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SOME EPIDEMIOLOGIC CHARACTERISTICS OF *BACILLUS ANTHRACIS* AS REVEALED THROUGH AFLP MARKERS

Hugh-Jones M.[1]*, Jackson P.[2], Keim P.[3], Kalif A.[1], Smith K.[1]

Cette recherche a porté sur le chromosome 4Mb en utilisant les marqueurs d'ADN AFLP, pour plusieurs raisons. D'abord, cela a permis de distinguer de manière fiable B. anthracis des espèces les plus proches ; ensuite, bien que les marqueurs AFLP puissent aisément établir la composition des plasmides pX01 et pX02 de chaque isolat, la réalité est que les plasmides sont facilement dispersés ; troisièmement, comme B. anthracis a potentiellement une faible diversité, la technologie permet d'évaluer une partie importante du génome pour des variations mineures ; et enfin, les fragments AFLP peuvent être changés en marqueurs satisfaisants spécifiques de locus pour l'analyse d'échantillons fixes ou de l'environnement. A ce moment, 31 fragments du chromosome polymorphe unique de B. anthracis ont été identifiés. Ceux-ci ont conduit à l'identification d'un petit groupe de souches « Afrique du sud », étroitement liées au protopathogène. Un groupe plus large a une distribution globale et est constitué de la série actuellement identifiée « Sterne-Ames », « Vollum-Kruger », « Western North America » ainsi que divers petits groupes comprenant un possiblement d'Argentine. Ils ont tous des distributions géographiques différentes.

Cette espèce est extrêmement monomorphe, ce qui suggère que l'hôte exerce une pression évolutive faible ou nulle sur cet agent létal. Ensuite, l'ontogénie peut être récente. Enfin, bien qu'il soit efficacement disséminé par les épizooties et par le commerce, parfois globalement, les différentes régions d'enzootie permanente ont probablement rendu maximaux les avantages d'un relativement faible nombre de changements dans l'organisme, reflétant peut-être uniquement des variables climatiques et régionales.

INTRODUCTION

Although the pathogenic differences of *Bacillus anthracis* isolates and resulting cultures have been known for over half a century and cultural preferences for much longer, it has until recently resisted attempts at fine definition of true 'strains'. Previous attempts at DNA fingerprinting revealed a lack of diversity.(Harrell et al, 1995; Henderson et al, 1994) Latterly however use of a M13 phage primer as an arbitrary PCR primer was able to demonstrate one DNA fragment difference between strains. This primer was used to isolate and sequence a variable number tandemly repeated (VNTR) sequence with a perfect 12-bp repeat in different copy numbers called the *vrrA* locus from *B.anthracis* and other *Bacillus* species.(Andersen et al, 1996; Jackson et al, 1997) Until very recently this was the only variable DNA sequence among *B.anthracis* strains. An additional advantage of *vrrA* analysis is that it can be used for characterising *B.anthracis* strains in histological tissues.(Jackson et al, 1997) Using amplified fragment length polymorphism (AFLP) DNA markers we have been able to uniquely identify the various closely related *Bacillus* species using 357 AFLP polymorphic fragments, to establish the presence or absence of the pXO1 and pXO2 plasmids, and using 31 polymorphic chromosomal regions to sort through an increasing number of *B.anthracis* strains.(Keim et al, 1997)

This research targetted the 4 Mb chromosome using AFLP DNA markers for a variety of reasons. Firstly it allowed us to reliably differentiate *B.anthracis* from its closest relatives; secondly, although the AFLP markers can readily establish the pXO1 and pXO2 plasmid composition of each isolate, the reality is that plasmids are shed naturally as well as in the laboratory, may be transferred between strains, and are therefore ephemeral; and thirdly as *B.anthracis* had potentially little diversity the technology could evaluate a very large percentage of the whole genome for numerically minor variations. Analysis and identification of the polymorphic AFLP fragments has demonstrated a low (some 3%) but very informative molecular variation, which has provided a high level of discrimination between *B.anthracis* isolates; at the same time 60% of the fragments differed between this organism and it nearest relatives, *B.cereus* and *B.thuringiensis*.

This research is on-going. A major thrust of the current epidemiologic studies is to identify the nature and causes of the normal variation within apparently stable geographic areas. Similarly, it is to begin to lay out the principles of what patterns of strain homogeneity and heterogeneity may be associated with generic type epidemiologic situations, e.g. with feed contamination, spread by insects and birds, variance in epidemics versus endemic or sporadic conditions, traditional trade routes and exchanges, and soils.

[1] Department of Epidemiology & Community Health, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803

[2] Environmental Molecular Biology, LS-7 M888, Los Alamos National Laboratory, Los Alamos, NM 87545

[3] Department of Biological Sciences, Northern Arizona University, Flagstaff, AR 86011-5640

METHODOLOGY

[1] Strains: To date over 230 cultures have been collected through the generosity of a number of individuals, of institutes and of national medical and veterinary services. Some isolates had been used for research; some had been archived since their initial identification, some over a significant number of decades and the oldest had survived serendipitously; and some were from recent outbreaks. In all but a few instances the year and place of the outbreak were known as was the species of the infected host. The donors supplied these data with the cultures.

[2] DNA analysis: The methodologies for isolate culture, DNA isolation and purification, PCR amplification and amplicon analysis, AFLP markers and analysis, DNA sequencing and analysis have been described elsewhere.

RESULTS

1. DENDOGRAM TO DATE:

The original dendogram of *B.anthracis* strains based on 78 isolates (Keim et al, 1997) has maintained its basic structure with a large global collection of strains (A) and of a more restricted series (B) found in Southern Africa, so far from Zimbabwe south. It has been suggested that this latter series is reflective of the original protopathogen. As more samples have been received and analysed it has been possible to perceive more structure within the A strains, but as yet not within the Southern African B.

