO1-*aat*, (Schupp et al., 2000) and Ceb-Bams (CB); CB-1, CB-3, CB-5, CB-7, CB-13, CB-15, CB-21, CB-22, CB-23, CB-24, CB-25, CB-28, CB-30 and CB-31 (Le Fleche et al., 2001). The PCR amplification was performed using the Phusion™ flash high fidelity PCR master mix (Finnzymes Oy, Finland). The reactions were performed in a final volume of 10 µl containing 5 µl Phusion flash PCR master mix, 0.5 µM of primer sets in 1 µl volume of each and 2 µl of PCR water. The Piko™ thermal cycler (Finnzymes Instruments Oy, Finland) was programmed at 95°C for 10 s for initial denaturation, followed by 35 cycles consisting of 95°C for 1 second, 58°C (changed according to the primers (Table 1)) for 5 s and 72°C for 15 s. Final extension was given 72°C for 1 minute. The thermal PCR-profile was used for all the VNTR markers. The amplicons were visualized on 1.5% agarose gel (MP Biomedicals, Eschwege, Germany), stained with ethidium bromide and evaluated under UV transilluminator. The estimation of the sizes of PCR products was done according to the migration pattern of a 100-bp DNA ladder.

RESULTS AND DISCUSSION

In this study, we used the MLVA genotyping comprising 21 VNTR markers to understand the relationship of 34 strains of *B. anthracis* isolated from soil (13 strains), hippopotamuses (16 strains) and human (5 strains) during an anthrax epidemic. Of these isolates, no difference was observed on a panel of 21 VNTR markers. The positive control isolate which is the vaccine strain (Sterne strain) had an observable difference with the outbreak isolates. The difference was observed with the CG3 marker where the Sterne vaccine was positive for CG3, while the outbreak strains were negative (Table 2). Further differences were noted with other markers which amplified a different molecular weight amplicon. The markers with different weight amplicons when compared with the Sterne vaccine strain were, CB-1, CB-3, CB-15, CB-21, CB-22, CB-23, CB-30 and CB-31. This MLVA analysis conducted on the isolates from human, soil and hippopotamus revealed relatedness using 21 VNTRs. The results indicated that all 34 isolates belonged to a single clone. The outbreak strains had the pXO1 and pXO2 plasmids required for virulence. Their presence indicates strain pathogenicity (Keim et al., 1997; Jackson et al., 1997; Habrun et al., 2011) as opposed to the vaccine strain which is negative for the pXO2 plasmid as expected. Analysis of other variable repeats found within *B. anthracis* provides a valuable tool to understand the epidemiology of anthrax outbreaks (Jackson et al., 1997). In this study, the confirmation through MLVA, indicates the outbreak may have been due to a single close. The results may also indicate that changes from one host to another as a result of genetic instability at the locus during the culture or infection process are rare. Such sequence stability suggests the utility of the VNTR regions as markers to determine the source of an infection or outbreak.

Conclusion

The cyclical nature of anthrax outbreaks determined by climatic factors such as extreme weather changes like drought followed by heavy rains, may lead to generation of high anthrax spore concentration causing disease in grazing animals such as hippopotamus which are bulky grazers. This outbreak indicated a single clonal source transmission, probably from soil to hippopotamuses and finally to humans through consumption of contaminated meat. The data presented here are limited and therefore call for continuous monitoring of human and animal clinical isolates and their environment (including soil) to

Table 1. Primers used in this study for MLVA analysis.

VNTR locus	Primer sequences (5' to 3')	Annealing temperature (°C)
vrrA	F: CACAACTACCACCGATGGCACA R: GCGCGTTTCGTTTGATTCATAC	60
vrrB1	F: ATAGGTGGTTTTCCGCAAGTTATTC R: GATGAGTTTGATAAGAATAGCCTGTG	61
vrrB2	F: CACAGGCTATTCTTTATCAAACTCATC R: CCCAAGGTGAAGATTGTTGTTGA	60
vrrC1	F: GAAGCAAGAAAGTGATGTAGTGGCA R: CATTTCCTCAAGTGCTACAGGTTC F:	62
vrrC2	CGAGAAGAAGTGGAACCTGTAGCAC R: GTCTTTCCATTAATCGCGCTCTATC F:	62
CG3	TGTCGTTTTATCTTCTCTCTCCAATAC R: AGTCATTGTTCTGTATAAAGGGCAT	59
pXO1	F: CAATTTATTAACGATCAGATTAAGTTCA R: TCTAGAATTAGTTGCTTCATAATGGC	57
CB-1	F: GTTGAGCATGAGAGGTACCTTGTCCTTTT R: AGTTCAAGCGCCAGAAGGTTATGAGTTATC F:	66
CB-3	GCAGCAACAGAAAACTTCTCTCCAATAACA R: TCCTCCCTGAGAACTGCTATCACCTTTAAC F:	64
CB-5	GCAGGAAGAACAAAAGAAACTAGAAGAGCA R: ATTATTAGCAGGGGCCTCTCCTGCATTACC F:	64
CB-7	GAATATTCGTGCCACCTAACAAAACAGAAA R: TGTCAGATCTCTAGTTGGCCCTACTTTTCCTC	63
CB-13	F: AATTGAGAAATTGCTGTACCAAACT R: CTAGTGCAATTTGACCCTAACTTGT	58
CB-15	F: GTATTTCCCCCAGATACAGTAATCC R: GTGTACATGTTGATTCATGCTGTTT	59
CB-21	F: TGTAGTGCCAGATTTGTCTTCTGTA R: CAAATTTTGAGATGGGAGTTTTACT	58
CB-22	F: ATCAAAAATTCTTGGCAGACTGA R: ACCGTTAATTCACGTTTAGCAGA F:	57
CB-23	CGGTCTGTCTCTATTATTCAGTGGT R: CCTGTTGCTCCTAGTGATTTCTTAC F:	62
CB-24	CTTCTACTTCCGTACTTGAAATTGG R: CGTCACGTACCATTTAATGTTGTTA F:	59
CB-25	CCGAATACGTAAGAAATAAATCCAC R: TGAAAGATCTTGAAAAACAAGCATT F:	56
CB-28	CTCTGTTGTAACAAAATTTCCGTCT R: TATTAAACCAGGAGTTACTTACAGC F:	59
CB-30	AGCTAATCACCTACAACACCTGGTA R: CAGAAAATATTGGACCTACCTTCC F:	61
CB-31	GCTGTATTTATCGAGCTTCAAAATCT R: GGAGTACTGTTTGTTGAATGTTGTTT	59

help draw sound conclusions on the exact spread of *B. anthracis*.

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Table 2. Genotyping results of *Bacillus anthracis* strains isolated from soil, hippopotamus and humans with the 21 VNTR locus.

Strain	VNTR markers																				
	vrrA1	vrrB1	vrrB2	vrrC1	vrrC2	CG3	pXO1	CB-1	CB-3	CB-5	CB-7	CB-13	CB-15	CB-21	CB-22	CB-23	CB-24	CB-25	CB-28	CB-30	CB-31
Outbreak isolates	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sterne Vaccine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

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