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Enhanced diagnostics, molecular epidemiology and disease ecology for anthrax

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Abstract

Outbreaks of anthrax occur sporadically in Australia commonly in the anthrax belt. However, little is known about the epidemiological links between *Bacillus anthracis* strains isolated from different outbreaks and the overall diversity of strains within Australia which makes outbreak prediction difficult. Molecular epidemiological studies were undertaken using the most differential genotyping techniques available. The diversity revealed within Australian *B. anthracis* strains is consistent with the historical records with the greatest diversity of isolates identified where the organism has been resident for the longest period of time. Results suggests that a single genotype was introduced into the Eastern states of Australia and that this introduction was followed by spread of the pathogen throughout the anthrax belt. Unexplained occurrences of disease in areas outside of the anthrax belt, which are associated with different genotypes, indicating separate introductions of *B. anthracis* into Australia. In order to better predict the potential ecological and geographic distribution of *B. anthracis* in Australia, an ecological niche model was developed. In addition, soil studies were undertaken that confirmed the longevity of anthrax spores in the environment. The distribution of immuno-chromatographic test kits around Australia has enabled the successful update and increased usage of these kits throughout Australia. This project has provided knowledge and tools to help optimise response strategies and assist in the prevention of anthrax within Australia.

Full abstract

Outbreaks of anthrax occur sporadically in Australia most commonly in the “anthrax belt”, a region which extends from Southern Queensland through the centre of New South Wales and into Northern Victoria. Little is known about the epidemiological links between *Bacillus anthracis* strains isolated from different outbreaks and the diversity of strains within Australia. This project aims to provide knowledge and tools to help optimise response strategies and assist in the prevention of anthrax within Australia. To enable these aims the project has focussed on improving our understanding of the epidemiology and ecology of Anthrax in Australia and development of enhanced diagnostics.

Data on outbreaks of disease (see Appendix 1) has been collated and temporally and spatially diverse strains of *B. anthracis* associated with recent outbreaks of disease collected and accessioned into the National Anthrax Reference Collection for use in molecular epidemiological studies. Genotyping of isolates for molecular epidemiological studies was undertaken using Multiple-Locus Variable-number tandem repeat Analysis (MLVA) employing 25 markers and Whole Genome Single Nucleotide Polymorphism (SNP) Typing (WGST). MLVA genotyping of 150 isolates of *B. anthracis* revealed 8 unique genotypes (MLVA groups, MG 1-8). The majority of isolates fell within two closely related genotypes (MG 1 and MG 2). Two genotypes (MG 7 and MG 8) were distinct from the major cluster of genotypes which was also reflected in their geographic origin outside of the anthrax belt. Genotyping of *B. anthracis* strains from outbreaks of disease in Victoria identified the presence of multiple genotypes associated with each outbreak. However, overall the genetic diversity of *B. anthracis* isolates from Australia is relatively low. Whole genome sequences were determined for 52 *B. anthracis* isolates which are representative of the isolates held within the National Anthrax Reference Collection. SNPs were identified between the whole genome sequences which were then used for WGST of these isolates. WGST was determined to be a more discriminatory method than Multi Locus VNTR analysis (MLVA) for genotyping *B. anthracis* isolates. The WGST method discriminated 4 genotypes, WGST genotypes B, C, D and E, within a MLVA genotype MG2 and two genotypes, WGST genotypes H and I, within MLVA genotype MG6. However, there was one case where WGST was unable to discriminate between two MLVA genotypes, MG 1 and MG3. It may be possible to discriminate these two MLVA genotypes using less stringent criteria for the definition of SNPs than that used in the present analysis. Overall the WGST analysis confirmed the MLVA typing of *B. anthracis* isolates but revealed previously undetected diversity within *B. anthracis* isolates from NSW. The diversity of *B. anthracis* in NSW is consistent with the historical record of *B. anthracis* within Australia with the greatest diversity of isolates identified where the organism has been resident for the longest period of time.

The geographical distribution of genotypes within Australia suggests that a single genotype was introduced into the Eastern states of Australia and that this introduction was followed by spread of the pathogen throughout the anthrax belt. Following the spread of the pathogen, localized differentiation into other closely related genotypes has occurred. In contrast, unexplained occurrences of disease in areas outside of the anthrax belt, which are associated with different genotypes, indicate separate introductions of *B. anthracis* into Australia.

The rapid lateral flow Immunochromatographic diagnostic test (ICT) kits are a highly valuable tool for active surveillance and disease diagnosis in areas with a history of anthrax and a routine tool in the investigation of sudden death in cattle. The kits are produced by the National Anthrax Reference Laboratory (NARL) have been distributed throughout Australia (Victoria, NSW, Western Australia, South Australia, Tasmania and Queensland). The number of kits distributed is increasing each year. The ability to produce these kits in-house at the NARL has provided Australia with the means to increase anthrax surveillance from the network of veterinarians and animal health officers. The simplicity of the kit and the ability to perform carcass-side has empowered veterinarians to implement greater caution when faced with sudden deaths on farm and provided a tool that can be used to justify to farmers the cascade of events that follow a diagnosis of anthrax.

The high sensitivity, high specificity and simplicity of the ICT have made this test an invaluable field tool for veterinarians and animal health officers throughout Australia.

In order to better predict the potential ecological and geographic distribution of *B. anthracis* in Australia an Ecological niche model (ENM) was developed . The ENM approach predicts the potential ecological and geographic distribution of the pathogen based on outbreak locations and environmental conditions including rainfall, temperature and soil pH. The ENM developed in this study predicted the region where *B. anthracis* is presently found in the “Anthrax Belt” stretching from southern Queensland through the centre of NSW and into Northern Victoria. However, early records of disease in Australia, particularly along the southern Victorian coastline were not predicted by the model. It is hypothesised that these early disease incidents were caused by consistent introduction and re-introduction of *B. anthracis* into these areas, most likely through movement of infected animals or importation of contaminated material (for example bone meal) through ports in these areas without long term establishment of disease.

The ENM analysis and WGST data is also consistent with the hypothesis that the unexplained occurrence of anthrax in Walpole in Western Australia and Rockhampton in Queensland were the result of recent introduction of the disease into these regions and not the exposure of animals to spores lying dormant in the soil. Overall, the consensus of the ENM with occurrences of anthrax in Australia gives confidence that a robust model has been developed to describe the distribution of the disease in Australia.

Executive summary

The overall objectives of this project were to build capability and preparedness for anthrax outbreaks and improve our understanding of the epidemiology and ecology of disease. This was achieved by

- Molecular genotyping of *B. anthracis* strains held within the National Anthrax Reference Laboratory (NARL) to assess the diversity of historical and newly collected *B. anthracis* isolates from multiple regions throughout Australia, predominantly Victoria and NSW.
- The development of simplified diagnostic genotyping scheme to allow the identification of genotypes of *B. anthracis* present in Australia.
- Determination that spores are able to persist in the soil environment long term on contaminated properties in the Goulburn Valley by analysing soil collected from these properties.
- Implementation of refinements to the conditions of manufacture and use of the Immuno-Chromatographic Test (ICT), evaluation of the performance of the ICT and increased utilisation of the anthrax ICT kits produced by the NARL.
- The generation of models to help inform the formulation of effective management strategies for prevention of anthrax based upon understanding the ecology of the disease.

Two genotyping methodologies, Multilocus VNTR analysis (MLVA) and Whole Genome SNP Typing (WGST), were employed to assess the genetic diversity of *B. anthracis* strains from Australia. WGST was determined to be the most discriminatory of the two methods. However, WGST is a very expensive and time consuming method in comparison to MLVA typing. Results of genotyping isolates from the same geographic area indicated that they are indistinguishable even when isolated years apart. Similarly, isolates from geographically distinct farms can be identical. These results support the hypothesis that *B. anthracis* remains dormant in the soil as a spore and under suitable conditions, the spore contacts and infects a susceptible animal and that little, if any, environmental cycling of the pathogen occurs. Furthermore, genotyping of isolates collected during an outbreak identified that greater than one genotype was associated with a disease outbreak. It is probable that environmental conditions at the time of the outbreak led to a series of parallel outbreaks. These results may lead to a change in thinking on the management strategies employed to control outbreaks in Australia. For example, a more extensive vaccination strategy may be required at the initiation of an unusual outbreak to encompass properties more distant to the initial infected property to account for parallel outbreaks of disease at more distant locations.

The most divergent genotypes identified were single isolates from Western Australia and Queensland. These genotypes are more divergent from other strains isolated in Australia than *B. anthracis* strains isolated from Japan and other areas of the world. It is hypothesised that these isolates represent recent introductions into Australia. WGST revealed greater genetic diversity within *B. anthracis* isolates from NSW compared to MLVA genotyping. This is consistent with NSW being the initial place of introduction of *B. anthracis* into Australia and that *B. anthracis* cycling between animals and the environment occurs more frequently in NSW (that is the disease occurs more regularly in NSW which would allow greater genetic variation to be introduced into the population). Overall, the genotyping conducted in this study provides a baseline survey of the genotypes of *B. anthracis* found within Australia and identifies the geographic location of these genotypes. This will be invaluable in epidemiological investigations of future outbreaks and will allow the identification of future incursions of *B. anthracis* into Australia.

As WGST and MLVA protocols employed in the molecular genotyping analysis of *B. anthracis* are time consuming and relatively expensive undertakings, a more rapid and simplified protocol was required for the differentiation of Australian *B. anthracis* strains. This project has developed a genotyping scheme for the identification of all genotypes known to be present in Australia based upon canonical SNPs (Single Nucleotide polymorphisms) present in the genomes of each WGST group identified. An allelic discrimination assay for the identification of one of these groups has been successfully developed. As a result, the NARL now has a suite of molecular tests which are available for application to future disease outbreaks. The rapid and almost continuous development of new molecular techniques means that the NARL will need to keep abreast of the changes and evaluate simplified methodologies that may become available in the future. Current

methods are still too complex to be undertaken as a routine test in laboratories that do not have specialist skills such those available in NARL. The simplified genotyping scheme developed in this project will be of use in the investigation of future outbreaks of disease in Australia and allow rapid genotyping of any future strains deposited in the collection.

Testing of soil samples determined that spores have survived on properties within the Goulburn Valley for over 7 years. This is consistent with research conducted overseas and the anecdotal evidence that disease recurrence on properties is due to spores which have been present in the environment for decades. Although the most sensitive method available for the isolation of *B. anthracis* from soil was employed, the failure to isolate *B. anthracis* from samples that tested positive by Bio-PCR indicates that improvements in sensitivity of isolation protocols used to detect the presence of *B. anthracis* spores are still needed, and may provide a better picture of spore contamination at these sites. This information may be useful not only in constructing and testing models predicting future anthrax outbreaks and their spread, but may also help in the focusing of control measures on the areas or properties identified to be contaminated with *B. anthracis* spores. Application of methods developed in this study for the identification of *B. anthracis* in soil will be invaluable in future research assessing the efficacy of decontamination protocols. Whilst current decontamination processes would significantly reduce the *B. anthracis* present in the environment, these studies have highlighted that some level of *B. anthracis* spores are surviving and remaining viable for years to come. The information on spore survival, and future research in this area, will inform the AUSVETPLAN for Anthrax.

The ICT, which now has an Australia-wide distribution, has proven to be a highly valuable tool for active surveillance and disease diagnosis in areas with a history of anthrax, and a routine tool in the investigation of sudden death in cattle. The ability to produce these kits in-house at the NARL has provided Australia with the means to control manufacture timeframes and to distribute the ICT throughout Australia giving greater coverage for all veterinarians and animal health officers. The NARL has received much positive feedback on the use of the ICT. As a result of this project there has been an increase in use of the test across a wider range of species and the test is being used in a great number of locations throughout Australia. The simplicity of the kit and the ability to perform carcass-side diagnosis has empowered veterinarians to implement greater caution when faced with sudden deaths on farm, and provided a tool that can be used to justify to farmers the cascade of events that follow a diagnosis of anthrax. The high sensitivity, high specificity and simplicity of the ICT have made this test an invaluable field tool for veterinarians and animal health officers throughout Australia. Refinements were recently made to the manufacturing process of the kits followed by a thorough comparison of multiple batches of kits which provided evidence in support of the modifications. Since the modifications have been implemented no aberrant results have been obtained with the field use of the kits. The NARL will continue to support the production, provision and use of the ICT kit.

An Ecological Niche Model (ENM) has been developed which is a robust prediction of the areas of Australia where *B. anthracis* spores are likely to survive. This model will aid in the formulation of effective management strategies for prevention of anthrax in. The identification of geographic areas where anthrax is likely to occur will enable focused surveillance and vaccination strategies to target at-risk animals before and during outbreak events. The predicted distribution of *B. anthracis* within Australia, as predicted by the ENM analysis, is consistent with the historical data and the current geographic range of disease in Australia but has improved the definition of the at-risk areas. The ENM analysis, as well as the genotyping data, is also consistent with the hypothesis that the unexplained occurrence of anthrax in Walpole in Western Australia and Rockhampton in Queensland were the result of a relatively recent introduction of the disease into these regions and not the exposure of animals to spores lying dormant in the soil, or from spread from a location within the anthrax belt. There is a still a need for additional research focusing on the complex environmental conditions which promote sporadic outbreaks of disease to help underpin the development of further predictive tools to enable control of anthrax in the Australian environment.

As a result of the National Anthrax Workshop held by the NARL, at the DEPI AgriBio site in Bundoora in May 2014, it was identified that there is a need to follow up with individuals knowledgeable and experienced in the field aspects of anthrax before these people retire. The NARL need to determine the most appropriate way to undertake this. Taken together, the work in

this project has significantly increased the knowledge of, and diagnostic capability for, anthrax within Australia. Continued research, particularly in the assessment of decontamination techniques, improvement of soil culturing methods and the refinement of models to allow better prediction of anthrax outbreaks, should lead to a comprehensive package of skills and knowledge to better protect Australian livestock from anthrax in the future.

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1 Background

Bacillus anthracis, the causative agent of anthrax, is a Gram positive spore forming bacterium with a worldwide distribution. Anthrax is a serious disease of livestock and humans, which can impact on the international trade of livestock and their products. Anthrax is endemic in many areas of the world, including Australia, where the disease is classed as an emergency animal disease. Outbreaks of anthrax have been recorded in Australia for over 150 years with the first occurrence of the disease recorded at Leppington, south-west of Sydney, New South Wales, in 1847 with the first reported outbreak of the disease in Victoria being in 1876 (Seddon, 1953; Seddon & Albiston, 1965). More recently outbreaks of anthrax in Australia, which have been largely confined to Victoria and New South Wales (Beveridge, 1983), have occurred in the "anthrax belt" a region which extends from Southern Queensland through the centre of New South Wales and into Northern Victoria. In Victoria, anthrax is mainly a disease of cattle and occurs primarily in the Goulburn Valley. In New South Wales anthrax occurs in the anthrax belt in the centre of the state and predominantly affects sheep (Durrheim *et al.*, 2009). Sporadic occurrences of disease outside of these areas in Victoria and NSW do occur, but, the occurrence of anthrax in other parts of Australia is rare. Anthrax has not been reported from the Northern Territory and no cases have been reported from South Australia and Tasmania since 1914 and 1933, respectively (Animal Health Australia, 2012). Western Australia was considered free of the disease until 1994, when cases occurred on three premises in a localised area around Walpole in the south of the state. No further cases have occurred in Western Australia since this time. There had been no cases in Queensland for over 70 years until one infected animal was detected on a single farm on the Marlborough Peninsula in 1993. A further outbreak occurred in Southern Queensland in 2002 (Animal Health Australia, 2012).

2 Project objectives

The overall objectives of this project were to build capability and preparedness for anthrax outbreaks and improve our understanding of the epidemiology and ecology of disease.

Below is a list of prioritised objectives for the project:

- Employ the molecular genotyping capability to assess the diversity of historical and newly collected *B. anthracis* isolates from multiple regions throughout Australia, predominantly Victoria and NSW.
- Determine spore survival and dispersal by analysing soil collected from contaminated farms.
- Formulate effective management strategies for prevention of anthrax based upon understanding the ecology of the disease.
- Increase the utilisation and evaluate the performance of the anthrax ICT and implement any refinement for conditions of manufacture and use.
- Development of simplified diagnostic genotyping protocols.

3 Assessment of genetic diversity of Australian *B. anthracis* isolates

3.1 Introduction

The genome of *B. anthracis* is highly monomorphic which makes differentiation between strains difficult, therefore it is important to target areas of the genome that are more rapidly evolving to

assess genetic diversity (Achtman, 2008). The most commonly used approach employs the use of variable-number tandem repeat (VNTR) loci to determine phylogenetic relationships among *B. anthracis* strains (Keim *et al.*, 2000). Multiple-locus VNTR analysis (MLVA) is one of the most discriminatory techniques available. The technique surveys the genome for mutations that occur during DNA replication which result in duplications of regions of the genome. The MLVA technique is portable and enables the comparison of Australian isolates with MLVA genotypes which have previously been determined for *B. anthracis* strains worldwide. MLVA markers are also highly stable which allows them to be used in improving our understanding the epidemiology of disease outbreaks (Beyer & Turnbull, 2009).

MLVA typing schemes using a minimum of 8 (MLVA8) (Keim *et al.*, 2000) to a maximum of 31 (Beyer *et al.*, 2012) VNTR markers have been successfully employed to discriminate between *B. anthracis* isolates. Although Australian isolates of *B. anthracis* isolates have been previously genotyped using the MLVA technique by Keim *et al*. (2000) and Van Ert *et al*. (2007a), only a small number, 30 isolates, have been typed using the MLVA8 and MLVA15 typing schemes respectively. These studies identified 3 genotypes within the collection of Australian isolates, but, the exact geographic location of the genotypes in Australia has not been defined with the exception that the majority of strains genotyped by Keim *et al*. (2000) and Van Ert *et al*. (2007a) were sourced from the unusual outbreak of disease in Victoria in 1997.

In this study we employed the MLVA25 technique described by Lista *et al*. (2006) to assess the genetic diversity of a collection of Australian *B. anthracis* isolates from various geographic locations. The MLVA25 technique was used in preference to the MLVA8 or MLVA15 typing schemes due to the increased number of loci employed and therefore the increased discriminatory power of this method (Keim *et al.*, 2004; Lista *et al.*, 2006). The availability of a relatively large database of MLVA25 genotypes makes the use of MLVA25 scheme a more valuable comparative method in comparison to the MLVA31 scheme for which a more restricted database is available.

Traditional spatial and temporal analysis can be used in combination with strain typing to reveal information about disease incidences where there is no key epidemiological linkage. Molecular typing methods such as MLVA25 have been successfully applied to genoptype isolates from natural outbreaks and have provided valuable insight into the introduction and dispersal patterns of *B. anthracis* in the environment.

Whole genome sequencing, facilitated by the advent of high-throughput approaches, brings the promise of single-base-pair resolution between isolates, making it the ultimate molecular typing method for bacteria (Achtman, 2008). Being able to distinguish between strains at the single nucleotide level, by comparing genomes in terms of single nucleotide polymorphisms (SNPs), drastically improves the discriminatory power of typing methods over conventional genetic typing methods such as MLVA. The terms Whole Genome Sequence Typing or Whole Genome SNP Typing (WGST) have been used to describe the approach of comparing whole genomes by extracting the SNPs from draft genome sequences. Several recent studies have shown that analysis of SNPs in bacterial genomes by WGST provides a means of determining the relatedness of epidemiologically linked isolates and allows tracking of bacterial evolution over periods of months to years (Biek *et al.*, 2012; Köser *et al.*, 2012; Lewis *et al.*, 2010; Schürch *et al.*, 2011).

WGST has been employed in *B. anthracis* to help in the forensic investigation of the so called “Amerithrax” attacks in the USA (Rasko *et al.*, 2011; Van Ert *et al.*, 2007b) but has not yet been applied to understand the epidemiology of outbreaks of disease where multiple isolates from infections that are attributable to a single focus are analysed.

This study employs both MLVA and WGST approaches to determine the diversity and evolutionary relationships of *B. anthracis* isolates from Australia including a set of isolates taken from a single outbreak of disease within the Goulburn Valley in Victoria.

3.2 Methods and materials

3.2.1 Multi-locus Variable-number-tandem-repeat Analysis (MLVA)

3.2.1.1 B. anthracis isolates

A total of 150 *B. anthracis* isolates from Australia, held at the National Anthrax Reference Laboratory (NARL), Department of Environment and Primary Industries, Victoria, were used in this study. Isolates were confirmed as *B. anthracis* on the basis of morphological characteristics when grown on sheep blood agar and polymyxin, lysozyme, EDTA, thallous acetate (PLET) agar (Knisely, 1966) at 37°C for 24h and 48h respectively. All strains were further characterised by PCR amplification using the PCR methods described by Antwerpen *et al*. (2008) and Berg *et al*. (2006).

All isolates were from outbreaks of disease which occurred between 1993 and 2009, with the exception of a single isolate from NSW that predated this time (Table 1). Isolates were representative of the geographic distribution of the disease in Australia and were collected from a range of sources (Table 1). The majority of isolates were from Victoria (101 isolates) but isolates from New South Wales (32 isolates), Queensland (12 isolates) and Western Australia (5 isolates) were also genotyped. The 101 isolates from Victoria included 37 isolates from an outbreak of disease in 1997 and 44 isolates from a disease outbreak in 2007. Isolates were routinely cultured on sheep blood agar plates at 37°C for 24h in order to prepare material for DNA extraction.

3.2.1.2 DNA isolation

DNA was extracted using either a QIAamp DNA Mini Kit as per manufacturers' instructions (Qiagen, Doncaster, Victoria, Australia) or by boiling of a cell suspension as described by Keim *et al*. (2000). Any spores contaminating the DNA extracts were removed using 0.1µm ultrafree-MC filter units (Millipore, Kilsyth Victoria, Australia) as described by Dauphin *et al*. (2009), the sterility of extracts was confirmed by plating 5µl of each extract onto a sheep blood agar plate followed by incubation at 37°C overnight.