A primary branch 'C' of the A series is clearly made up of isolates closely related to type cultures of Sterne and Ames, and has therefore been labelled 'Sterne-Ames'.

The large secondary branch 'D' is made up of geographically widely dispersed series of strains — 'Vollum - Kruger' with the former of Asiatic recoveries and the latter covering a broad swath of Africa from western Zambia to Mozambique; 'Western North America' with isolates from west of the Mississippi and from the Texas counties bordering Mexico to the North West Territory of Canada; while the residua have yet to declare themselves and the historic origins of branch D, and therein may be a series representative of Argentina.

2. MAJOR GEOGRAPHIC DISTRIBUTIONS:

a. Southern Africa: African isolates have been from South Africa, Namibia, Zambia, Zimbabwe, the Kruger National Park, and Mozambique. We are actively requesting isolates from other African countries but until they are received and analysed it is impossible to know how far north this series is distributed within Africa. European and American isolates of 'Southern Africa' are assumed at this time to reflect infections following upon exposure to imported contaminated materials, such as hides and bone meals.

b. Sterne-Ames: To date this 'C' branch of A is made up of (1) a small group of isolates closely related to the Sterne-type strain with representatives from the Lebanon and western Europe, and as yet none from Natal or other parts of Africa. (2) A similar 'Ames' group with representatives from Indonesia, southern India, Pakistan, and northern Turkey, plus Australia, USA, Germany, France, and Jamaica; interestingly this disease is no longer seen in Jamaican and the Jamaican strain is identical with one from Pakistan indicating its possible origin. It is expected that 'Ames' will be the characteristic strain for Central Russia and the western countries of the previous USSR. (3) The rest of this series has, so far, been most commonly identified among isolates from Namibia as well as from Zambia, Zimbabwe, Mozambique, and South Africa, with singular isolates from the UK, Germany, and the USA.

c. Vollum-Kruger: This is made up of the following components:

i: Vollum; to date these are all versions of the original Vollum culture isolated from a dead cow in south Oxfordshire in 1936. We have yet to identify its specific source. All other isolates related to the Vollum type-strain have come largely from Asia ... South Korea, Indonesia, Pakistan, and with isolates from Ireland, Switzerland, Germany, Norway, and New Hampshire (USA).

ii: Kruger; This is made up of three groups of related isolates from the Kruger National Park but, interestingly, all with a *vrrA* VNTR of 6, which has not been seen in any other field isolates in our collection to date; from two related groups from western Zambia (Mongu) including an isolate from a dead elephant in the nearby Caprivi Strip; and from southern Mozambique.

d. Western North America (WNA): The largest group in this series is of essentially identical isolates from Texas (USA) to Northern Alberta (Canada) with isolates from Iowa, Alberta & Saskatchewan, Colorado, Nebraska (and Turkey), with closely related isolates from Germany (and Texas), Turkey, and Norway (and Iowa); the later presumably reflecting contaminated exports from North America. There is a second group of strains which with two exceptions, two ranches near Edmonton, have only been recovered from Wood Buffalo in northern Alberta and the adjoining North West Territory. The isolates from South Dakota and California have been coherent but specific to each state. The rest of the WNA series has yet to develop a clear identity. One group is of isolates from Pakistan and Italy, probably reflecting the importation of wool and hides from the former country, of Namibia and Slovakia (bones? Imported by the latter country), and of a cluster of isolates from Argentina, Florida, Texas, and Maryland.

3. ENDEMIC CHARACTERISTICS:

Based upon collections from North America and the Kruger National Park the major characteristics of the endemic situation are strain consistency over a number of decades and the absence of a wide heterogeneity.

4. LIVESTOCK FEED-SOURCE CHARACTERISTICS:

The characteristics of feed-related outbreaks are the sudden appearance of strains characteristic of other places, distant or near, not seen before in the affected area, and thus of strain variance over time. Logically

these 'new' strains should make epidemiological sense, e.g., in relation to bone meal from Namibia or India where the 'imported' strain is common, and to trace-back investigation results. This strain heterogeneity may extend to the variant strains found within a batch of feed as displayed on different farms in the same outbreak or even sometimes within a single herd or flock. However within a single outbreak all isolatescan be the same.

DISCUSSION

As this pathogen is so successful, it routinely kills in a few days, it clearly has little need to be flexible in relation to host defences for itsown survival. In that studies now indicate that more animals may be infected than die there are certainly advantages in minimising the ID_{50}, LD_{50}, and vegetative generation times, and maximising germination rates needed to infect and kill the host. Similarly in maintaining an hypoallergenic spore for latent infections. At this time we have noted no obvious host preference for the various strains that cannot be explained by local epidemiology — for example the presence of the *vrrA* VNTR 6 Kruger strain in the Kruger National Park, where kudu are the most afflicted species, and the absence of this VNTR so far elsewhere may be coincidence. The existence of a meaningful extrinsic vegetative cycle outside the host is now widely doubted if for no other reason that it has yet to be demonstrated in the absence of a highly enriched environment and in the presence of competing organisms. If it were to occur, however rare and extraordinary the circumstances, the natural variance of the other closely related free-living *Bacillus* species would suggest that this might be a route for generating new strains. Extrinsicly the major component is certainly the survival of the spore. The more that is known about spores, the more complex they are seen to be and therefore with a large capacity for differences, whether advantageous or merely non-deleterious. The general conservatism of *B.anthracis* strains would imply that any environmental pressures for change are simple and gross, and not subtle and complex.

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