3.2.1.3 Multiple locus variable-number tandem repeat analysis

MLVA analysis using 25 loci was carried out as outlined by Lista *et al*. (2006) and modified by Ted Hadfield, Midwest Research Institute, Florida (Pers. Comm.). Primers were labelled with one of four fluorescent dyes (6-carboxyfluorescein, VIC, NED or PET). PCR amplification of variable number tandem repeat (VNTR) alleles was performed on a C1000 thermal cycler (Bio-Rad, Gladesville, New South Wales, Australia) using a Type-it Microsatellite PCR Kit (Qiagen, Doncaster, Victoria, Australia) as per the manufacturers' instructions. A 2µl aliquot of each amplification product was subjected to electrophoresis on a 2% agarose gel containing Sybr® Safe (Invitrogen), and visualised by UV transillumination, to ensure that the VNTR markers had amplified prior to separation by capillary electrophoresis on a 3730xl DNA analyser at the Australian Genome Research Facility (AGRF). Fragment sizes were estimated using GeneMapper version 3.7 software by comparison to a GeneScan™ 1200 LIZ® Size Standard (Applied Biosystems, Mulgrave, Victoria, Australia).

Table 1. Information on strains used for Multilocus VNTR Genotype Analysis

MLVA Genotype	**Geographic location State** (Town or area)	**Dates isolated**	**Number of Strains**	**Sources**
MG 1	**Victoria** (Tatura)	1997	30	Ovine, Bovine, Soil/Water, Flies
	Victoria (Tatura)	2003	1	Bovine
	Victoria (Tatura)	2005	1	Bovine
	Victoria (Tatura)	2007	5	Bovine
	Victoria (Kyabram)	2009	1	Bovine
MG 2	**Victoria** (Tatura)	1997	3	Flies, Ovine, Soil
	Victoria (Swan Hill)	2002	1	Bovine
	Victoria (Stanhope, Wyuna)	2007	35	Bovine, Soil
	Victoria (Tatura)	2007	4	Bovine
	Victoria (Stanhope)	2009	1	Bovine
	NSW (Narrabri)	1994	1	Bovine
	NSW (Narrandera)	2002	1	Ovine
	NSW (Deniliquin)	2003	1	Bovine
	NSW (Narrandera)	2004	2	Ovine
	NSW (Yenda, Berrigan)	2005	3	Ovine, Bovine
	NSW (Narrandera)	2010	11	Ovine
	Qld (Wandoan, Dirranbandi)	2002	9	Bovine
MG 3	**Victoria** (Tatura)	1997	3	Bovine, Flies
	Victoria (Harston)	2004	13	Bovine
	Victoria (Harston)	2008	2	Bovine
MG 4	**Victoria** (Tatura)	1997	1	Bovine
MG 5	**Qld** (Dirranbandi)	2002	1	Bovine
MG 6	**NSW** (Moree)	1979	1	Bovine
	NSW (Hunter Valley)	2007/2008	3	Bovine
	NSW (Cumnock)	2008	9	Bovine, Soil
MG 7	**WA** (Walpole)	1994	5	Bovine
MG 8	**Qld** (Rockhampton)	1993	2	Bovine

Table 2. Multilocus genotypes of Australian *B. anthracis* isolates and diversity indices for each marker locus for the *B. anthracis* isolates typed

Marker	MLVA Genotype[a]								Diversity	Confidence	K	max
Loci	MG 1	MG 2	MG 3	MG 4	MG 5	MG 6	MG 7	MG 8	Index[b]	Interval		(pi)
CG3	2	2	2	2	2	2	2	2	0	0.000 - 0.069	1	1
Bams44	8	8	8	8	8	8	8	8	0	0.000 - 0.069	1	1
Bams3	30	30	30	30	30	30	30	30	0	0.000 - 0.069	1	1
VrrB2	7	7	7	7	5	7	7	7	0.02	0.000 - 0.057	2	0.99
Bams5	6	6	6	6	6	6	7	7	0.129	0.044 - 0.214	2	0.93
Bams15	45	45	45	45	45	45	45	45	0	0.000 - 0.069	1	1
Bams1	16	16	16	16	16	16	16	16	0	0.000 - 0.069	1	1
VrrC1	33	33	33	33	33	33	57	57	0.129	0.044 - 0.214	2	0.93
Bams13	70	70	70	70	70	70	73	70	0.094	0.018 - 0.170	2	0.95
VrrB1	16	16	16	16	16	16	12	12	0.129	0.044 - 0.214	2	0.93
Bams28	14	14	14	14	14	14	14	14	0	0.000 - 0.069	1	1
VrrC2	17	17	17	17	17	17	17	17	0	0.000 - 0.069	1	1
Bams53	8	8	8	8	8	8	8	8	0	0.000 - 0.069	1	1
Bams31	64	64	64	40	64	64	64	64	0.02	0.000 - 0.057	2	0.99
VrrA	10	10	10	10	10	10	10	10	0	0.000 - 0.069	1	1
Bams25	13	13	13	13	13	13	13	13	0	0.000 - 0.069	1	1
Bams21	10	10	10	10	10	10	9	9	0.129	0.044 - 0.214	2	0.93
Bams34	8	8	8	8	8	8	8	8	0	0.000 - 0.069	1	1
Bams24	11	11	11	11	11	11	11	11	0	0.000 - 0.069	1	1
Bams51	9	9	7	9	9	9	9	9	0.178	0.085 - 0.272	2	0.90
Bams22	17	16	17	17	16	16	13	13	0.557	0.513 - 0.601	3	0.51
Bams23	11	11	11	11	11	11	11	11	0	0.000 - 0.069	1	1
Bams30	57	57	57	57	57	57	54	57	0.094	0.018 - 0.170	2	0.95
pX01	6	6	6	6	6	6	8	8	0.129	0.044 - 0.214	2	0.93
pX02	8	8	8	8	8	9	9	9	0.194	0.099 - 0.289	2	0.89

[a] MLVA25 genotype allele numbers indicate the number of repeat units for each VNTR. Shaded numbers indicate alleles which vary between genotypes using MLVA genotype MG 1 as a reference.

[b] Diversity is based upon Simpson's index calculated using all 99 isolate the greater the value, the greater the sample diversity.

A representative of each VNTR allele size was reamplified using unlabelled primers and amplicons were sequenced to confirm the amplicon size and VNTR repeat number using ABI Prism BigDye Terminator Sequencing at the AGRF. An allele number string, based upon the number of tandem repeats present in each of the 25 VNTR markers, was assigned to each isolate (See Table 2)

3.2.1.4 Data analysis

The allele number string representative of each isolate was imported into Bionumerics version 6.1 (Applied Maths, Sint-Martems-Latem, Belgium) and compared to publicly available data from the *B. anthracis* MLVA database at MLVAnet (http://minisatellites.u-psud.fr/MLVAnet/). Data were analysed using categorical values and UPGMA analysis to generate trees from allelic profile data. Individual marker diversity within the population studied was assessed by determining Simpson's index of diversity using the VNTR Diversity and Confidence Extractor (V-DICE) available at the The Health Protection Agency (Colindale, London, United Kingdom) website (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl). The index of discrimination was calculated from the distribution of types with the Discriminatory Power Calculator (http://insilico.ehu.es/mini_tools/discriminatory_power/index.php)

3.2.2 Whole genome SNP Typing

3.2.2.1 B. anthracis isolates

A total of 51 *B. anthracis* isolates from Australia, held at the National Anthrax Reference Laboratory, Department of Environment and Primary Industries, Victoria, were used in this study (**Table 3**). Isolates were confirmed as *B. anthracis* on the basis of morphological characteristics when grown on sheep blood agar and polymyxin, lysozyme, EDTA, thallous acetate (PLET) agar (Knisely, 1966) at 37°C for 24h and 48h respectively. All strains were further characterised by PCR amplification using the PCR methods described by Antwerpen *et al*. (2008) and Berg *et al*. (2006).

All isolates were from outbreaks of disease which occurred between 1993 and 2009, with the exception of a single isolate from NSW that predated this time (**Table 3**). Isolates were representative of the known genetic diversity (assessed by MLVA genotyping, geographic distribution of the disease in Australia and were collected from a range of sources (**Table 3**). The majority of isolates were from Victoria (41 isolates) but isolates from New South Wales (8 isolates), Queensland (1 isolates) and Western Australia (1 isolates) were also genotyped. The 42 isolates from Victoria included 10 isolates from an outbreak of disease in 1997 and 23 isolates from a disease outbreak in 2007. Isolates were routinely cultured on sheep blood agar plates at 37°C for 24h in order to prepare material for DNA extraction.

3.2.2.2 DNA isolation

DNA was extracted from overnight cultures on SBA using either a Promega Wizard® Genomic DNA Purification Kit according to the manufacturer's instructions. Any spores contaminating the DNA extracts were removed using 0.1µm ultrafree-MC filter units (Millipore, Kilsyth Victoria, Australia) as described by Dauphin *et al*. (2009), the sterility of extracts was confirmed by plating 5µl of each extract onto a sheep blood agar plate followed by incubation at 37°C overnight. Filtered DNA was quantified and quality assessed using a NanoDrop 2000 spectrophotometer.

3.2.2.3 Genome sequencing

Construction of Illumina libraries were conducted using a TruSeq™ DNA Sample Preparation Kit according the to the manufacturers' protocol with minor variation. In brief: A 120µl volume of genomic DNA (approximately 2µg) in TE buffer was loaded into a Covaris microTube, and subsequently sheared to fragments of an average length of 400 bp using a Covaris S220 (Covaris) using the following settings: duty cycle of 2%, intensity of 4, for 200 cycles of 15s duration to produce 1500bp fragments followed by a duty cycle of 10%, with an intensity 4 for 200 cycles of 55s duration to produce an average fragment size of 400bp. Shearing was followed by end repair, A-tailing and adapter ligation. Initial enrichment PCR (with indexed primers for individual libraries)

was performed using Phusion High Fidelity DNA polymerase (Finnzymes) with the following cycling conditions: initial denaturation for 30 sec at 98°C, followed by 30 cycles of denaturation for 10 s at 98°C, annealing for 20 s at 65°C and extension for 1 min at 75°C to determine the optimal enrichment PCR cycle number for each library (to avoid skewing the representation of the library). Enrichment PCR was then repeated using the optimal cycle number for each library with index primers, quantified using a KAPA Library Quantification Kit (Kapa Biosystems) and run on a DNA 1000 BioAnalyzer chip (Agilent) to determine size distribution and purity. Libraries were then pooled, quantified using a KAPA Library Quantification Kit (Kapa Biosystems) and quality checked on a DNA 1000 BioAnalyzer chip (Agilent). Libraries were sequenced on either an Illumina HiSeq 2000 after a cBot cluster generation using a TruSeq PE Cluster Kit v3-cBot-HS kit or an Illumina MiSeq sequencer using a MiSeq Reagent Kit v2 (2 x 150 sequencing protocol) according to the manufacturer's instructions.

3.2.2.4 Genome mapping

Paired end Illumina reads were mapped to the genome sequence of Bacillus anthracis str. A0248 (GenBank Accession Number CP001598) using the Burrows-Wheeler Alignment Tool. Samtools was used to convert, sort, index and write a consensus file from aligned data for each of the *B. anthracis* strains sequenced.

3.2.2.5 SNP identification and filtering

Samtools was used to call variants from the aligned data and for initial SNP filtering. SNPs were excluded from the analysis if they had a Samtools mpileup quality score <999, were within a repeat region (as identified by equicktandem (600 max repeat size, 20 theshold score)), were within a region of ambiguous assembly, were A/T or G/C transversions, were within 50bp of a gap in assembly or were located in non-coding sequence.

3.2.2.6 Data analysis

The SNPs identified were concatenated into a single string of SNPs stored as a character set in Bionumerics version 7 (Applied Maths). The evolutionary history of Australian *B. anthracis* isolates in comparison to a set of *B. anthracis* genomes representing the diversity of *B. anthracis* worldwide was inferred using the Maximum Parsimony (MP) method as implemented in MEGA5 (Tamura *et al*., 2011). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 0 in which the initial trees were obtained by the random addition of sequences (10 replicates). Phylogenetic trees from representative Australian strains of *B. anthracis* were generated using Minimum Spanning Algorithm using default settings in Bionumerics version 7 (Applied Maths).

3.2.2.7 Mapping of genotype Information

The Latitude and Longitude data of genotyped isolates within the collection was mapped using QGIS 2.0 (Quantum GIS Development Team (2013). Quantum GIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.osgeo.org).

3.3 Results

3.3.1 Multi-locus Variable-number-tandem-repeat Analysis (MLVA)

3.3.1.1 Marker diversity

All VNTR markers were amplified from all isolates tested, However, only 12 of the 25 VNTR markers were informative for the population of *B. anthracis* isolates assessed in this study with diversity indices varying from 0 to 0.557 (Table 2.2). The VNTR marker with the greatest discriminatory power was the Bams22 marker (DI = 0.557) which discriminated 3 alleles within the 151 isolates.

3.3.1.2 Genetic diversity of *B. anthracis* strains in Australia

The 150 Australian *B. anthracis* isolates genotyped fell into 8 distinct MLVA25 genotype patterns, MG 1 – MG 8 (Figure 1, Figure 2, Table 1). All 8 MLVA patterns related to strains within the previously defined A3 genotype based upon the MLVA8 typing system (Keim *et al.*, 2000; Lista *et al.*, 2006) (Figure 2.2). The majority of the isolates genotyped (143 of 150; Table 1) belonged to 6 (MG 1 – MG 6) of the 8 genotypes and form a monophyletic cluster (Figure 2). The remaining strains fell into two related genotypes (MG 7 and MG 8) which cluster with a previously genotyped strain from Australia (Lista45; Figure 2). A total of 110 isolates fell into two MLVA genotypes, MG 1 and MG 2 (Table 1). Genotype MG 1 contained 38 isolates from Victoria which were isolated in 1997, 2003, 2005, 2007 and 2009 (Table 1). Genotype MG 2 contained 72 isolates from Victoria (in 1997,2002, 2007 and 2009) NSW (in 1994, 2002, 2003, 2004, 2005 and 2010) and Southern Queensland (in 2002) (Table 1). Isolates in MG 3 were isolated from Victoria during 1997, 2004, and 2008 and MG 4 from Victoria in 1997. The single isolate which comprised genotype MG 5 was from Southern Queensland in 2002 and the 13 isolates in genotype MG 6 were isolated from NSW in 1979, 2007 and 2008. The 5 isolates in genotype MG 7 were from an incident of disease in Walpole, Western Australia in 1994 and the 2 isolates in MG 8 were from a disease incident on the Marlborough Peninsula north of Rockhampton in Queensland in 1993. The strains most closely related to Australian *B. anthracis* strains originate from Japan (Okutani *et al.*, 2010) (Figure 2).

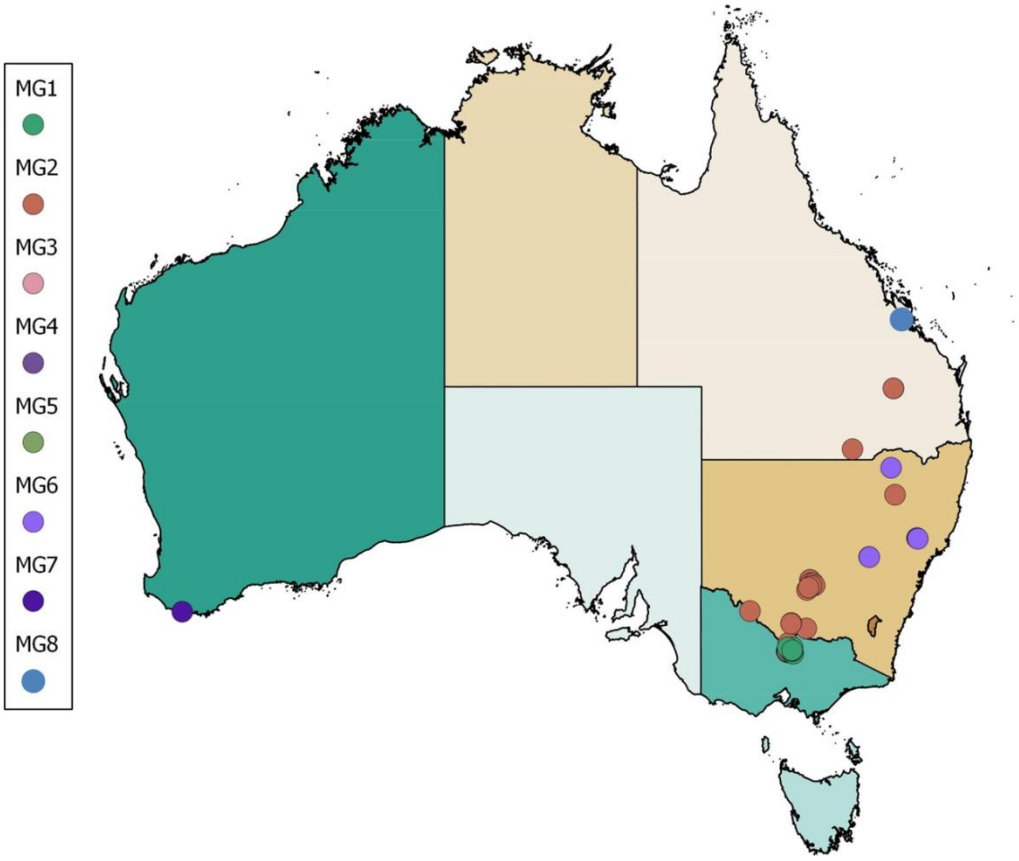

Figure 1. Geographical location of isolates colour-coded for genotype within the National collection.

Analysis of epidemiologically linked isolates from outbreaks throughout Australia revealed that isolates responsible for each outbreak belonged to between 1 and 4 MLVA25 genotypes. The 37 isolates genotyped from the outbreak of anthrax in Victoria in 1997 were found in 4 genotypes; MG 1 (30 isolates), MG 2 (3 isolates), MG 3 (3 isolates) and MG 4 (1 isolate) (Table 2.1). The 44 strains from the outbreak in 2007 belonged to two genotypes; MG 1 (5 isolates) and MG 2 (39 isolates) (Table 2.1). Isolates from an outbreak of disease in Queensland in 2002 belonged to two genotypes MG 2 (9 isolates) and MG 5 (1 isolate). All other epidemiologically linked isolates from outbreaks of disease fell within a single genotype.

3.3.2 Whole genome SNP Typing

3.3.2.1 Genetic diversity of *B. anthracis* strains in Australia

A total of 289 SNPs described the variation between Australian strains of *B. anthracis*. In comparison, employing the same SNP discover methodology, a total of 1773 SNPs were identified in set of genomes representative of the known diversity of *B. anthracis* worldwide, including the Australian isolates. Comparison of Australian isolates to genome sequences available in GenBank revealed that all Australian WGST genotypes are related to strains within the previously defined A3 genotype (Keim *et al*., 2000; Lista *et al*., 2006) (**Figure 3**).

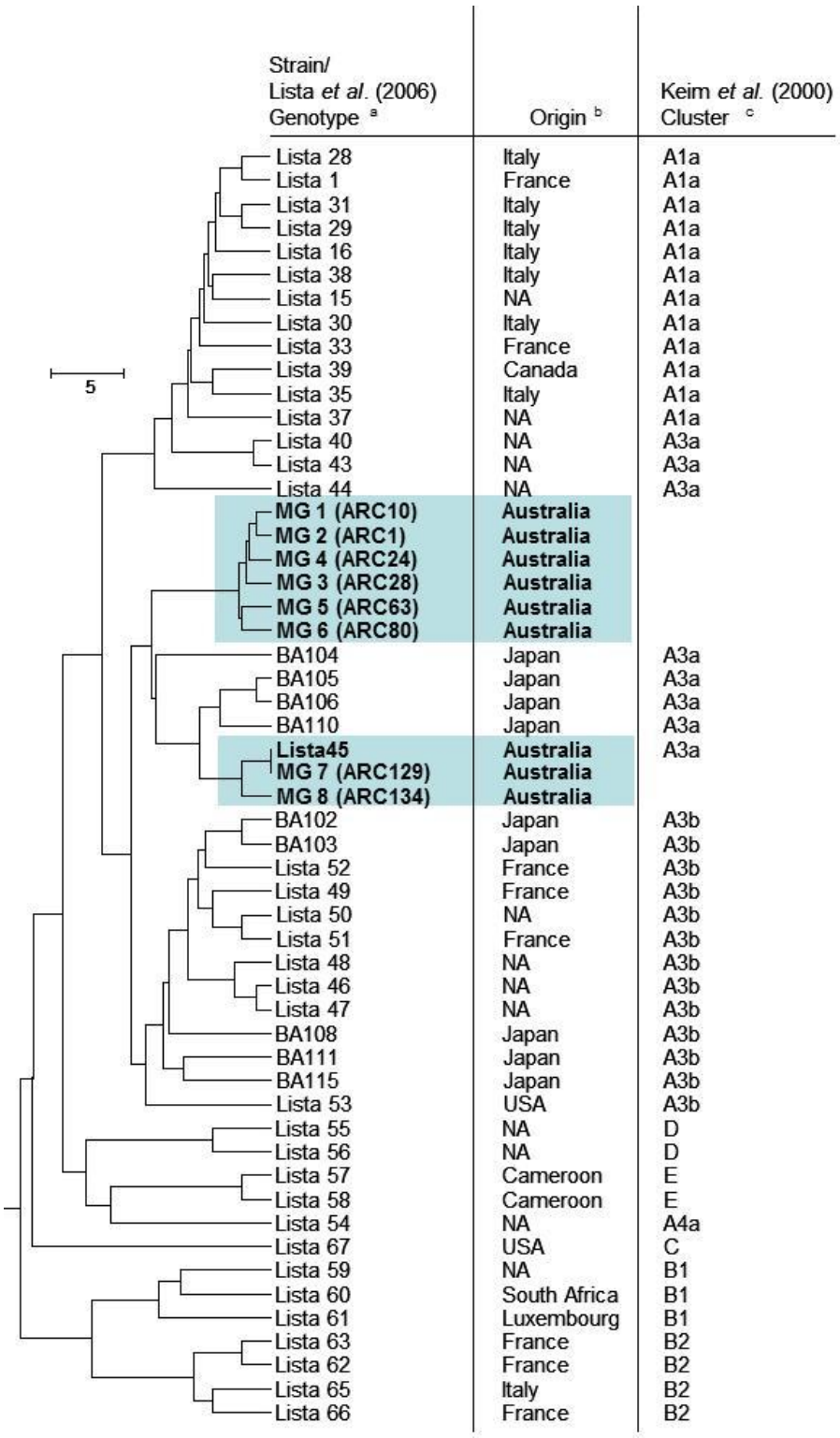

Figure 2. Phylogenetic relationship of *B. anthracis* strains including Australian strains (Bold faced type in blue box) assessed by MLVA25 genotyping.[a] Strains prefixed by "Lista" refer to the MLVA25 genotypes described by Lista *et al*. (2006). Strains prefixed by "BA" are strains representing MLVA25 genotypes described by Okutani *et al*. (2010). Strains prefixed MG are strains representing genotypes described in this study[b] Country of origin of strains where known, NA indicates that the origin is unknown.[c] Cluster of strains as defined by Keim *et al*. (2000) and adapted by Lista *et al*. (2006) and Okutani *et al*. (2010)

The 51 Australian *B. anthracis* isolates genotyped fell into 10 distinct WGST genotypes, A –H and J and K (**Table 3**, **Figure 3** and **Figure 4**). A WGST genotype was defined as genome sequences that differ by less than 2 SNPs. Therefore, WGST genotypes A, B and H contain strains that vary by 1 or 2 SNPs (**Figure 4**). The majority of the isolates genotyped (46 of 51) form a monophyletic cluster and belong to 6 (A, B, C, D, E and F) of the 10 genotypes recognised within Australian

strains (**Table 3** and **Figure 3**). The remaining 5 isolates genotyped fell into the other 5 genotypes (H, I, J and K). A total of 41 isolates belonged to two WGST genotypes, A and B (**Table 3**). WGST genotype A contains 18 isolates from Victoria which were isolated in 1997, 2004, 2005, 2007 and 2009 (**Table 3**, **Figure 5**, **Figure 6**). WGST genotype B contains 22 isolates from Victoria in 2007, 2008 and 2009 and NSW in 2005 (**Table 3**, **Figure 5**, **Figure 6** and **Figure 7**). WGST genotype C contains 2 isolates one from Victoria in 2002 and one from NSW in 2003 (**Table 3**, **Figure 5**, **Figure 6** and **Figure 7**). WGST genotypes D and E contain single isolates from NSW isolated in 2002 and 2004 respectively (**Table 3**, **Figure 5** and **Figure 7**). A single isolate from an outbreak of disease in Southern Queensland in 2002 constitutes WGST genotype F (**Table 3** and **Figure 5**). Genotype H consists of two isolates from the 2007/2008 outbreak of disease in the Hunter Valley area of NSW (**Table 3** and **Figure 5**, **Figure 7**). Genotype I is related to genotype H and consists of a single isolate from Moree in 1979. Genotypes J and K are the most divergent (**Figure 5**) and consist of strains from incidents of disease in Walpole in Western Australia and a disease incident on the Marlborough Peninsula north of Rockhampton in Queensland respectively (**Table 3** and Figure 1.3).

Comparison of WGST and MLVA genotyping revealed the power of whole genome sequencing as a tool to discriminate between *B. anthracis* isolates belonging to the same MLVA group. WGST genotypes B, C, D and E contain isolates that belong to MLVA genotype MG 2. Strains which clustered into MLVA genotype MG 6 belonged to the related WGST genotypes H and I which can be differentiated from each other by 18 SNPs (**Table 3**). In contrast, WGST genotype A contained strains that belonged to two MLVA genotypes (MG 1 and MG 3) (**Table 3**).

Table 3. Information on strains used for Whole Genome Sequence Typing

WGST Genotype	Geographic location State (Town or area)	Date(s) isolated	Number of Isolates	Sources	MLVA Genotype
A	Victoria (Tatura)	1997	6	Bovine, Soil	MG1
	Victoria (Tatura)	2007	4	Bovine	MG1
	Victoria (Tatura)	2005	1	Bovine	MG1
	Victoria (Byrneside)	1997	1	Bovine	MG1
	Victoria (Mooroopna)	1997	1	Bovine	MG1
	Victoria (Harston)	1997,2004	4	Bovine	MG3
	Victoria (Kyabram)	2009	1	Bovine	MG1
B	Victoria (Stanhope)	2007	17	Bovine	MG2
	Victoria (Stanhope)	2008	1	Bovine	MG2
	Victoria (Stanhope)	2009	1	Bovine	MG2
	Victoria (Wyuna)	2007	2	Bovine	MG2
	NSW (Berrigan)	2005	2	Bovine	MG2
C	Victoria (Swan Hill)	2002	1	Bovine	MG2
	NSW (Deniliquin)	2003	1	Bovine	MG2
D	NSW (Narrandera)	2002	1	Ovine	MG2
E	NSW (Narrandera)	2004	1	Ovine	MG2
F	Qld (Wandoan)	2002	1	Bovine	MG5
H	NSW (Rouchel)	2007/2008	2	Bovine	MG6
I	NSW (Moree)	1979	1	Bovine	MG6
J	WA (Walpole)	1994	1	Bovine	MG7
K	Qld (Rockhampton)	1993	1	Bovine	MG8

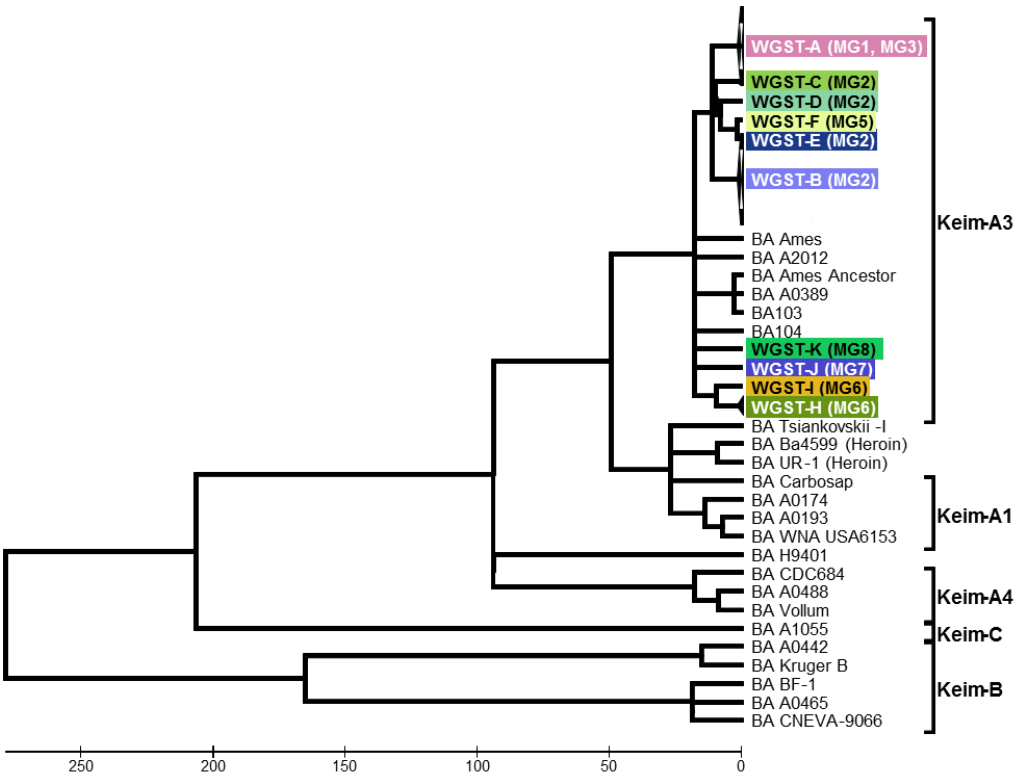

Figure 3. Maximum Parsimony analysis of WGST genotype taxa. The WGST genotypes are represented as WGST-A through to WGST-H. Genotype beginning with the code "BA" represent genome sequences available in GenBank. The evolutionary history was inferred using the Maximum Parsimony method. The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. The analysis involved 75 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1773 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011). The previously determined MLVA genotype is indicated in parentheses after the WGST genotype.

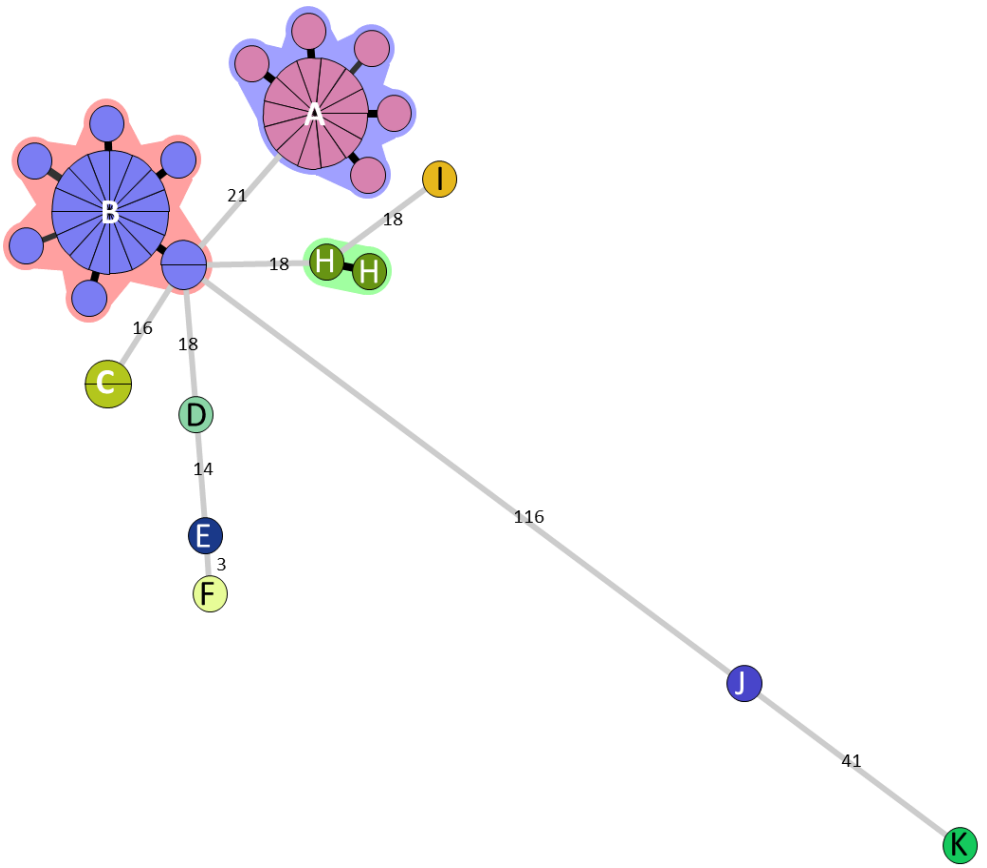

Figure 4. Fully parsimonious minimal spanning tree of 289 SNPs for 51 isolates of B. anthracis from Australia coloured by SNP group, letters within the nodes indicate the WGST genotype as defined in Table 2.1. Numbers on lines between nodes indicate the number of SNPs separating the nodes. Nodes that are separated by only one or two SNPs are indicate by black lines without a numeral (Nodes within WGST genotypes A, B and H).

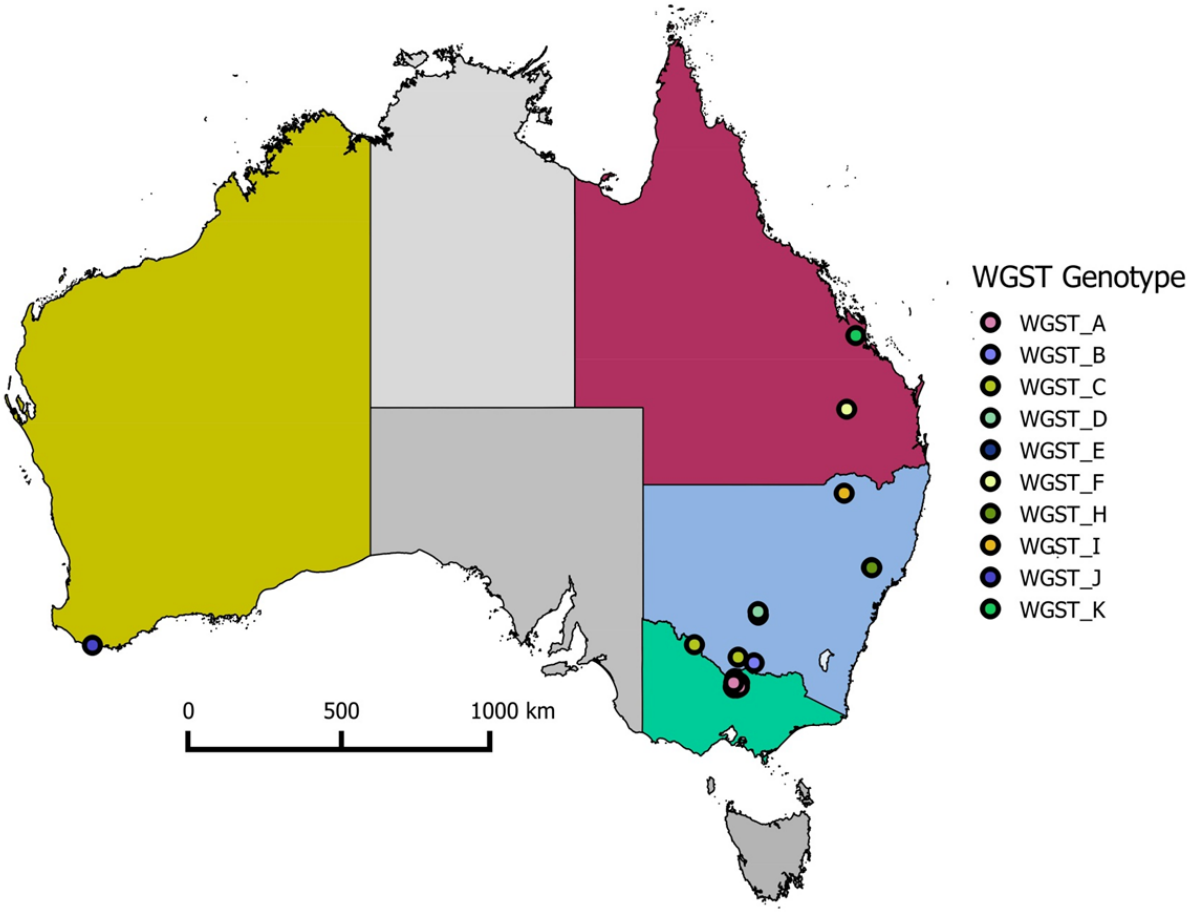

Figure 5. Geographical location of isolates colour-coded for WGST genotype within the National reference collection.

Figure 6. Geographical location of isolates within the Goulburn Valley area of Victoria colour-coded for WGST genotype within the National reference collection.

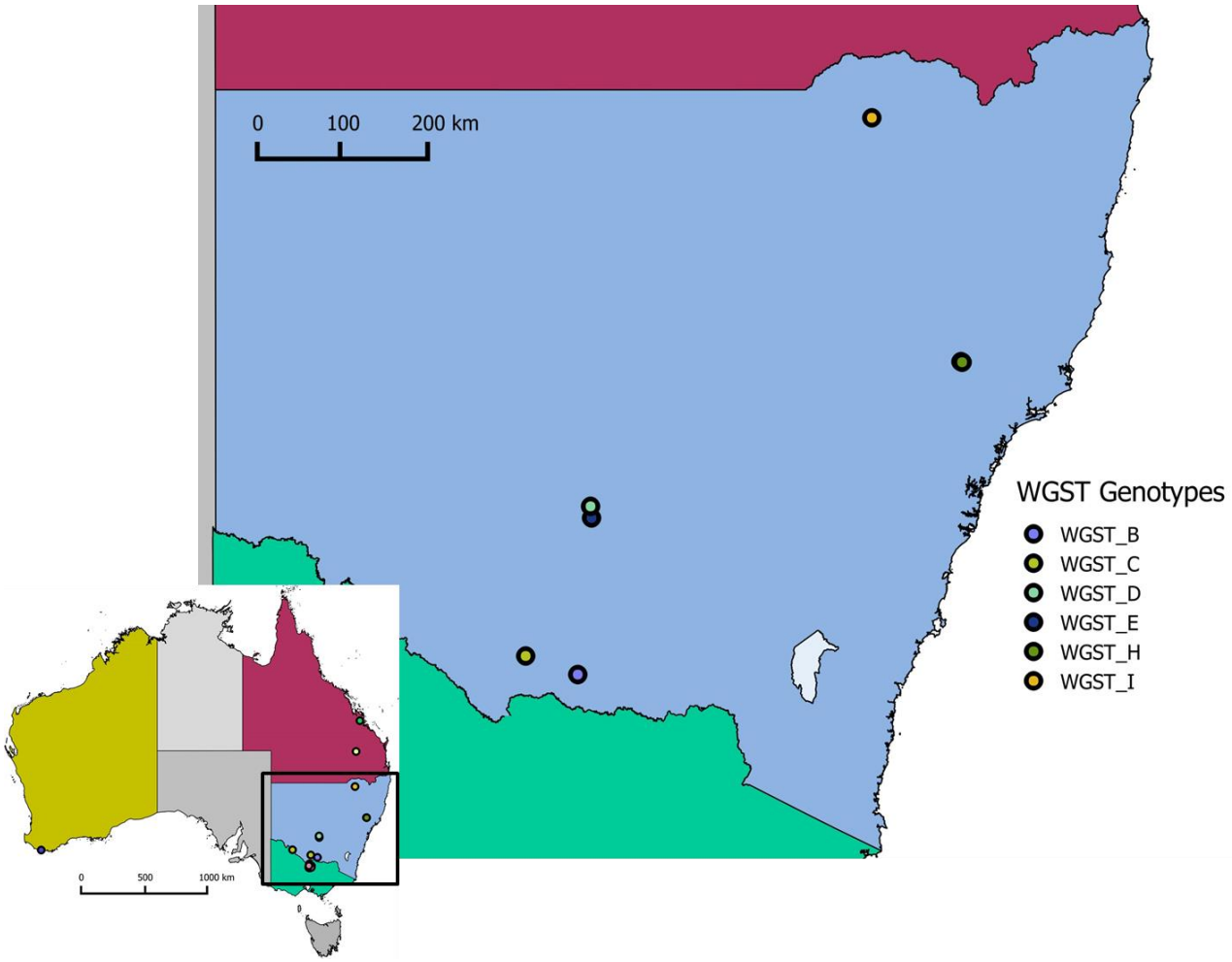

Figure 7. Geographical location of isolates within NSW colour-coded for WGST genotype within the National reference collection.

Table 4. Information on strains and genotypes from 2007 outbreak in Stanhope, Victoria

Property	Location	No. Strains Genotyped	WGST Genotype	MLVA Genotype
i	Stanhope	9	B1(7), B3(1), B4(1)	MG2
ii	Stanhope	1	B2	MG2
iii	Stanhope	2	B1	MG2
iv	Tatura	4	A1(2), A2(1), A3(1)	MG1
v	Stanhope	2	B1	MG2
vi	Stanhope	1	B1	MG2
vii	Stanhope	1	B5	MG2
viii	Stanhope	1	B1	MG2
ix	Wyuna	2	B1	MG2

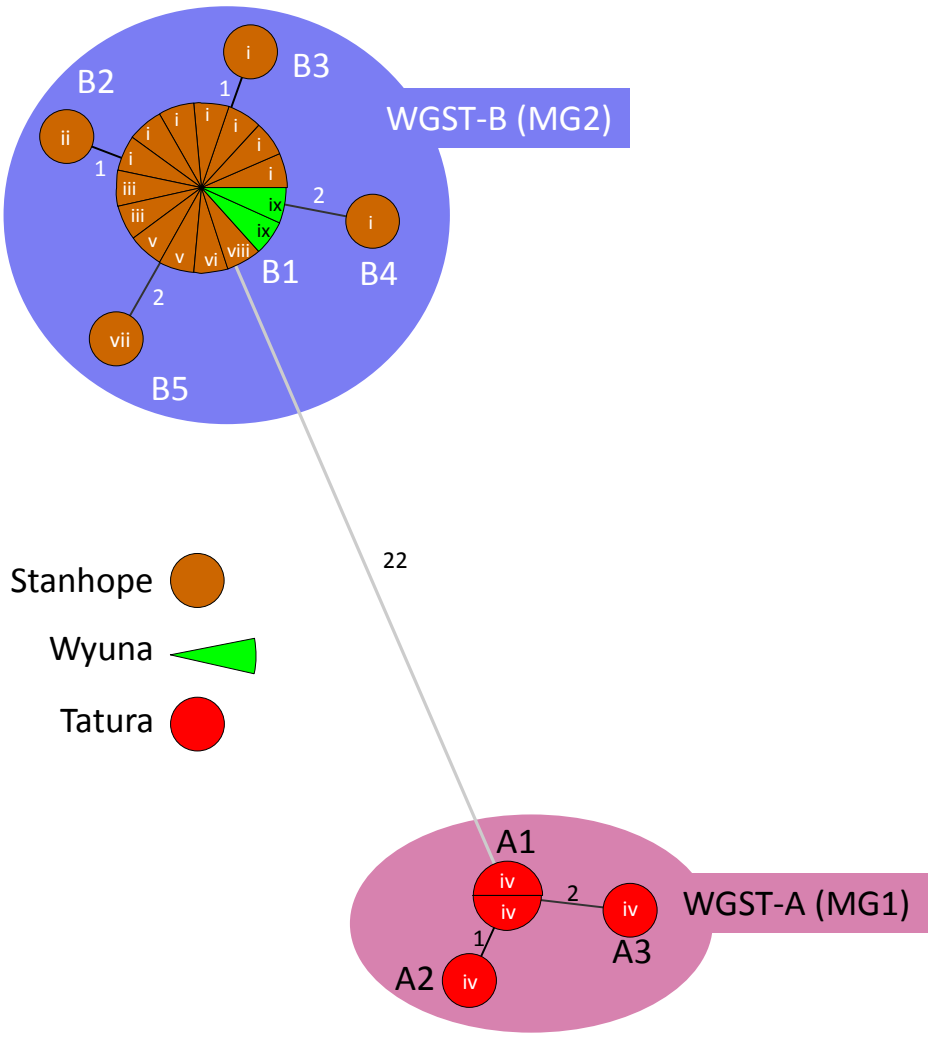

Figure 8. Fully parsimonious minimal spanning tree of 26 SNPs for 23 isolates of B. anthracis from the 2007 outbreak of disease coloured by origin of the strains (Stanhope – orange, Wyuna – green and Tatura – red). Numbers on lines between nodes indicate the number of SNPs separating the nodes. The roman numerals associated with each node indicate the property from which the strain was isolated (see **Table 4** for details).

A total of 23 isolates were genotyped by WGST from an outbreak of disease in Stanhope and Tatura in Victoria in 2007 (**Table 4**). All isolates from Stanhope (19 of 23) belonged to WGST genotype B with the remaining 4 isolates from Tatura belonging to genotype A. Within WGST genotype B are strains collected from 8 properties during the outbreak (**Table 4**, **Figure 8**) including isolates from property ix in Wyuna which is geographically distinct from the main centre of the outbreak in Stanhope.

3.4 Discussion

The Australian *B. anthracis* isolates genotyped in this study belong to the A3 group which has a worldwide distribution and has been described as the most important group of *B. anthracis* strains due to its wide distribution and prevalence (Keim *et al.*, 2000).

The majority of isolates genotyped using both MLVA and WGST fell into a monophyletic cluster of closely related isolates. MLVA genotypes within this monophyletic differ in one or two of the VNTR markers tested and the majority of isolates fall into two genotypes, MG 1 and MG 2, which differ by a single repeat unit in a single marker, BAMS22 (Table 2). One of these MLVA genotypes, MG 2, has a wide distribution within Australia being isolated from throughout the Anthrax belt. The closely related genotypes MG1, MG3 and MG4 are only found in Northern Victoria (**Figure 1**, Figure 9), MG5 in Southern Queensland and MG6 in the Hunter Valley, Moree and Cumnock (**Figure 1**). Given that *B. anthracis* was first introduced into NSW in 1847 (Seddon & Albiston, 1965) the

genotyping data suggests that a progenitor of this monophyletic group was introduced at that time and was spread throughout the anthrax belt along the stock routes. Overall, the low level of genetic diversity of *B. anthracis* isolates within the anthrax belt, identified by both MLVA and WGST appraces, is consistent with the relatively recent introduction of *B. anthracis* into Australia in 1847 in comparison to the relatively high level diversity of strains found in other areas of the world where the organism has been endemic for a longer period of time (Beyer *et al.*, 2012; Kenefic *et al.*, 2009; Smith *et al.*, 2000).

Based upon MLVA genotyping the greatest diversity of *B. anthracis* isolates is found in Northern Victoria and not NSW. Generally the diversity of a pathogen is accepted to be greatest in the area where the pathogen has been resident for the longest time which, based upon the history of anthrax within Australia, would suggest that *B. anthracis* isolates from within NSW should exhibit the greatest genetic diversity. However, only one MLVA genotype, MG2, was recognised within NSW. Genotyping of *B. anthracis* isolates employing the WGST approach was able to discriminate between isolates belonging to the MLVA MG2 genotype. All 4 of the WGST genotypes within the MLVA MG2 genotype are found in NSW and only one has been found in Victoria. Therefore, contrary to the MLVA genotyping data, WGST identified that the genetic diversity of isolates in NSW is greater than isolates from Victoria. Hence, the insight into the genetic diversity of *B. anthracis* in Australia allowed by WGST is consistent with the historical records which indicate *B. anthracis* was initially introduced into NSW in 1847. The WGST genotyping data is also consistent with the spread of the pathogen along stock routes in NSW (Seddon & Albiston, 1965) followed by limited localized differentiation within NSW, and later in Victoria, into closely related genotypes within the Anthrax Belt.

In contrast to the situation in NSW where MLVA genotype MG 2 could be subdivided into 4 WGST groups MLVA genotypes MG 1 and MG3 were indistinguishable clustering into a WGST A. Employing MLVA genotyping four genotypes were recognised in the Goulburn Valley region of Victoria (MG 1, MG 2, MG 3 and MG 4). It is important to note that due to technical difficulties the isolate belonging to MLVA genotype MG 4 was not genotyped using WGST. It is therefore unknown if this isolate would represent a third WGST in Victoria or alternatively if the WGST of this isolate would place it into one of the two WGST identified in Victoria, as was the situation with MLVA genotype MG 3 which was not able to be discriminated from MG 1 isolates by WGST. The four MLVA genotypes appear to be restricted to different geographic locations within the region (**Figure 9**). MLVA genotype MG 1 (WGST A) appears to be restricted to the area around Tatura and MG 2 (WGST B) to the area around Stanhope, MG 3 to the area around Harston and the single isolate belonging to the MG 4 genotype was isolated from Rushworth. The reason for this geographic stratification is unclear but may be related to the lack of movement of infected animals within the area after the diversification of the 4 MLVA genotypes.

MLVA genotyping also indicates that there have been other introductions of *B. anthracis* into Australia.

The most divergent genotypes identified within the Australian isolates sequenced were WGST genotypes J and K equivalent to MLVA genotypes MG 7 and MG 8 respectively. WSGT genotype J is represented by a single isolate from a disease incident in Walpole in WA and WSGT genotype K is represented by a single isolate from a disease incident Rockhampton in Queensland. To put the diversity of these strains in a worldwide context WGST genotype B strains vary by 14 SNPs from strain BA104 (Figure 1.1) which was isolated in Japan and by 40 SNPs from the Ames ancestor strain (Figure 1.1) which was isolated in China (results not shown). In contrast Genotype B and Genotype J are discriminated by 116 SNPs (Figure 1.2). Therefore, WGST genotype J (and K) is more divergent from other strains isolated in Australia than strains isolated from Japan and China. Isolates belonging to these genotypes differ considerably from the other six genotypes found in the collection and it is considered unlikely that these genotypes arose within Australia from the ancestral genotype postulated to have been introduced into Australia in the mid-1800s. Isolates belonging to these genotypes are also geographically isolated from Anthrax Belt being found in the south of Western Australia (Forshaw *et al.*, 1996) and the central coast of Queensland (Animal Health Australia, 2012). Anthrax has not been reported from these areas before or after these events and the sources of these outbreaks are unknown, although contaminated feed has been implicated in the Queensland outbreak (Animal Health Australia, 2012).

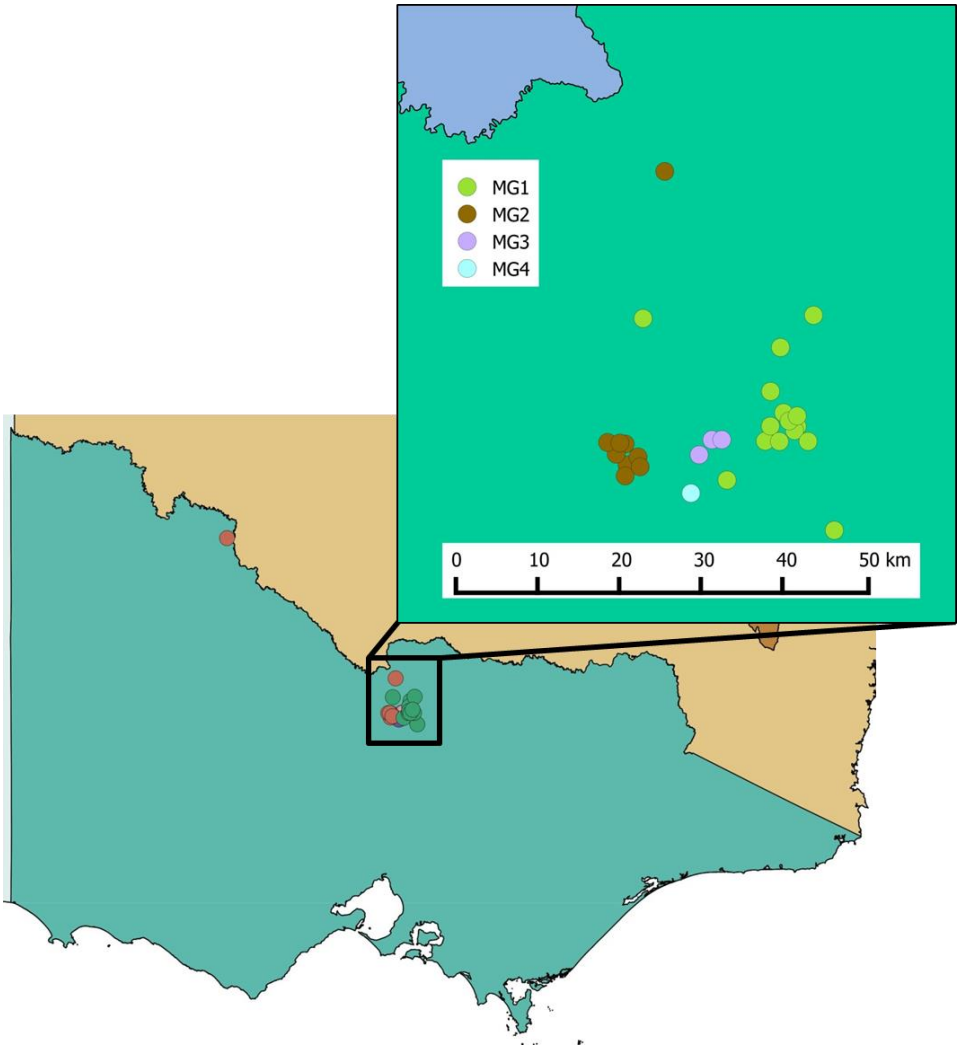

Figure 9. Distribution of MLVA genotypes within the Goulburn Valley region of Victoria

In 1997 a large disease outbreak occurred in a relatively small area of the Goulburn Valley in Victoria (Turner *et al.*, 1999b). It has also been reported that the outbreak was potentially initiated at a point source followed by spread of the disease by the social cohort of the dead animals licking and sniffing around the carcass (World Health Organization, 2008). The MLVA8 genotyping results of Keim *et al*. (2000) have been seen as supporting this hypothesis as the isolates genotyped from the outbreak area belonged to the same genotype (World Health Organization, 2008). However, Turner *et al* (1999a) noted that the available epidemiological evidence, gathered at the time of the outbreak, indicated that the spread of anthrax between farms was not the major generator of the outbreaks on individual farms. Therefore the epidemiological evidence indicated that the prevailing environmental conditions may have led to the emergence of *B. anthracis* from soil reservoirs to cause a series of concurrent parallel outbreaks as has previously been hypothesised for outbreaks of *B. anthracis* elsewhere (Keim *et al.*, 2004; Smith *et al.*, 2000). The presence of four MLVA25 genotypes associated with the outbreak suggests that the 1997 outbreak was a series of concurrent parallel outbreaks of disease. Indeed, a set of isolates collected from multiple farms at the same time during the 1997 outbreak were found to belong to multiple genotypes (results not shown). Smith *et al*. (2000) has also identified the existence of multiple genotypes in outbreaks of disease in Kruger National Park as has Beyer *et al*. (2012) in the Okaukuejo region of Etosha National Park in Namibia. In contrast Kenefic *et al*. (2008), and Garofolo *et al*. (2010) have found only a single genotype to be responsible for outbreaks in South Dakota and Southern Italy, respectively. Therefore, it is probable that environmental conditions at the time of the unusual outbreak led to a series of parallel outbreaks during 1997. These results may lead to a change in thinking on the management strategies employed to control such unusual outbreaks in Australia.

For example, it may be that a more extensive vaccination strategy is be required at the initiation of an unusual outbreak to encompass properties more distant to the initial infected property to account for parallel outbreaks of disease at more distant locations.

The MLVA and WGST typing data also questions the origin of the strain Australia94 (equivalent to strain 29/32, The Center for Applied Microbiology and Research, Porton Down, United Kingdom; K4834 (Keim *et al.*, 2000) and strain A0039 (Van Ert *et al.*, 2007a)) Australia94 is the genome sequence representative of the A.Br.Aust94 genetic group described by Keim *et al* (2000). Previous publications have indicated that this strain, and therefore by extension this genotype, originated in Victoria (Rasko *et al.*, 2005; Tourasse *et al.*, 2006). The MLVA25 genotype of Lista45, derived from the genome sequence of Australia94, is equivalent to MG 7 and WGST J in this study, this genotype is only found in Western Australia and not in Victoria. Tracing back the origin of this strain has revealed that the strain does originate from Western Australia (Martin Hugh-Jones, Louisiana State University and Peter Turnbull Pers. Comm.). Therefore the strain Australia94 (29/32, K4834, A0039) which has the same MLVA25 genotype as the MG 7 genotype described in the current study originated from Western Australia as would be expected for a strain of this genotype.

Of the two genotyping methods employed in this study WGST is more discriminatory than MLVA typing. WGST uses the entire genome to distinguish between strains at the single nucleotide level in comparison to the MLVA technique previously used which scans only 25 loci on the genome. WGST analysis of *B. anthracis* isolates was able to identify 4 genotypes (WGST genotypes B, C, D and E) within a single MLVA genotype (MG2) and two genotypes (WGST genotypes H and I) within MLVA genotype MG6. However, there was one case where WGST was unable to discriminate between 2 MLVA genotypes (MG 1 and MG3), it may be possible to discriminate these two MLVA genotypes using less stringent criteria for the definition of SNPs than that used in the present analysis.

The two most common WGST genotypes identified in this study were WGST A (18 isolates) and WGST B (22 isolates) as these genotypes contained the isolates from the major outbreaks of disease in Victoria in 1997 and 2007. WGST genotype A contained isolates primarily from the outbreak in Tatura in 1997 but isolates from the same region in 2004, 2005, 2007 and 2009. Similarly, isolates from the 2007 outbreak of disease in Stanhope and Wyuna predominate in WGST genotype B but isolates from the Stanhope area in 2008 and 2009 are also present in this genotype. Therefore, results from the WGST analysis have shown that organisms isolated from the same geographic area and, in some cases the same property, are indistinguishable by the WGST approach even when isolated years apart. This supports the hypothesis that *B. anthracis* remains dormant in the soil as a spore and is bought to the surface where it infects a susceptible animal (Hugh-Jones & Blackburn, 2009) and that little, if any, environmental cycling occurs. Isolates from one property (property i – **Table 4**) in 2007 and 2009 had exactly the same WGST SNP genotype indicating that there has been no change in the genome of these epidemiologically linked isolates during this time. Similarly, isolates from the 1997 outbreak in the Tatura region belong to WGST genotype A and isolates belonging to the same genotype were isolated from an affected property (property iv - **Table 4**) in 2007, indicating that over the period of 10 years there has been no change in the genome of these epidemiologically linked organisms. Therefore, based upon WGST as a measure of evolutionary diversity, there has been no evolution of *B. anthracis* within a defined geographic area over a defined time period. Hence, there has presumably been little, if any, vegetative growth or interaction with the soil microbial community which would be expected to drive evolutionary change. Also arguing for the maintenance of a stable genotype over time and space is the presence of isolates causing disease during the 2007 outbreak from geographically distinct properties in Stanhope (**Table 4** – properties i, iii, v, vi and viii) and Wyuna (**Table 4** – property ix). There is no known epidemiological link between the outbreaks of disease on these properties and it is therefore unlikely that disease on these properties is due to movement of contaminated material or animals. Therefore isolates without any identifiable variation caused disease in two geographically distinct regions at the same time indicating that this genotype is widely distributed and that animals on both of these properties independently came into contact with spores present in the soil. Similarly there was no identifiable variation within the genomes of isolates from Swan Hill in 2002 and Deniliquin in 2003 which belong to WGST genotype C and the isolates causing these outbreaks are therefore separated in both time and space. In contrast, strains from two

properties during the 2007 outbreak (properties i and iv in **Table 4**, **Figure 8**), while belonging to the same WGST genotype, exhibit a very small degree of SNP variation. It is possible that these SNP sub-genotypes were generated during the infection of the animal, but it is also possible that these SNP variants were already present in *B. anthracis* spores in the soil, or were generated during the minimal subculture of the strains which occurred during isolation and preparation of the genomic DNA. Therefore, if it is accepted that environmental cycling of *B. anthracis* in uncommon, the presence of these variants does indicate that SNPs may be introduced in a relatively short evolutionary time scale.

In conclusion, this work employing MLVA and WGST genotyping schemes has revealed that *B. anthracis* isolates from Australia are more genetically more diverse than previously thought. This study establishes a baseline of the genetic diversity of *B. anthracis* strains found in Australia that can be used for future epidemiological and forensic analyses allowing the identification of any further introductions of *B. anthracis* into Australia. The overall genetic diversity identified within the Australian population of *B. anthracis* is relatively low and the geographic distribution of related genotypes indicates that following introduction of a single genotype into New South Wales in the mid-1840s this bacterium was spread throughout the anthrax belt in Australia along the stock routes, as hypothesised by Seddon and Albiston (1965). The spread of the pathogen was then followed by limited localized differentiation into other closely related genotypes within the Anthrax Belt. However, unexplained occurrences of disease in areas outside of this anthrax belt, which are associated with different genotypes, indicate that separate introductions of *B. anthracis* into Australia have occurred.

4 Development of simplified diagnostic genotyping protocols

4.1 Introduction

Molecular subtyping schemes to differentiate between different strains of a bacterial pathogen require the detection of differences in the genome of isolates of the pathogen. As indicated above *B. anthracis* is a genetically monomorphic pathogen which requires highly discriminatory molecular genotyping techniques to differentiate strains. While it is possible to use the MLVA and WGST techniques described above to discriminate between *B. anthracis* isolates, this is time consuming and expensive and a more cost effective and rapid method is required. In order to enhance our ability to respond to emerging outbreaks of disease and enable real time identification of the genotype to which a *B. anthracis* isolate belongs simplified genotyping protocols are required.

Single nucleotide polymorphisms (SNPs) are the most common genetic variation found in genomes of all species. SNPs also represent important biologically informative DNA markers extensively used to elucidate phylogenetic relationships among worldwide strains, due to their evolutionary stability (Pearson *et al.*, 2004). By querying a large number of SNPs against collections of diverse strains, a set of canonical SNPs (canSNPs), that define major clades within the *B. anthracis* species, has been identified and used for subdividing all isolates into three major lineages (Van Ert *et al.*, 2007a). All strains of *B. anthracis* within the National Anthrax Reference Collection belong to the A.Br. Aust94 canSNP group defined by van Ert *et al* (2007a) (see **Figure 10**).Employing the comparative genomics approach focused on SNPs discovery described in section 3 above diagnostic SNPs were identified that specifically discriminate regional sub-groups of strains within the Australian population of *B. anthracis*. These diagnostic SNPs have been used to form the basis of an extended canSNP typing scheme to allow discrimination of the 10 WGST groups recognised within the Australian *B. anthracis* population (**Figure 10**).

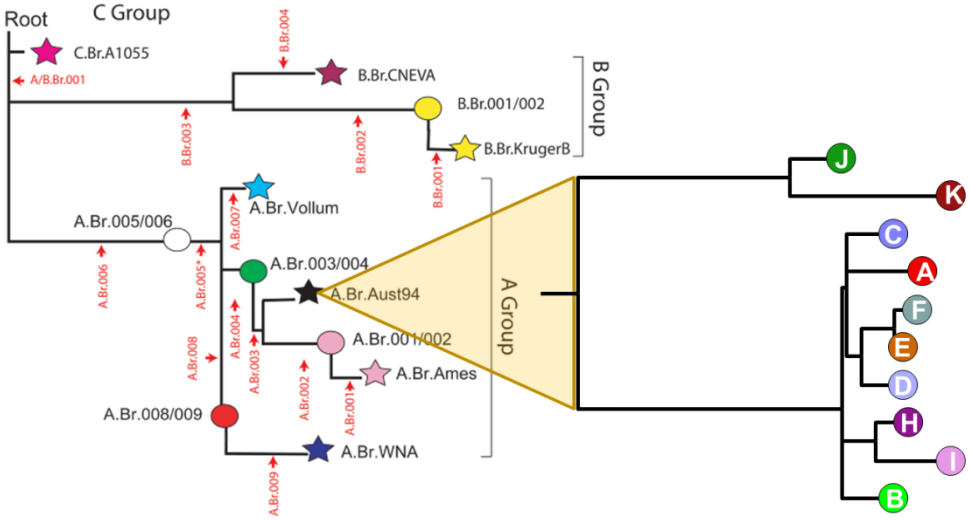

Figure 10. Genotyping scheme developed for the identification of Australian *B. anthracis* strains. As shown in the figure this scheme is an extension of the canSNP genotyping scheme proposed by Van Ert *et al*. (2007a).

4.2 Methods and materials

4.2.1 Identification of diagnostic single nucleotide polymorphisms

The comparative genomic sequence analysis carried out for the WGST (section 3 above) for the identification and filtering of SNPs was used to identify Australian *B. anthracis* specific canonical SNPs.

4.2.2 Allelic discrimination assay development

An allelic discrimination assay for the SNP-J+K (**Table 5**) was developed using the RealTimeDesign software available from Biosearch Technologies (Petaluma, CA, USA; https://www.biosearchtech.com/realtimedesign). Probe and primer combinations were designed with the probes labelled with either FAM or CAL Fluor Orange 560 and quenched using BHQ-1 *plus*. The forward primer SNP J+K_F (AAGAAATCATCACCAACTGCTACA) and reverse primer SNP J+K_R (TGCAGAGCAGTATTTGGGTAAA) were designed to amplify a 190bp fragment from all *B. anthracis* isolates. The SNP J+K_Allele 1 probe (FAM-TAACACCCG***C***TAACTGT- BHQ-1 *plus*) was designed to hybridise to *B. anthracis* isolates belonging to WGST-J and WGST-K. The SNP J+K_Allele 2 probe (CAL-Fluor-Orange-560 - AACACCCGTTAACTGTC - BHQ-1 *plus*) was designed to hybridise to all other *B. anthracis* isolates (note: probes are designed to hybridise to the antisense DNA strand). Each PCR was conducted in a total volume of 10µL containing 1 x ABI Fast Universal Master Mix, 100 nM of each probe, and 450 nM of each forward and reverse primer and 1 µL of template DNA. The thermal cycling parameters were 50°C for 2min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 65°C for 1 min. Thermal cycling and determination of endpoint fluorescent data was conducted on an ABI 7500 Fast real-time PCR machine.

4.3 Results

A total 125 SNPs which are specific for the different lineages recognised within the Australian population of *B. anthracis* were identified. A set of 11 SNPs were chosen which were descriptive for each lineage (**Table 5**, **Figure 11**).

Table 5. Australian *B. anthracis* specific canonical Single Nucleotide Polymorphisms identified for the differentiation of the 10 WGST recognised by whole genome sequencing.

	Single Nucleotide Polymorphism[a]										
SNP Group/ Lineage	SNP-A 4680141	SNP-B 1849607	SNP-C 74760	SNP-D 731194	SNP-E+F 324694	SNP-F 1657942	SNP-H+I 429101	SNP-H 2076082	SNP-I 773524	SNP-J+K 497550	SNP-K 162789
WGST-A	**G**	C	C	C	T	T	C	A	T	A	C
WGST-B	A	**T**	C	C	T	T	C	A	T	A	C
WGST-C	A	C	**T**	C	T	T	C	A	T	A	C
WGST-D	A	C	C	**T**	T	T	C	A	T	A	C
WGST-E	A	C	C	C	**C**	T	C	A	T	A	C
WGST-F	A	C	C	C	**C**	**C**	C	A	T	A	C
WGST-H	A	C	C	C	T	T	**T**	**G**	T	A	C
WGST-I	A	C	C	C	T	T	**T**	A	**C**	A	C
WGST-J	A	C	C	C	T	T	C	A	T	**G**	C
WGST-K	A	C	C	C	T	T	C	A	T	**G**	**T**

[a] The number below the SNP designation indicates the position on the reference chromosome (GenBank number - CP001598)

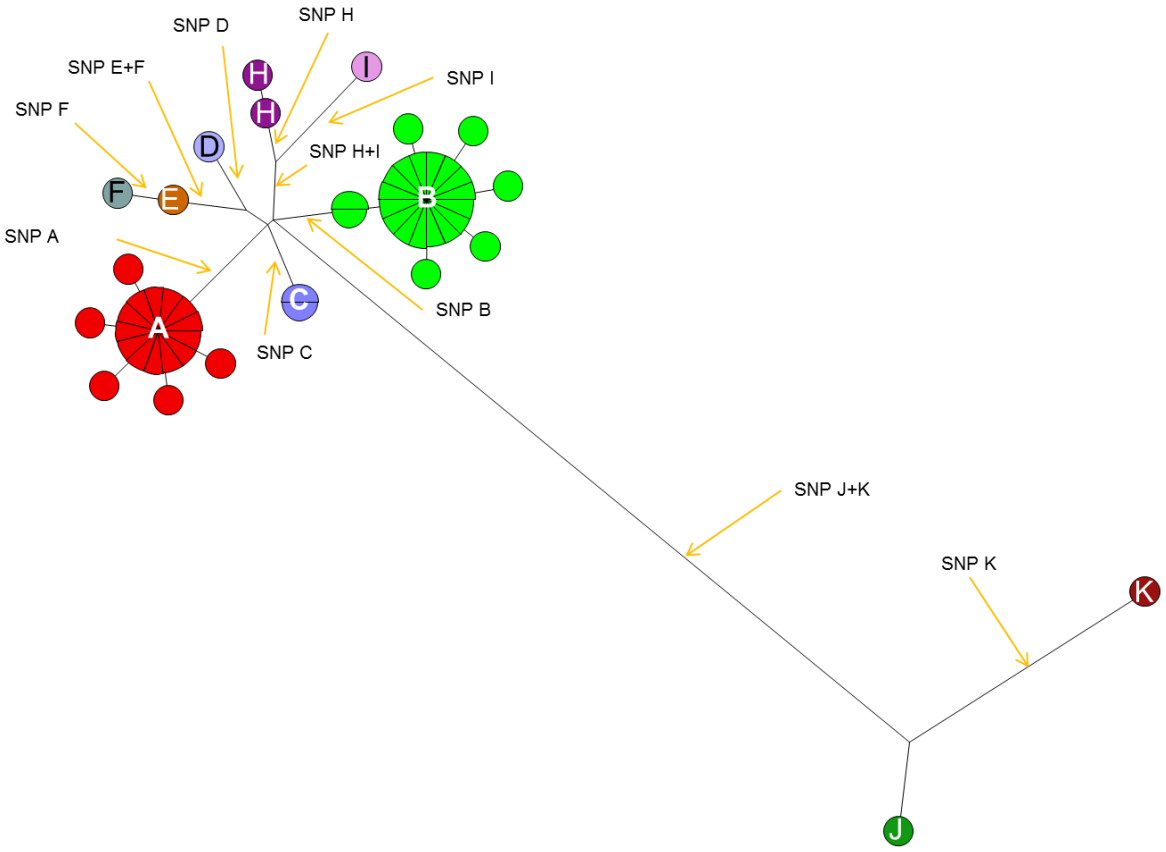

Figure 11. Representation of the Australian *B. anthracis* specific canonical Single Nucleotide Polymorphism scheme. Each Australian canSNP is mapped onto a maximum parsimony phylogenetic tree indicating the branch for which each SNP is specific.

The Allelic discrimination assay that was developed to identift isolates belonging to the WGST J and K was able to discriminate between the isolates belonging to the WGST-J and K and representatives of all other WGST groups.

4.4 Discussion

While it is possible to use the MLVA and WGST techniques to discriminate between *B. anthracis* isolates, Application of these techniques to a large number of strains is time consuming and expensive. Therefore the development of simplified, more cost effective and rapid methods to accurately genotype *B. anthracis* strains from Australia is required.

Van Ert *et al*. (2007a) have shown that a select number of SNPs representative of specific branches and nodes in a SNP-based phylogenetic analysis of *B. anthracis* are sufficient to accurately determine the phylogenetic position of any newly isolated *B. anthracis* strain. Therefore, a small number of canSNPs which define key phylogenetic junctions along the *B. anthracis* SNP tree are able to replace the tedious and costly genome-wide SNP analysis. The canSNP scheme for Australian *B. anthracis* isolates is an extension of the canSNP scheme of Van Ert *et al*. (2007a). Under the canSNP scheme described by Van Ert *et al*. (2007a) all *B. anthracis* isolates within the National Anthrax Reference Collection fall into the A.Br.Aust94 canSNP group. Unfortunately this group is not as well defined as other canSNP groups as there is no specific canSNP associated with the A.Br.Aust94 canSNP group (see **Figure 10**). Hence, the strains that cluster in this group are more diverse than is the case for other canSNP groups. The large diversity encompassed within the A.Br.Aust94 group is exemplified by the 116 SNPs which separate WGST-J and K and the remaining WGST groups (**Figure 4**).

The identification of single SNPs which uniquely define the genotype of Australian *B. anthracis* isolates, as recognised by WGST, enables the development of single assays for each group negating the need to complete more costly and time consuming full genotype analysis. This has been shown for the WGST J+K branch where an allelic discrimination assay has been developed. This assay can be completed within 3 hours instead of the days to weeks required for a full genotype analysis to be completed.

The simplified diagnostic genotyping scheme proposed here, for the differentiation of Australian *B. anthracis* strains, will be of use in the investigation of future outbreaks of disease in Australia and allow rapid genotyping of any future strains deposited in the collection. Rapid identification of strain genotype is an important step in establishing the origin of the strain by determining if the strain is known to be ecologically established within the region, represents a movement of a strain from within Australia or potentially the introduction of a completely new exotic strain.

The application of these simplified techniques will enhance our ability to respond to emerging outbreaks of disease and enable real time identification of the genotype to which a *B. anthracis* isolate belongs.

5 Determination of spore survival and dispersal by analysing soil collected from contaminated farms.

5.1 Introduction

B. anthracis is relatively easily isolated from fresh clinical specimens by culture onto sheep blood agar and identification by the formation of typical colonial morphology and the lack of haemolysis. However, isolation from environmental samples can be notoriously difficult due to the presence of other microorganisms especially closely related species such as *Bacillus cereus*, *Bacillus mycoides* and *Bacillus thuringiensis* (Turnbull, 1999). The large and diverse microbial load of environmental samples, especially in soil, and the likelihood that *B. anthracis* spores are present in low numbers, necessitates the use of heat treatment or ethanol shock to eliminate other vegetative bacteria and select for spores prior to plating on media (Dragon & Rennie, 2001). However, even after such treatments, the isolation of *B. anthracis* is still difficult because of the

presence of spores of other *Bacillus* spp. (Marston *et al.*, 2008). Therefore, selective media are generally employed to improve the efficiency of isolation of *B. anthracis* culture from soil samples (Dragon & Rennie, 2001). Heat or ethanol shocking of soil samples form the isolation of *B. anthracis* from soil serves the dual purpose of inducing spore germination and destroying vegetative cells of other contaminating bacteria. Despite culture and isolation being labour intensive and more time consuming in comparison to the direct application of molecular methods, culture is still considered by many to be the most sensitive method for detection of *B. anthracis* from soil (Gulledge *et al.*, 2010). Following culture, the identification of *B. anthracis* by morphology and biochemical profiling alone is also problematic due to the presence of morphologically and biochemically similar *Bacillus* spp. Therefore, molecular tools targeting virulence genes that are encoded on plasmids pXO1 and pX02 (Wielinga *et al.*, 2011) are essential for identification of *B. anthracis*.

The most commonly used method for the isolation of *B. anthracis* from soil is the OIE standard method (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.01_ANTHRAX.pdf). More recently Fasanella *et al*. (2013) have described the Ground Anthrax Bacillus Refined Isolation (GABRI) method for isolation of *B. anthracis* from the soil environment. The GABRI method is reported to be more sensitive than the OIE method was therefore used as a basis for the isolation of *B. anthracis* from soil in Australian studies.

The purpose of this study was to confirm iv viable *B. anthracis* spores were still present in soil collected from farms involved in the 2007 outbreak of Anthrax in Stanhope.

5.2 Methods and materials

5.2.1 Sampling sites

Soil was sampled from 35 sites on 4 properties (

Table 6) which were involved in the 2007 outbreak of anthrax in the Goulburn Valley. Soil samples had previously been collected from these sites 6 months after the outbreak of disease and tested for the presence of *B. anthracis* spores, and a number of these sites tested positive for the presence of *B. anthracis* spores (NARL, Unpublished,

Table 6). The GPS coordinates of these sites had also been established in 2007 making relocation of these sites possible.

Where possible a 3m x 3m area was sampled for each site. This area was broken into 9 x 1m squares. From each 1m square a corer was used to collect 5, 2 x 10cm, cylindrical soil cores which were pooled (approximately 200-300g of soil per 1m square). A total of 9 x 200-300g soil samples were collected per site (Figure 10). Due to the position of some sites it was not always possible to collect 9 samples per site. Also, at times further samples were taken from a site when the evidence on the ground (such as the presence of a depression) suggested that the GPS position may not be entirely accurate. Overall a total of 306 soil samples were processed (

Table 7).

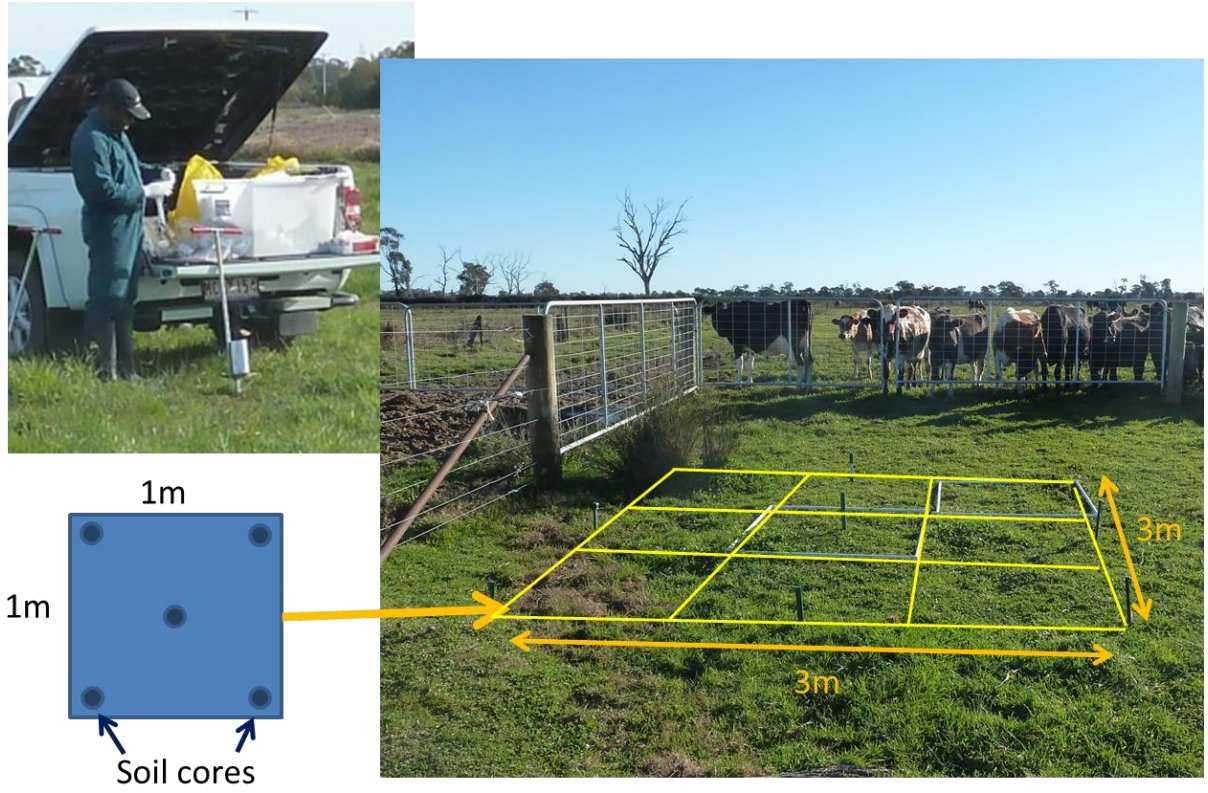

Figure 12: Soil sampling methodology

5.2.2 Modified GABRI method for isolation of *B. anthracis* from soil

The GABRI method for the isolation of *B. anthracis* from soil was initially used as described by Fasanella *et al*. (2013). However, initial testing of soils spiked with *B. anthracis* Sterne strain indicated that the level of overgrowth of the semi-selective medium was too great to allow isolation of *B. anthracis*. In order to decrease the level of overgrowth by competing organisms, modifications to the procedure were trialled. Initially sheep blood was omitted from the semi-selective TMSP medium in order reduce the nutrient available for the growth of competing microorganisms. The removal of sheep blood from the medium did not affect the growth of *B. anthracis* Sterne strain but did inhibit the growth of the competing soil flora. Following this three further modifications were trialled; increasing the heat treatment from 64°C to 75°C, removing the heat inactivation step and introducing a 100% ethanol wash in its place, removing the heat inactivation step and introducing a 100% ethanol wash in its place followed by centrifugation and re-suspension of the pellet in sterile water. Of these modifications the simplest and most effective was the introduction of an ethanol wash (**Appendix 6**) and the final modified procedure employed to isolate *B. anthracis* is outlined in **Figure 13**. Once putative *B. anthracis* colony forms were identified on the initial isolation plate they were subcultured onto sheep blood agar plates and incubated for 24h at 37°C. The presence of *B. anthracis* in a soil sample was confirmed by the formation of typical *B. anthracis* colony forms on sheep blood agar and a positive PCR result using the PCR described by Wielinga *et al*. (2011).

To improve the sensitivity of the detection of *B. anthracis* further a Bio-PCR step was also included. Once the 10 plates of the semi-selective TMSP medium had been examined for the presence of typical colonies of *B. anthracis* and these had been subcultured, growth from all 10 plates was resuspended in approximately 10 mL of sterile water. A 1 mL aliquot of the cell suspension was then boiled for 10min and centrifuged at 15000 rpm in a microcentrige to remove

the cell debris. A 2µL volume of this boiled cell lysate was then used as template in the PCR described by Wielinga *et al*. (2011).

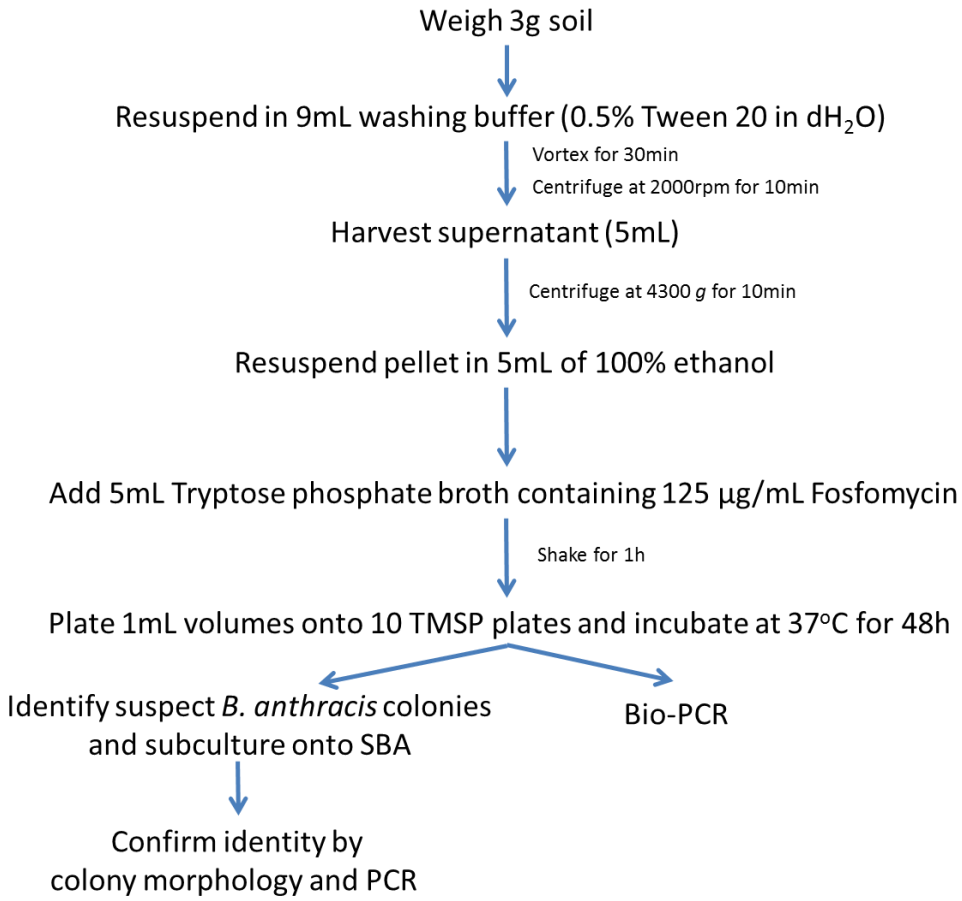

Figure 13. Outline of the Modified GABRI method for detection and isolation of *B. anthracis* from soil.

5.3 Results

Four properties were targeted in this study. A number of sites were sampled on each four properties targeted in this study. Property 2 had 20 sites sampled, property 3 had 6 sites sampled, property 6 had 4 sites sampled and property 7 had 5 sites sampled (

Table **6** and

Table 7). At least 1 site from all 4 properties from which soil samples were obtained tested positive for the presence of *B. anthracis* (

Table **6** and

Table 7). From property number 7 *B. anthracis* was isolated from one (site 48) of the 5 sites sampled. The strain isolated from site 48 tested negative for the pX02 marker by PCR (Wielinga *et al.*, 2011) indicating that this strain is non-pathogenic. Two of the three sites sampled on property 6 tested positive for the presence of *B. anthracis* (sites 36 and 37). However, *B. anthracis* was only successfully cultured from site 37, site 36 tested positive by Bio-PCR only. Similarly two of the 6 sites on property 3 tested positive for *B. anthracis* (sites 26 and 30). However, *B. anthracis* was only successfully isolated from one of these two sites (site 30) with site 26 being positive by Bio-PCR only. Of the 20 sites tested on property 2, *B. anthracis* was successfully cultured from 3 sites (Sites 6, 14, 18 and 52) and a further 3 sites tested positive by Bio-PCR only (sites 5, 10 and 21) (

Table **6** and

Table 7). The number of samples positive within each site varied from zero to ten positive samples for each site (

Table 7 and **Appendix 5**). When a site was positive generally the samples which tested positive clustered together (**Appendix 5**). For example for sites 36 and 37 which were adjacent death and burn sites for the same animal in 2007 (

Table 6) adjacent 1m squares tested positive for the presence of *B. anthracis* (see **Appendix 5**)

Sites on these four properties were targeted for study as previous work, employing the OIE method for isolation of *B. anthracis* from soil had been performed 6 months after the 2007 anthrax outbreak. Results from the 2007 sampling indicated that a number of sites on these properties were contaminated with *B. anthracis*. (see

Table 6). However, many of the sites that tested positive for *B. anthracis* in 2007 were found to be negative for the presence of *B. anthracis* in 2013 (for example sites 27 and 47;

Table 6). Conversely, sites which tested negative for *B. anthracis* in 2007 subsequently tested positive for *B. anthracis* in 2013 (for example sites 14 and 30;

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Table 6)

Table 6: Site description and comparison of results of soil testing carried out in 2007 and 2013/2014

Property	Site number	Death date	Site type	Description of Site	2007[a] Results	2013[b] Results
7	47	6/02/2007	Death	Death site for #254	POS	NEG
	48	6/02/2007	Burn	Burn site for death	NEG	POS
	49	12/02/2007	Death	Death site for #492 Hereford heifer yearling estimate of death site from GPS	NEG	NEG
	50	12/02/2007	Death	Death site for AngusX steer; estimate of death site from GPS	NEG	NEG
	51	12/02/2007	Burn	Burn site for #492 and AngusX steer	NEG	NEG
6	36	2/02/2007	Death	Death site for Friesian cow 798	POS	POS
	37 & 37a[c]	2/02/2007	Burn	Burn site for Friesian cow 798	POS	POS
	38	Unknown	Burn	Burn site for Friesian cow 865 (Death occurred in dam and carcase found 06/02	NEG	NEG
3	26	31/01/2007	Carcase contact	Carcase Jersey cow 990 brought here for pickup by knackery	POS	POS
	27	Unknown	Burn	Burn site for Jersey cow 989	POS	NEG
	29	12/02/07	Burn	Burn site for cow that died	NEG	NEG
	30	12/02/2007	Death	Estimate of death location evidence of soil scraping large area	NEG	POS
	31	17/02/07	Burn	Burn site	NEG	NEG
	32	17/02/07	Death	Death site. GPS point some metres away; but went with large soil scraped area adjacent to burn site	NEG	NEG
2	2	28/01/2007	Death	Death site for Jersey heifer calf 053 or Jersey bull calf 143	NEG	NEG
	3	27/01/2007	Death	Death site for Jersey heifer calf 053 or Jersey bull calf 143	POS	NEG
	4	19/01/2007	Death	Death site for Jersey cow 413	NEG	NEG
	5	19/01/2007	Death	Death site for Jersey cow 331	POS	POS
	6	Unknown	Burn	Burn site	POS	POS
	8	23/01/2007	Death	Death site for Jersey/Friesian cow 301	NEG	NEG
	9	20/01/2007	Death	Death site for Jersey/Friesian cow. Estimate (from farmer's memory). Cow died in general area.	NEG	NEG
	10	27/01/2007	Death	Death site for Jersey/Friesian cow 303	POS	POS
	12	24/01/2007	Death	Death site for Jersey cow 105	NEG	NEG
	13	22/01/2007	Death	Death site for Jersey cow 116	NEG	NEG
	14	26/01/2007	Death	Death site for Jersey cow 319	NEG	POS
	16	29/01/2007	Death	Death site for Jersey X cow 320	NEG	NEG
	18	18/01/2007	Death	Death site for Jersey cow 98	NEG	POS
	19	30/01/2007	Death	Death site for Jersey cow 143	NEG	NEG
	20	18/01/2007	Death	Death site for Jersey/Friesian cow 314. Area not sprayed with formalin	NEG	NEG
	21	18/01/2007	Death	Death site for Jersey/Friesian cow 315	NEG	POS
	22	18/01/2007	Death	Death site for Jersey cow 97. Area not sprayed with formalin	NEG	NEG
	23	21/01/2007	Death	Death site for Jersey cow 419	NEG	NEG
	24	20/01/2007	Death	Death site for Jersey/Friesian cow 302	NEG	NEG
	52	2009	Death	Death Site	Not Tested	POS

[a] POS= Site positive for the presence of *B. anthracis*; NEG= Site negative for the presence of *B. anthracis*.

[b] Sites highlighted in red tested positive by culture and Bio-PCR. Sites highlighted in orange tested positive by Bio-PCR only. The site highlighted in blue tested positive by culture only, no-Bio-PCR was performed, and the strain isolated was non-pathogenic lacking the pX02 plasmid.

[c] Site 37a is an extra 1m square from which soil was sampled 1m east of the site 37. This site was targeted due to the presence of a depression indicating this may have been a “dig out” associated with decontamination of the site in 2007

Table 7. Results of soil testing carried out in 2013/2014

Property	Site number	Number of samples	Overall Site Result	Sample Result[a]										
				1	2	3	4	5	6	7	8	9	10	11
7	47	11	Negative	n	n	n	n	n	n	n	n	n		
	48	9	**Positive**	n	n	n	n	n	n	n	n	C*[b]		
	49	9	Negative	n	n	n	n	n	n	n	n	n		
	50	9	Negative	n	n	n	n	n	n	n	n	n		
	51	9	Negative	n	n	n	n	n	n	n	n	n		
6	36	9	**Positive**	n	n	n	P	n	n	n	n	P		
	37	11	**Positive**	P	P	P	P/C	P/C	P/C	P	P/C	P/C	P	n
	37a	1	**Positive**	P/C										
	38	11	Negative	n	n	n	n	n	n	n	n	n	n	n
3	26	4	**Positive**	n	n	P	n							
	27	9	Negative	n	n	n	n	n	n	n	n	n		
	29	9	Negative	n	n	n	n	n	n	n	n	n		
	30	9	**Positive**	n	n	n	P/C	P	n	P	P/C	n		
	31	9	Negative	n	n	n	n	n	n	n	n	n		
	32	9	Negative	n	n	n	n	n	n	n	n	n		
2	2	8	Negative	n	n	n	n	n	n	n	n			
	3	9	Negative	n	n	n	n	n	n	n	n	n		
	4	9	Negative	n	n	n	n	n	n	n	n	n		
	5	9	**Positive**	P	n	P	n	P	P	P	P	P		
	6	9	**Positive**	P	P	P	P	P	P	P	P	P/C		
	8	9	Negative	n	n	n	n	n	n	n	n	n		
	9	9	Negative	n	n	n	n	n	n	n	n	n		
	10	9	**Positive**	n	n	n	n	P	n	n	n	n		
	12	9	Negative	n	n	n	n	n	n	n	n	n		
	13	9	Negative	n	n	n	n	n	n	n	n	n		
	14	9	**Positive**	n	n	n	n	n	n	P/C	n	P/C		
	16	9	Negative	n	n	n	n	n	n	n	n	n		
	18	9	**Positive**	n	P/C	P/C	P/C	P/C	n	n	n	n		
	19	9	Negative	n	n	n	n	n	n	n	n	n		
	20	9	Negative	n	n	n	n	n	n	n	n	n		
	21	9	**Positive**	P	P	P	n	n	n	n	n	n		
	22	9	Negative	n	n	n	n	n	n	n	n	n		
	23	9	Negative	n	n	n	n	n	n	n	n	n		
	24	9	Negative	n	n	n	n	n	n	n	n	n		
	52a[2]	9	**Positive**	n	n	n	n	n	n	n	n	P/C		

[a] Sample results: n=negative, P=Bio-PCR positive, P/C=Bio-PCR and Culture positive, C*=Culture positive but no Bio-PCR performed on sample, [b] Extra site from animal that Died in 2009 on Farm 2

5.4 Discussion

The isolation of *B. anthracis* from death, burn and carcase contact sites from cattle that died in the 2007 outbreak of disease in the Goulburn Valley indicates that spores have remained viable at these sites for at least 7 years and that these sites may be important sources of *B. anthracis* for

future reinfection of animals leading to new disease outbreaks. The results also indicate that decontamination procedures carried out at the time were not totally successful even though it would be assumed that the initial load of *B. anthracis* in the environment was significantly reduced.

The inability to isolate *B. anthracis* from some sites that previously tested positive for *B. anthracis* six months after the 2007 outbreak may indicate that *B. anthracis* was not able to survive for long periods in this soil. However, it is also possible that the inability to detect the presence of *B. anthracis* in these samples is due to a sampling problem, where the organism is unevenly distributed in the soil and the sampling protocol employed did not collect soil containing the *B. anthracis* spores. It is also possible that the GPS co-ordinates for some of the sites may have been incorrect or the accuracy of the GPS unit used to identify the sites was not sufficient to accurately locate the site. It is known that the GPS units employed are accurate to within approximately 2m which may mean that the soil sampling site was not well targeted and the actual site of interest was not sampled. Alternatively it is possible that the number of *B. anthracis* spores present in the soil may fluctuate over time and the ability to detect the presence of spores in soil is affected by the time that soil samples are collected. Lindeque and Turnbull (1994) performed longitudinal sampling of soil from death sites over a 14 to 60 month period. At times during the sampling period *B. anthracis* was not able to be cultured but was able to be cultured at a subsequent time point, similar to the results obtained in the present study.

The isolation of a non-pathogenic *B. anthracis* strain from site 48 on property 7 can be attributed to the loss of the pX02 plasmid and this is not an uncommon observation. Turnbull *et al*. (1992) identified a number of *B. anthracis* strains isolated from soil where the pX02 plasmid was lacking indicating a progressive loss of the pX02 plasmid over a 5 to 8 year period.

The use of Bio-PCR led to the identification of some soil sites and samples that were positive for *B. anthracis* which would not have been identified if culture alone was used. However, the Bio-PCR results should be interpreted with some caution. The Bio-PCR will amplify template DNA from non-viable spores and a positive result does not indicate that all three markers are co-located in a single organism only that they are present in the boiled cell lysate of the total growth present on the isolation plates. For example when a soil sample, spiked with the Stern strain which lacks the pX02 plasmid, was tested using Bio-PCR all three markers, including the pX02 plasmid marker, were amplified. Taken in isolation this result would indicate that this sample contained a fully virulent *B. anthracis* strain. However, further testing of this sample, without the sample being spiked with the *B. anthracis* Sterne strain, indicated that the growth on the GABRI semi-selective plates contained an organism which cross-reacted with the PCR for the pX02 marker. Therefore the results of Bio-PCR directly on the culture lysate can only be taken as indicative of the presence of virulent *B. anthracis* in the soil samples. Even taking this potential issue into account the Bio-PCR step is a valuable adjunct to the isolation of *B. anthracis* from soil using the GABRI method. It is also considered that a Bio-PCR positive, but culture negative, sample is indicative of the presence of *B. anthracis* in the sample at a level below that for which it is possible to isolate *B. anthracis* from the sample. The Ct values obtained for samples where no cultured representative was identified were higher than for samples from which a cultured representative was isolated (results not shown). This indicates that there is a lesser amount of starting template in these samples and therefore potentially a lower number of spores present. It is also apparent from the pattern of Bio-PCR positive samples in relation to samples from which *B. anthracis* was isolated (**Appendix 5**) that Bio-PCR positive, but culture negative, sites surround the culture positive sites (**Appendix 5**) this may be interpreted to indicate that *B. anthracis* is present in these sites but below the level of detection by the GABRI method.

In conclusion, despite the relatively low proportion of positive sites, there are sites on each property that have been contaminated with viable *B. anthracis* spores for at least 7 years after the last outbreak. The long-term survival of spores in the environment continues to pose a risk of exposure of livestock to *B. anthracis* once the environment becomes contaminated with the pathogen. For this reason, it is important to determine environmental contamination by the spores so as to enable better assessment of the risk of exposure to animals. Further sampling from these high-risk areas and improvements in sensitivity of protocols used to detect the presence of *B.*

anthracis spores may provide a better picture of spore contamination at these sites. Molecular typing of isolates collected from these sites may also yield a more detailed picture of the epidemiology of *B. anthracis* strains in the Goulburn valley area. The isolation of *B. anthracis* from soil samples is essential in linking cases of anthrax to their source (Marston *et al.*, 2008). This information may be useful not only in constructing models predicting future anthrax outbreaks and their spread, but may also help in focusing control measures on the areas or properties identified to be contaminated with *B. anthracis* spores.

6 Increase the utilisation and evaluate the performance of the anthrax ICT and implement any refinement for conditions of manufacture and use

6.1 Introduction

In the veterinary and medical setting diagnostic test results form the basis on which many important decisions are made. In the case of anthrax, acquiring rapid test results enables prompt commencement of quarantine, tracing and control of stock movements, and the implementation of timely control strategies to minimise the spread and impact of disease. For anthrax, time to disease diagnosis is important due to the peracute nature of disease, zoonotic potential, persistence of spores in the environment and trade implications surrounding outbreaks. It is also important for occupational health and safety, to rule out anthrax as a potential cause of death before in-field necropsy of the carcass. The anthrax Immunochromatographic test (ICT) is a rapid, simple and accurate in-field diagnostic test that detects the protective antigen component of the anthrax toxin in the blood of an animal that has died from anthrax. The ICT was developed by the United States Naval Medical Research Center (USNMRC) (Burans *et al.*, 1995; Muller *et al.*, 2004) and were provided to us through transfer agreements and collaborative arrangements since 2000.

To determine the performance of the ICT, clearly defined groups of diseased and non-diseased animals were identified that were representative of the end population for which the test would be used. Whole blood samples were collected from 916 cattle sourced from Victoria and New South Wales over a period of 10 years (2001 to 2010). Each sample was tested using the ICT, in-field and blood samples sent to the National Anthrax Reference Laboratory (NARL) for confirmatory bacterial culture. The diagnostic specificity and sensitivity of the test is estimated to be 100% [*Sp* 100% (99.56 to 100%; 95% CI) and *Se* 100% (91.72 to 100%; 95% CI)] (Muller *et al*. 2014, submitted).

The ICT is a lateral flow device that produces a clear positive and negative results (Refer to

Figure **14**) which has definite advantages over the traditional and molecular methods for diagnosis. The test can be performed in-field, has no demands for specialised equipment, requires only basic training in its use, and has results available within 15 minutes.

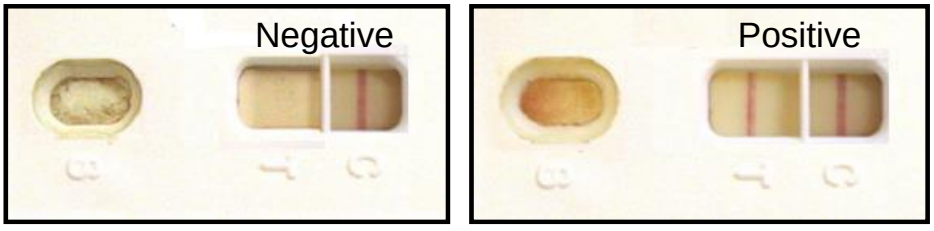


Figure 14. The anthrax immunochromatographic test, demonstrating both a negative and positive result.

Since 2009, the NARL has been granted the rights to produce the anthrax ICT for distribution throughout Australia. Since 2010 the NARL has been producing these kits in-house and providing

them to our Australian veterinary colleagues on a cost recovery basis for the in-field diagnosis of anthrax. During the term of this project one batch (AU3) of kits has been produced and distributed and a further batch (AU4) has been produced and will be distributed later this year.

6.2 Increased distribution of ICT kits to field veterinarians throughout Australia

The ICT kits produced by the NARL have been distributed to Victoria, New South Wales, South Australia, Tasmania, Western Australia and Queensland (**Table 8**). This represents a significant increase in the uptake of the field kit since 2010, where supply was almost exclusively limited to Victoria. The distribution of kits throughout Australia has increased in the last 4 years and has expanded in the last 2 years indicating a change in surveillance for anthrax in Australia. Given the areas in which anthrax is most commonly seen, distribution in Victoria and NSW is much greater than anywhere else in Australia.

The number of kits distributed across Australia is approximately 600 kits per year. Kits generally have a shelf-life of 18 months from the time of production, although past experience with the kits indicates that this is a highly conservative timeline. With the current batch of AU3 kits, the use-by date was extended for a further 6 months after successful quality control testing. Typically, if the analytical sensitivity of the kits has not altered during the first year of circulation and there have been no issues with the kits, for example no false positive results have been recorded during this period, the expiry date of the batch of kits is extended. The recent production of AU4 kits is in the final stages of quality control testing and once completed these kits will replace the AU3 kits that are currently in circulation.

When a new batch of kits is produced, kits are distributed to areas in Victoria through key contacts in each of the 4 regions (North East, North West, South West and Gippsland) and to other jurisdictions through the organisations listed in **Table 8**. At the time of distribution of new kits, all unused kits from the previous batch are returned if their use-by date has been reached or the batch has been recalled. In either case the batch should not be used. When this occurs NARL staff contact each jurisdiction and organise the recall of the kits.

Table 8. Distribution of ICT kits throughout Australia for 2012-13 and 2013-14

Jurisdiction	Organisation	Approximate number of kits per year
Victoria	North East Region	200
	North West Region	25
	Gippsland Region	100
	South West Region	100
NSW	State Veterinary Diagnostic Laboratory Department of Primary Industries NSW	100-150
SA	Gribbles Veterinary Pathology, Glenside SA	20
Tas	Animal Health Laboratory, DPIPWE, Tas	10
WA	Department of Agriculture and Food, Animal Health Laboratory	30
QLD	University Of Queensland, School of Veterinary Science diagnostic and teaching bacteriology laboratory	4[a]

[a] Only for 2012-13

The ICT is a rapid and simple field based test that requires very basic training in its use and interpretation which makes it an easy and ideal tool for all field veterinarians in ruling out anthrax as a cause of sudden death on farm. All States that have used the kits, with the exception of QLD, have expressed a need for the continuation of the supply for 2014-2015. The feedback from field users of the ICT has been extremely positive, with a distinct focus on the benefits of an in-field test both in terms of limiting occupational risks and environmental contamination, and the ability to show farmers immediate results which helps to justify the subsequent actions that must be taken with an anthrax case.

6.3 Field results from all jurisdictions Summer/Autumn 2012-2014

In Victoria a total of 29 diagnostic investigations were carried out using the anthrax ICT kits in 2012-13 and a further 64 in 2013-14. All results from testing samples with ICT kits were negative for anthrax. Further testing of 11 (2012-13) and 7 (2013-14) of these diagnostic investigations within the laboratory confirmed the ICT result (**Table 9**). These samples were investigated further as there was a degree of suspicion that anthrax may have been responsible for the death of the animal even though the ICT produced a negative result.

A detailed account of all ICT results over the Summer/Autumn of 2012-2013 and 2013-2014 can be found in **Appendix 7**.

In NSW there were a total of 30 (2012-13) and 51 (2013-14) diagnostic investigations conducted using the anthrax ICT. For 2012-13, twenty five of the samples tested by the ICT were negative with 7 of these being confirmed within the laboratory by Polychrome Methylene Blue (PCMB) smear and/or PCR. There were 5 positive results of which 4 were confirmed positive in the laboratory (PCMB smear and PCR) including one ovine sample from Central West, a bovine sample from Lachlan and 2 bovine samples from the North West (**Table 9**).There was one positive ICT conducted in the field from one of the North West properties that was not confirmed within the laboratory using PCMB smear and PCR. This result prompted a subsequent sample submission from the same property which tested positive by PCMB smear at the laboratory. This suggests that the original ICT field result was correct and that the PCMB smear and PCR were unable to detect anthrax in the sample that was provided to the laboratory for confirmation. In this instance the age of the carcass and the resulting sample that was tested is unknown. However, it is known that when animals have been dead for >24 h that although the anthrax ICT kit is able to detect a positive case the capsule that surrounds the vegetative bacilli may not be readily detected in a blood smear (Office International des Epizooties, 2008) and the sample may be incorrectly diagnosed as being negative for the presence of *B. anthracis* on a PCMB smear. Given that PCR is derived from scrapings off the PCMB smear and no culture was performed it is possible that a positive animal may have been mis-diagnosed in the lab but detected by the ICT conducted onsite. Luckily the result was able to have be confirmed on the repeat sample.

For 2013-14, forty six of the samples tested by the ICT were negative with 25 of these being confirmed within the laboratory by PCMB smear and/or PCR. There were 5 positive ICT results all from the Central West region derived from 3 animals. Of the 3 animals tested, 2 were confirmed positive in the laboratory (PCMB smear and PCR) including one ovine and one bovine sample, the third bovine sample from a separate property and investigation had a faint positive on 3 separate ICT's. The faint positives were the result of swabs taken from the nose, neck and leg of a heavily decomposed bovine that had been dead for at least 6 days before sampling was undertaken(**Table 9**). This result was not confirmed in the laboratory (PCMB smear and PCR) and subsequent testing using a dehydrated ear received by the NARL a further 10 days later was not able to be confirmed using culture methods. The circumstances surrounding this case were difficult due to the death occurring on a neighbour's property and the owner of the cow not in residence at the time, hence the long delay between death and testing. In this instance the age of the carcass was known to be well outside of recommendations for the use of PCMB smear and the ICT. Appropriate samples

Table 9. Summary of ICT field results Summer/Autumn 2012-2014

Region	Year	No. of Kits	ICT Result	Overall Result
Victoria				
South West	**2012-13**	3	Neg	Neg
	2013-14	15	Neg	Neg
North East	**2012-13**	22	Neg	Neg
	2013-14	46	Neg	Neg
Gippsland	**2012-13**	4	Neg	Neg
	2013-14	3	Neg	Neg
North West	**2012-13**	0	No testing required	
	2013-14	0	No testing required	
New South Wales 2012-13[a]				
Central North	**2012-13**	2	Neg	Neg
Central West	**2012-13**	11	Neg	Neg
		1	Pos	Pos
Hume	**2012-13**	3	Neg	Neg
Lachlan	**2012-13**	1	Pos	Pos
North Coast	**2012-13**	1	Neg	Neg
North West	**2012-13**	6	Neg	Neg
		3	Pos	Pos
Riverina	**2012-13**	2	Neg	Neg
New South Wales 2013-14[a]				
Central Tablelands	**2013-14**	2	Neg	Neg
Central West	**2013-14**	16	Neg	Neg
		2	Pos	Pos
		3	Weak Pos	Incl[b]
Hunter	**2013-14**	1	Neg	Neg
Murray	**2013-14**	1	Neg	Neg

Region	Year	No. of Kits	ICT Result	Overall Result
North Coast	**2013-14**	3	Neg	Neg
North West	**2013-14**	14	Neg	Neg
Northern Tablelands	**2013-14**	2	Neg	Neg
Riverina	**2013-14**	3	Neg	Neg
Western	**2013-14**	2	Neg	Neg
Western Australia				
South Perth	**2012-13**	3	Neg	Neg
	2013-14	2	Neg	Neg
Albany	**2012-13**	5	Neg	Neg
	2013-14	0	No testing required	
Tasmania				
Southern	**2012-13**	1	Neg	Neg
	2013-14	0	No testing performed	
Northern	**2012-13**	1	Neg	Neg
	2013-14	0	No testing required	
South Australia				
Glenside	**2012-13**	Test was not supplied to SA for until 2013		
	2013-14	3	Neg	Neg

[a] NSW Regions have been redefined and merged so a compilation between the two years was not able to be performed

[b] Inconclusive result due to carcass in state of advanced decomposition and samples provided not adequate for culture and confirmation of the result

such as swabs of the nose, anus and environmental samples were not taken, these are the OIE recommended samples for old and decomposed cases where isolation of *B. anthracis* involves a search for relatively few organisms amongst a background of other flora (Office International des Epizooties, 2008). Given that all three samples, taken from different areas of the animal, were positive in the ICT it seems unlikely that this is a false positive case. However further clarification with environmental culture is not possible and as such this case is not able to be classified as an anthrax positive. This case demonstrates the importance of using appropriate testing for different situations and the importance of collecting the most appropriate sample for different tests.

In Western Australia there were a total of 8 (2012-13) and 2 (2013-14) diagnostic investigations conducted all of which reported negative results for the anthrax ICT. All but 1 of the 2013-14 cases were confirmed at the laboratory using culture (**Table 9**).

For Tasmania there were a total of 2 (2012-13) diagnostic investigations conducted all of which reported negative results for the anthrax ICT. These were all confirmed at the laboratory using culture and PCMB smear. For 2013-14 no ICT's were performed (**Table 9**).

For South Australia there were a total of 3 (2013-14) diagnostic investigations conducted all of which reported negative results for the anthrax ICT. These were all confirmed at the laboratory using PCMB smear (**Table 9**).Two of these were ovine and the third was from a pig. We do not recommend the use of the ICT in pigs as they are known to be relatively resistant to anthrax. Anthrax manifests significantly differently in pigs in comparison to ruminants and terminal bacteraemia frequently not marked (Office International des Epizooties, 2008). For unknown reasons pigs have a low number of *B. anthracis* detectable in the blood at death and as such PCMB smears may not reveal the capsulated bacilli. Therefore, it is not expected that the blood taken from a pig, that died from anthrax, will contain the anthrax toxin at a level that would be detectable within the ICT (World Health Organization, 2008). This feedback has been passed on to our South Australian colleagues.

The University Of Queensland, School of Veterinary Science diagnostic and teaching bacteriology laboratory requested 4 kits in April 2013 in preparedness for emergency animal disease management. However information pertaining to the use of these kits has not been provided. Work will continue to be pursued with QLD to increase collaborations and potential for expansion of the use of these kits with regional veterinarians throughout QLD.

Proficiency panels have been provided to all states where required as part of the compulsory training for all new users of the ICT. South Australia desired a large number of panels due to the geographic isolation of the veterinarians involved in using the tests and independent training required. As a result "train the trainer" panels were distributed, results were confirmed for each and competence established.

6.4 Refinement of conditions of use or manufacture for anthrax ICT test

Since the capacity to produce kits in Australia was established in 2009, three batches of kits have been produced by DEPI Victoria. The three batches have been designated AU1, AU2 and AU3. The current batch, AU3, has been modified to optimise the sensitivity and specificity of the kits for the Australian environment.

Refinement of the manufacturing process is ongoing and, as indicated above, changes were made to the manufacture of the AU2 ICT kits and a further change introduced in the AU3 batch of kits that are in current distribution. These changes were introduced to reduce the level of cross reactions observed with the initial batch of kits produced in Australia (the AU1 batch). Even though the level of cross reaction observed in the AU1 batch was low, this was not tolerated well by users. The robustness of the AU3 kits in comparison with previous versions produced in Australia (AU1 and AU2) and kits provided by the USNMRC, were assessed by testing all batches of kits against a panel of 115 blood samples obtained from dead animals from the Stanhope knackery. These blood samples were obtained from cattle that died of causes other than anthrax and were therefore expected to test negative with the ICT kit. The 115 samples were tested on site at the knackery with the help of Dr Chris Werner a District Veterinary Officer within the DEPI, Victoria. All AU3 kits used in the study produced negative results, as did all AU2 kits and the US Navy kits. A total of 9 (7.8%) of the 115 AU1 kits tested produced a weak line generally interpreted as a false positive result. This rate of false positives is consistent with that experienced in the field for the AU1 kits in 2009/2010 which led to production modifications for the manufacture of subsequent batches of test kits. The recent testing undertaken on all batches of kits available confirm that the refinements in production of the AU3 kits, which are in full distribution throughout Australia, eliminated the incidence of weak false positive results that had been experienced in early production lots. All testing conducted to date has confirmed that the AU3 kits are fit for use and therefore subsequent

batches, such as AU4, which will be manufactured using the same methodology, should have eliminated any problems associated with earlier batches of the kits.

The fourth batch of kits AU4 has recently been produced and has passed the standard analytical sensitivity and analytical specificity testing this batch is currently undergoing stability testing. Successful completion of testing of the AU4 batch of kits is expected by the end of May 2014. Distribution of these kits will be undertaken to replace all kits in circulation by the end of June 2014 which will coincide with the expiration of AU3 kits.

The latest batch of AU4 kits should therefore be of equivalence in reliability as the AU3 kits when used with appropriate samples and within the timeframe of 48h post-mortem as recommended. However as with every production of kits careful monitoring and consulting with field users of the kits is undertaken on an ongoing basis so that any issues that arise can be investigated and resolved promptly.

6.5 Conclusion

The ICT is a highly valuable tool for active surveillance and disease diagnosis in areas with a history of anthrax and a routine tool in the investigation of sudden death in cattle. The ability to produce these kits in-house at the NARL has provided Australia with the means to manufacture to meet demand and to distribute ICT's throughout Australia giving greater coverage for all veterinarians and animal health officers. The simplicity of the kit and the ability to perform carcass-side has empowered veterinarians to implement greater caution when faced with sudden deaths on farm and provided a tool that can be used to justify to farmers the cascade of events that follow a diagnosis of anthrax. The high sensitivity, high specificity and simplicity of the ICT have made this test an invaluable field tool for veterinarians and animal health officers throughout Australia, and the NARL has received numerous accounts of positive feedback from users of the kits.

One reason for continuing the evaluation of the performance of the ICT throughout Australia was to generate more data on the use of the kit on samples collected from species other than Bovines. During the period of study in this project the kit has been used to test Ovines, Equines and even Camelids as well as Bovines (**Appendix 7**) and has detected positive cases in sheep as well as cattle.

7 Epidemiological analysis of anthrax outbreaks

7.1 Introduction

Since the first reported occurrence of anthrax in Australia at Leppington, near Sydney, in 1847 the disease has spread along the eastern seaboard probably via travelling stock.

At present anthrax occurs commonly in NSW and Victoria. In Queensland the last recorded outbreak was in Southern Queensland in 2002 and in Western Australia the first and only outbreak was recorded in 1994 in Walpole to the South West of Perth. The disease has never been reported in the Northern Territory and the last cases in South Australia and Tasmania were recorded in 1914 and 1933 respectively (Animal Health Australia, 2012; Seddon & Albiston, 1965). The Northern Territory, South Australia and Tasmania are all considered to be free from anthrax (Animal Health Australia, 2012). Therefore the data collected for analysis in this project is from Victoria, NSW, Queensland and Western Australia.

Although anthrax has been known in Australia since the mid-19th Century detailed information on all disease incidents prior to 1990 is either not complete, not reliable or not available at all. Prior to 1990 only the information on the larger outbreaks of disease even approaches completeness. A

lack of detailed information on outbreaks and accompanying data on the environmental variables makes it impossible to accurately analyse data from anthrax outbreaks prior to 1990. Representative isolates of *B. anthracis* are also lacking from disease outbreaks prior to 1997 in Victoria and NSW, therefore there is an absence of genotyping data from strains from outbreaks prior to 1990 making analysis of epidemiological data in combination with strain genotyping data impossible. Consequently, this has meant that only Anthrax outbreak data post 1990 can be considered in detail.

The ecology and epidemiology of pathogens such as *B. anthracis* can be explored using tools similar to those used for species distribution modelling. Ecological niche modelling (ENM) has been used to predict the potential ecological and geographic distribution of pathogens based on outbreak locations (Blackburn *et al.*, 2007; Joyner *et al.*, 2010a; Nakazawa *et al.*, 2010), presence of disease vectors (Adjemian *et al.*, 2006; Costa *et al.*, 2002) and disease reservoirs (Townsend Peterson *et al.*, 2002). ENM approaches identify non-random associations between a species' locality data and environmental parameters. Ecological niche modelling experiments of *B. anthracis* are particularly useful due to the potential association between spore survival and ecological conditions (Hugh-Jones & Blackburn, 2009). Results from the application of ENM to study *B. anthracis* may be applied to improve our understanding of the potential disease risk in an area as well as to focus surveillance and vaccination strategies to target at risk animals before and during outbreak events (Blackburn *et al.*, 2007; Mullins *et al.*, 2011). ENM has been applied to improve understanding of the ecology and epidemiology of anthrax in the United States and Kazakhstan by using locations of reported outbreaks (Blackburn *et al.*, 2007; Joyner *et al.*, 2010a).

The ENM analysis of outbreak data was conducted in collaboration with Dr Jason Blackburn and Alassane Barro at the University of Florida. Dr Blackburn is recognised as a world expert in the area of ENM, particularly with respect to the application of methodology to aid in the understanding of the ecology and epidemiology of anthrax. This collaboration was facilitated by a visit by Dr Blackburn to DEPI funded by DEPI Victoria under the "Visiting Fellows" Program.

7.2 Methods and materials

7.2.1 Environmental data

Coverage of six environmental variables were downloaded from the WorldClim website http://www.worldclim.org for use in the ENM approach (

Table 10). The WorldClim variables are calculated from interpolation of monthly temperature and precipitation measurements recorded at stations located worldwide between 1961 and 2000. Monthly values are transformed by WorldClim into 19 bioclimatic variable grids that describe annual trends, seasonality and potentially limiting ecological parameters such as temperature of the coldest and warmest months. Two satellite-derived environmental variables describing temperature and vegetation measures were obtained from the Trypanosomiasis and Land Use in Africa (TALA) research group (Oxford, United Kingdom) (Hay *et al.*, 2006). Four soil variables were added to the set of eight environmental layers, soil variables were derived from the Harmonized World Soil Database and were available at 1 km^2 resolution (FAO/IIASA/ISRIC/ISSCAS/JRC: Harmonized World Soil Database Rome, Italy and IIASA, Laxenburg, Austria; 2012). All coverages were re-sampled to 8 km^2 and clipped to the boundaries of Australia in ArcView (Environmental Systems Research institute, Redlands, CA).

7.2.2 Ecological niche modelling

This study used the Genetic Algorithm for Rule-Set Prediction (GARP) to perform the ecological niche modelling (Anderson *et al.*, 2003). Models were developed in Desktop GARP v.1.1.3 (http://www.lifemapper.org/desktopgarp/), which gives the user the option to write out the rule sets for each model. Briefly, GARP is a presence only modelling technique that detects non-random associations between species localities and specific environmental variables. GARP is an iterative algorithm that searches for non-random relationships between point occurrence data and environmental coverages (raster files of climatic or environmental data). GARP develops a series of if/then logic statements, called rules that use one of four types (range, negated range, atomic, or logistic regression) to describe presence or absence of the target species in ecological space. Each individual GARP model is a set of 50 rules that are randomly generated, tested and modified. The user sets a maximum number of models to be created in a single experiment. A best subsets procedure within GARP then selects a set of optimal models based on user defined omission and commission criteria (Anderson *et al.*, 2003). The algorithm is a two-step process, where first relationships are defined in variable space through a random walk and then applied to the geographic landscape where those conditions are met. GARP therefore has the benefit of being able to project rule sets onto the environmental layers of a different landscape and has been shown to be robust in this application (Townsend Peterson *et al.*, 2007).

GARP models were evaluated with *post hoc* accuracy tests using independent Australian outbreak data withheld from the modeling experiments. Due to the iterative nature of GARP, the rule-set approach does not arrive at a single solution (McNyset, 2005). Because of this model performance can be affected by variation in input data. We developed a suite of 10 GARP experiments to evaluate the effect of input variability on model output, each with a different random subset of training and testing data. We used the geospatial modeling environment (GME, www.spatialecology.com) to randomly select 10 independent evaluation data sets of 30% of the occurrence points (n = 22) to withhold from GARP experiment to calculate accuracy metrics (Anderson *et al.*, 2002; Fielding & Bell, 1997; McNyset, 2005) since it is preferable to use an independent subset of data rather than re-substitution to assess model accuracy (Fielding & Bell, 1997). The remaining 70% of the occurrence points (n = 48) were used for model building. The data used for this purpose were taken from outbreaks and incidents of disease occurring between 2000 and 2013 because of the greater reliability of this data set. DesktopGARP internally partitions training/testing data for model building and testing, which was set at 70% and 30%. To maximize GARP performance, model runs were set to a maximum of 1,000 iterations or until convergence of 0.01. The best subset procedure was used to select the 20 best models under a 10% hard omission threshold and a 50% commission threshold (Anderson *et al.*, 2003) for a final 10-model best subset for each GARP experiment. Each 10-model best subset was summated in ArcMap using the raster calculator to create a composite prediction (Lim *et al.*, 2002; Peterson *et al.*, 2006).

Predictive accuracy for each GARP experiment was assessed using the independent dataset (i.e., the 30% of occurrence points withheld from model building) (Peterson *et al.*, 2006). We evaluated each best subset using the area under the curve (AUC) in a Receiver Operating Characteristics (ROC) analysis of the 30% independent datasets (Kostelnick *et al.*, 2007; McNyset, 2005; Wiley *et*

al., 2003). The AUC has been used extensively in species distribution modeling, and measures the ability of a model to discriminate between sites where a species is present, versus those where it is absent (Hanley & McNeil, 1982). The AUC measures how well the 10-best model set predicts the points in the independent dataset; an AUC of 1 indicates a perfect model while an AUC of 0.5 defines a model that predicts no better than random (Wiley *et al.*, 2003).

Because AUC scores alone may not describe the accuracy of a model (McNyset, 2005), we also calculated measures of omission and commission. Total and average omission was calculated from the 10-best models subsets and the independent test data (McNyset, 2005). Total omission was calculated as the total number of independent test points predicted absent by the summated grid of all 10 best models. Average omission was calculated as an average omission across each of the 10 best models. Total and average commission were also calculated. Total commission was calculated as the total number of pixels predicted present across all 10 models divided by the total number of pixels in the study area. Average commission was calculated as the average of the total number of cells predicted present divided by the total number of pixels in the study area on a model-by-model basis for each of the 10-best models in the subset.

For this study we calculated accuracy metrics for each of the 10 randomly subset datasets and ranked experiments by AUC and total omission, selecting the experiment that balanced a high AUC and low omission. The best model was selected to describe the ecological niche characteristics and potential geographic distribution of *B. anthracis* in Australia.

Table 10. Environmental coverage used for GARP models

Environmental Variable (unit)	Name	Source
Elevation (m)	Altitude	WorldClim*
Annual Temperature Range (°C)	BIO7	WorldClim
Annual Mean Temperature (°C)	BIO1	WorldClim
Precipitation of Driest Month (mm)	BIO14	WorldClim
Precipitation of Wettest Month (mm)	BIO13	WorldClim
Annual Precipitation (mm)	BIO12	WorldClim
NDVI Amplitude (no units)	wd1014 a1	TALA†
Mean NDVI (no units)	wd1014 a0	TALA
Soil pH (-log(H^+))		HWSD‡
Topsoil Calcium (% weight)		HWSD
Topsoil Organic Content (% weight)		HWSD
Subsoil Base Saturation (%)		HWSD

*http://www.worldclim.org[46]

† Trypanosomiasis and Land Use in Africa (TALA) research group (Hay *et al.*, 2006)

‡Harmonized World Soil Database (FAO/IIASA/ISRIC/ISSCAS/JRC: Harmonized World Soil Database Rome, Italy and IIASA, Laxenburg, Austria; 2012)

7.3 Results

The modelling process reached convergence of accuracy (0.01) prior to the maximum setting of 1000 iterations in each experiment run for this study. Model number 1 had the highest AUC score of all 10 experiments run using random selections of testing data. Table 2.2 summarizes the accuracy metrics and rank of all 10 experiments. The AUC score from the ROC analysis for Model 1 was 0.97 and was significantly different from a line of no information ($p<0.001$). Average omission for Model 1 was 2.73% and total omission was 0%, meaning that the vast majority of *post hoc* testing data were successfully predicted by all models in the best subset. The predicted geographic distribution of *B. anthracis* across Australia is illustrated in **Figure 15**.

The predicted distribution of *B. anthracis* is primarily restricted to a corridor from south eastern Queensland through the centre of NSW and into southern Victoria (**Figure 15**). The model also predicts that the ecological signals are favourable for *B. anthracis* survival around Port Augusta in South Australia and on the South Coast of Western Australia. The model predicts that the majority of Australia will not support the survival of *B. anthracis* (**Figure 15**).

Most disease outbreaks and disease incidents post 1990 are predicted to fall within the *B. anthracis* distribution area predicted by the model. The exceptions to this are the disease incidents in Walpole WA in 1994 and Rockhampton in Queensland in 1993 (**Figure 16**). All incidents of disease post 1990 in NSW, including the outbreak in Rouchel in the Hunter Valley in 2007/2008, are within the distribution zone predicted by the model (**Figure 17**). Similarly, all Victorian outbreaks post 1990 are within the distribution zone predicted by the model (**Figure 18** A and B). However, many of the historical reports of the disease in Victoria (pre-1990) fall outside of the predicted zone. This is especially true of disease reports from between 1914 and 1963 where there are many reported events occurring along the south coast of Victoria (**Figure 18**A). Exceptions to this are the larger outbreaks of disease that occurred in 1968 and 1972 in the Shepparton-Yarrawonga and Leitchville-Gunblower areas respectively which do fall in the predicted zone.

As expected all isolates genotyped using WGST fell within the predicted zone as they were all collected post 1990 (**Figure 19**). The most divergent WGST genotypes, J and K, fall outside of the predicted zone (**Figure 19**). Within NSW all isolates genotyped fell within the predicted zone with genotype H from the outbreak of disease in Rouchel being on the eastern limit of the zone (**Figure 20**). In Victoria all WGST genotypes A, B and C fall within the predicted zone (**Figure 21**).

Table 11. Summary statistics for 10 GARP modelling experiments using randomly subset testing and training data to evaluate model variability due to changes in input data. Experiments are ranked by Area Under the Curve (AUC) score and omission values.

Model	AUC	SE	Z	Total Omission	Average Omission	Total Commission	Average Commision
1	0.97	0.026	11.98	0.00	2.73	5.43	10.77
2	0.96	0.030	11.03	0.00	4.10	6.44	11.94
3	0.94	0.035	11.40	4.50	1.10	6.63	11.06
4	0.96	0.029	11.27	0.00	4.10	5.93	11.53
5	0.94	0.035	11.94	4.50	0.70	7.06	11.25
6	0.90	0.044	12.33	13.60	7.40	4.93	8.57
7	0.97	0.027	11.78	0.00	4.10	4.94	10.29
8	0.95	0.032	11.68	0.00	7.30	7.72	11.77
9	0.94	0.035	11.48	4.50	1.10	6.38	11.51
10	0.96	0.028	10.93	0.00	0.00	7.52	12.68

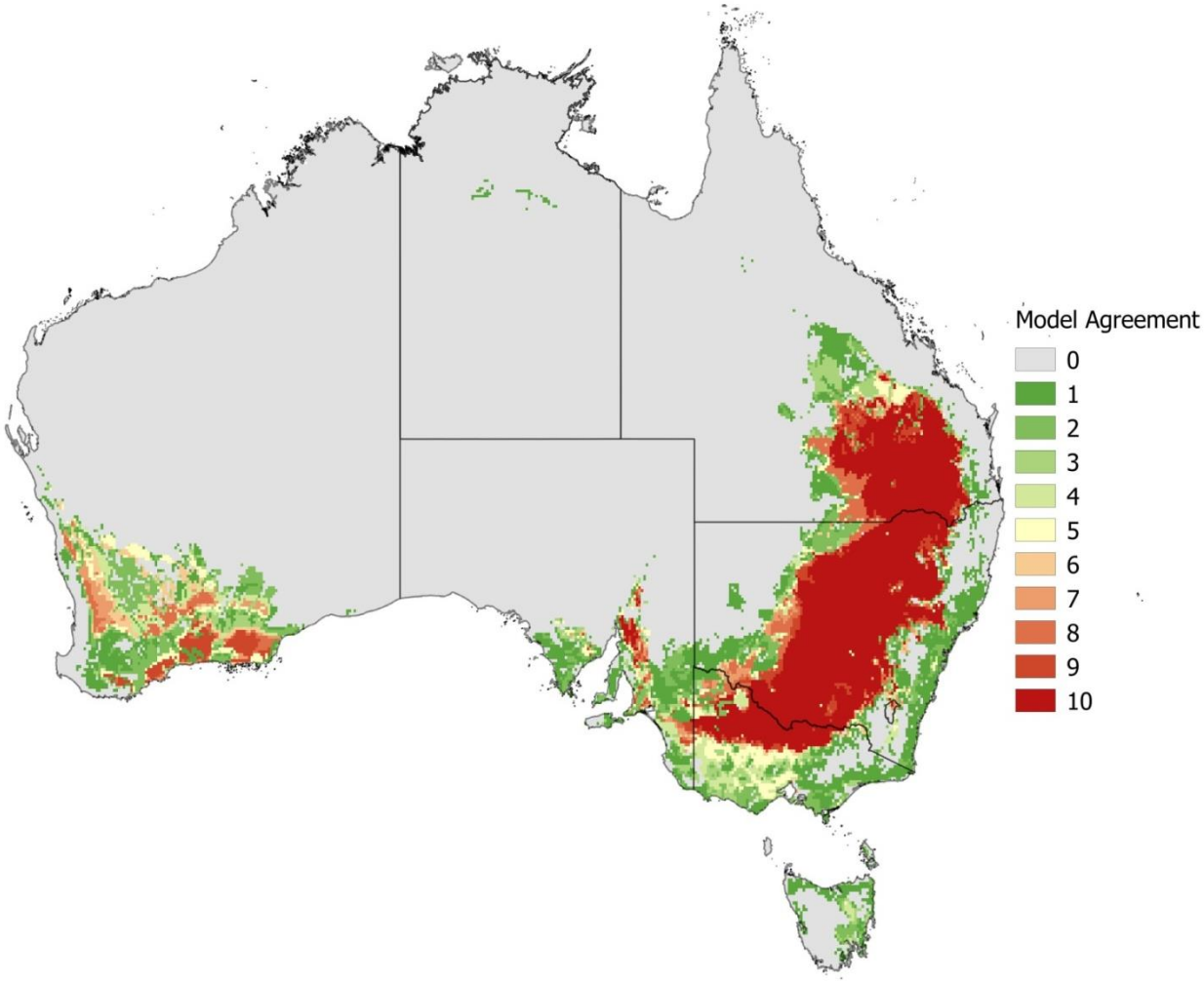


Figure 15. Potential geographic distribution of *Bacillus anthracis* across Australia based on a GARP ecological niche modelling experiment. Darker red colours represent higher levels of individual model agreement in the best subset of 10 individual models within the highest ranking experiment

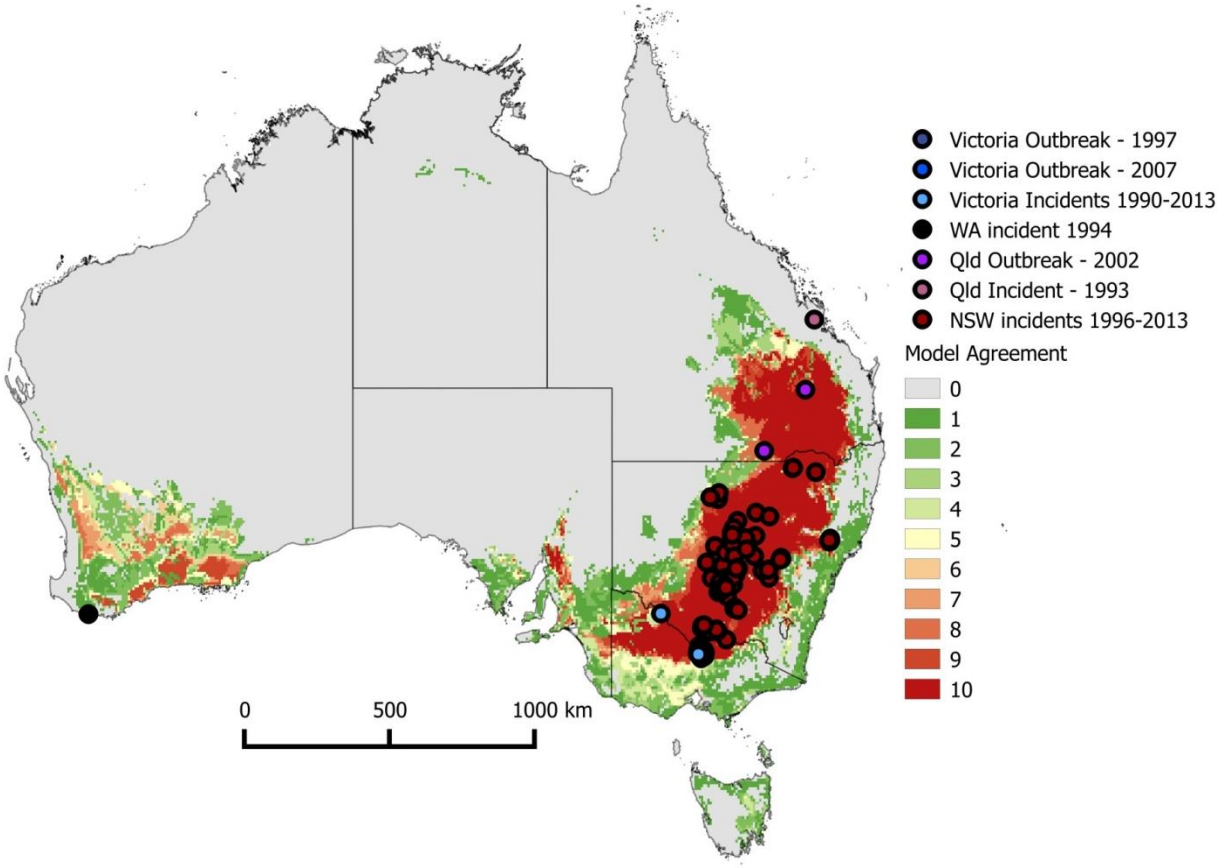


Figure 16. Predicted distribution of *Bacillus anthracis* in Australia based on the genetic algorithm for rule-set prediction. Anthrax outbreaks and Incidents from 1990 to 2013 are marked.

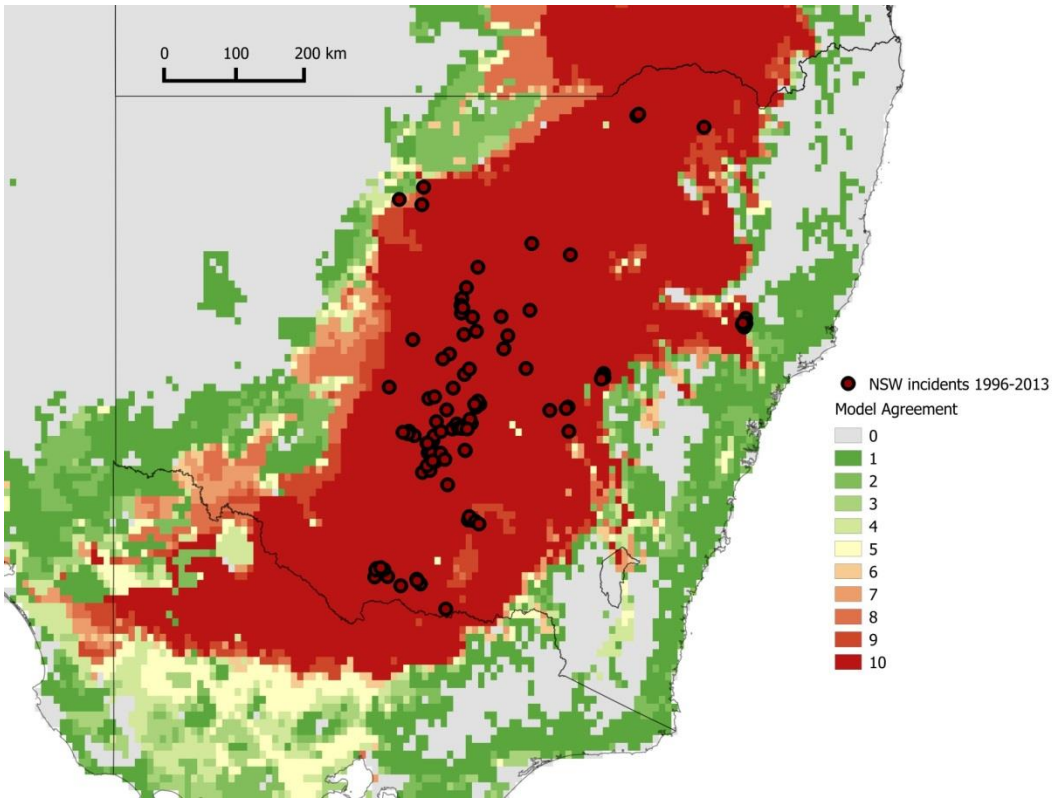


Figure 17. Predicted distribution of Bacillus anthracis in NSW based on the genetic algorithm for rule-set prediction. Anthrax outbreaks and Incidents from 1996 to 2013 are marked.

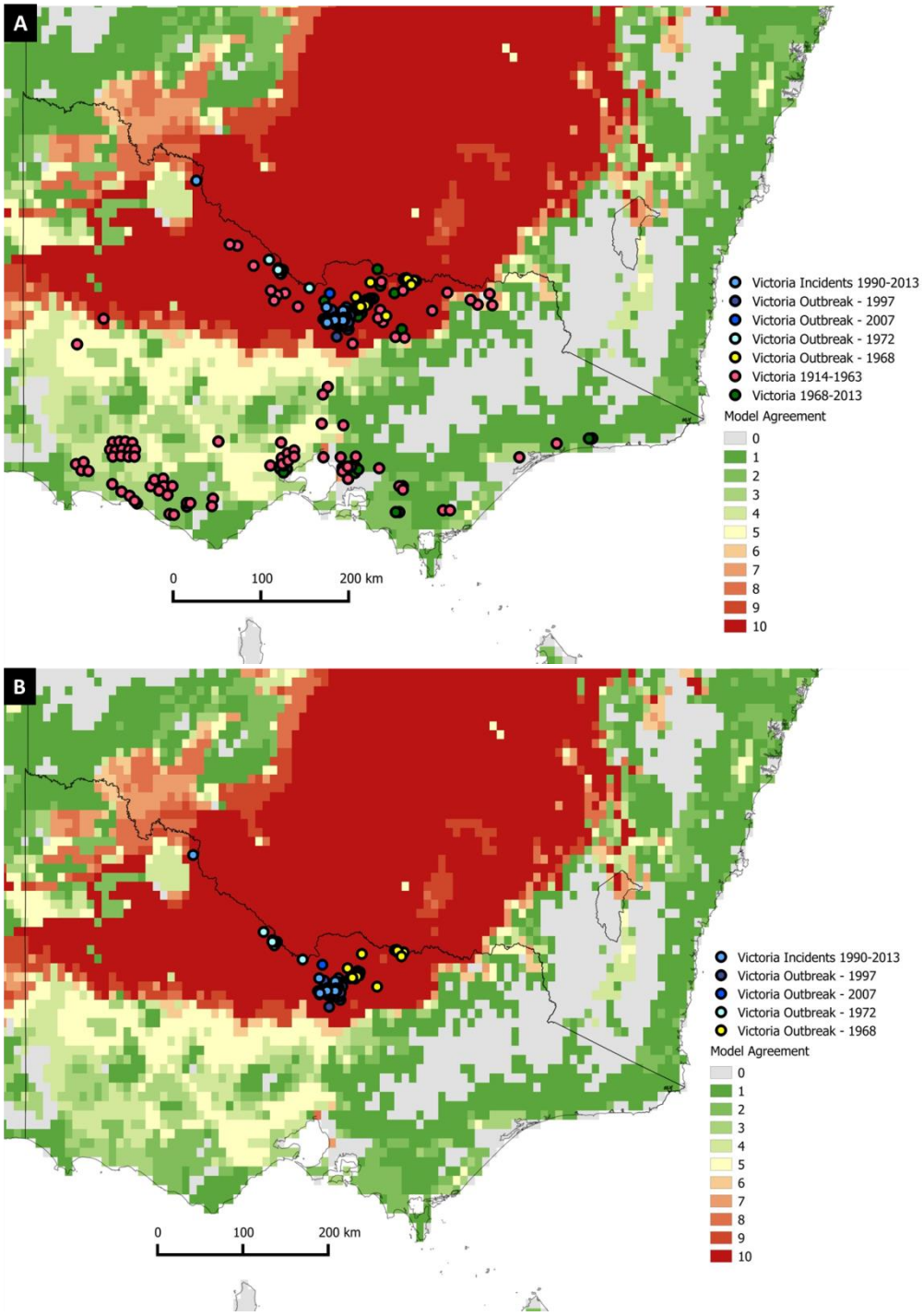


Figure 18. Predicted distribution of *Bacillus anthracis* in Victoria based on the genetic algorithm for rule-set prediction. A – Anthrax outbreaks and incidents in Victoria from 1914 until 2013 are marked. B - Anthrax outbreaks and Incidents from 1990 to 2013 are marked.

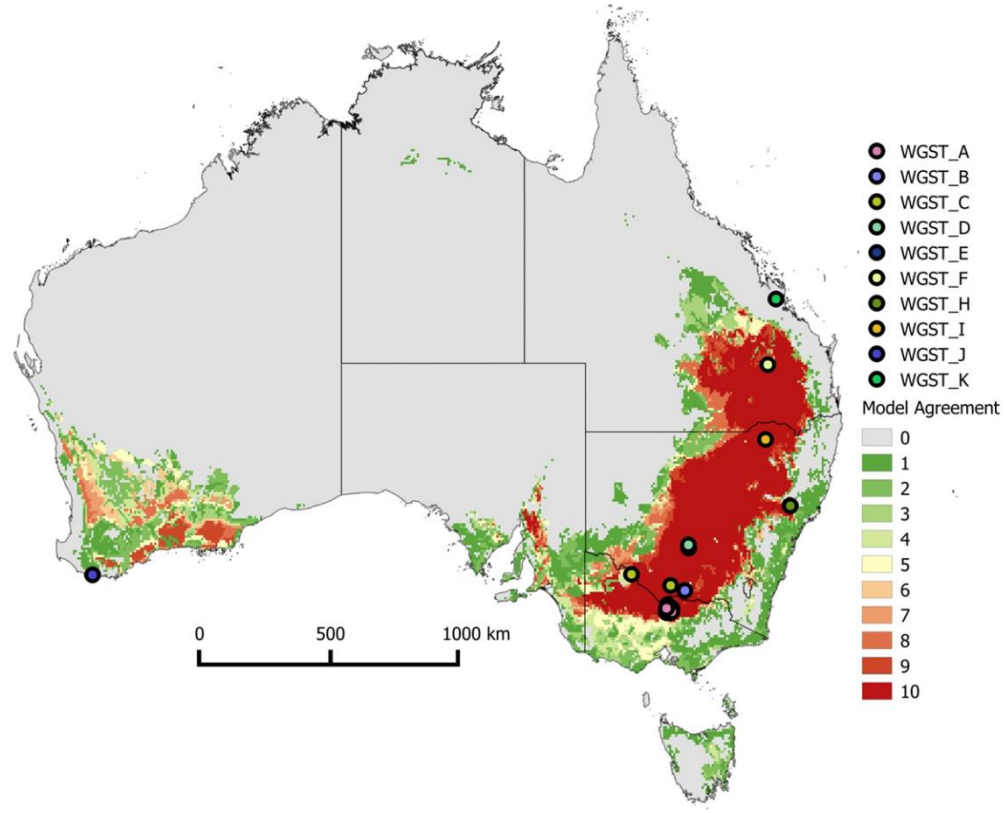


Figure 19. Distribution of WGST genotypes (WGST genotype A – K) and their relationship to the predicted distribution of *Bacillus anthracis* in Australia based on the genetic algorithm for rule-set prediction.

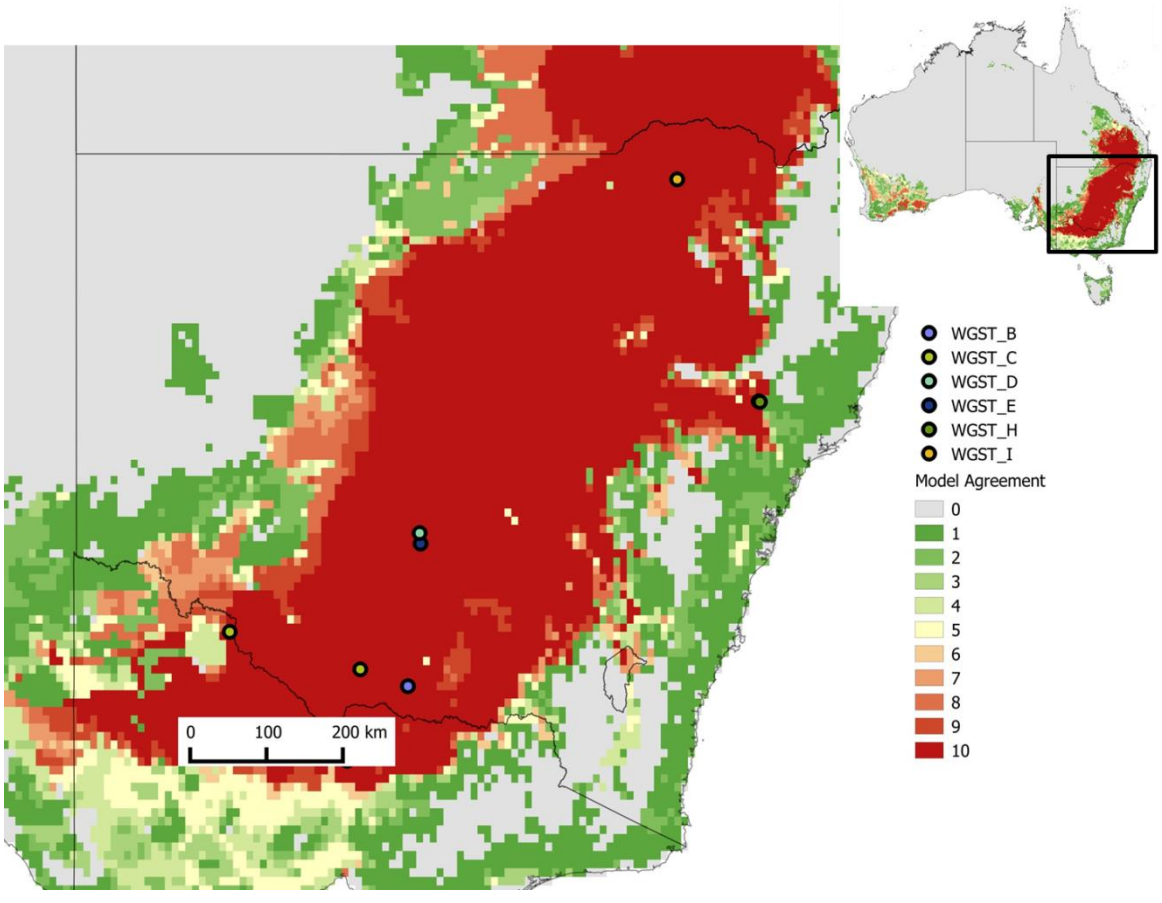


Figure 20. Distribution of WGST genotypes (WGST genotype B – I) and their relationship to the predicted distribution of *Bacillus anthracis* in NSW based on the genetic algorithm for rule-set prediction.

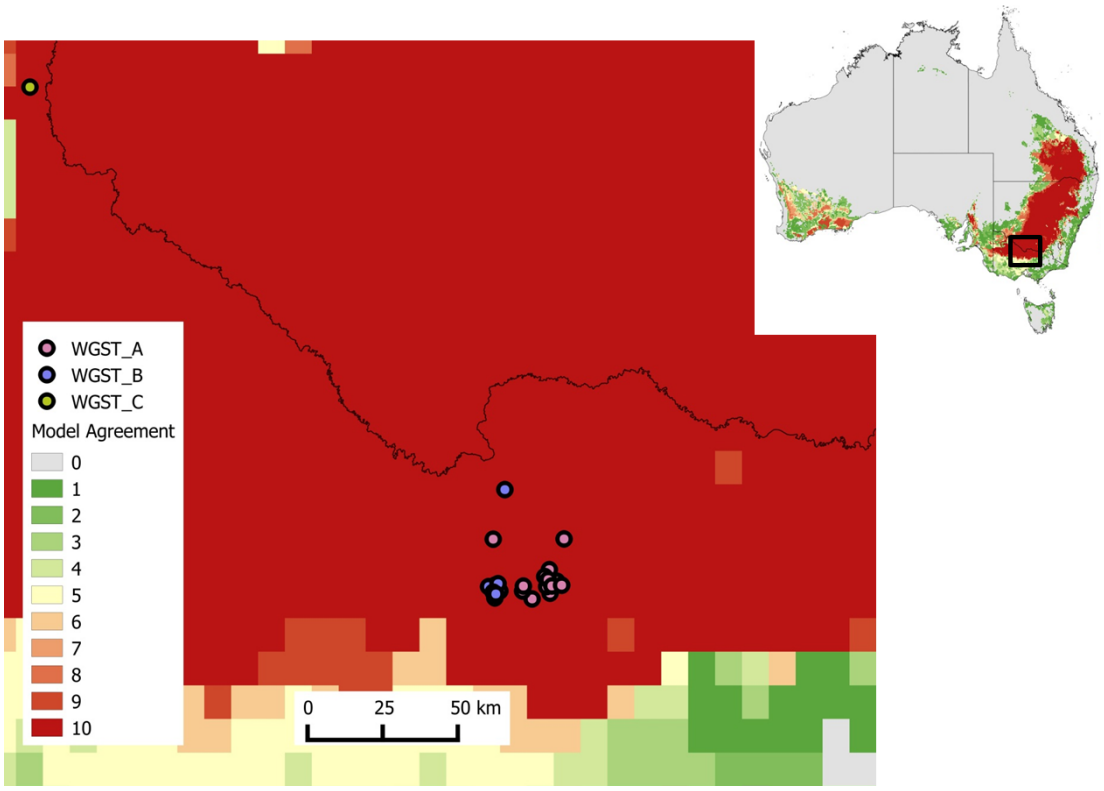


Figure 21. Distribution of WGST genotypes (WGST genotype A – C) and their relationship to the predicted distribution of *Bacillus anthracis* in Victoria based on the genetic algorithm for rule-set prediction.

7.4 Discussion

The distribution of *B. anthracis* predicted by the ENM approach stretches from southern Queensland though the centre of NSW and into Northern Victoria (**Figure 15**). The recent history of *B. anthracis* outbreaks within Australia predicts the distribution of *B. anthracis* within Australia to be restricted to the area which is commonly referred to as the Anthrax Belt, a belt running through the centre of New South Wales (Durrheim *et al*., 2009) which has been defined to extend from between Bourke and Moree in the north to Albury and Deniliquin in the south (Robinson & Maloney, 2006). The predicted distribution of *B. anthracis* in Australia by the ENM approach predicts the Anthrax belt, as defined above, but extends the area further east in NSW to Inverell and also predicts the area of the Hunter Valley where the outbreak of disease occurred in 2007/2008. The predicted area stretches into Southern Queensland through Warwick and up to Biloela and Blackwater and into Victoria through Wodonga in the east to Swan Hill in the west and down to the Shepparton area including Tatura and Stanhope, where the most recent outbreaks of disease have occurred within Victoria, in the south.

The region predicted by ENM matches the expected distribution of the disease based upon the historical accounts of introduction and spread of the disease in the southern states of Australia with the exception of the presence of the disease in the southern coastal regions of Victoria. Historically, anthrax was first identified within Australia at Leppington in 1847, and was spread to the north, west and south by travelling stock. By 1950 the disease had been carried north to Liverpool and beyond, and south to Camden. The movement of livestock in large numbers between Sydney and the country districts resulted in the spread of anthrax throughout practically the whole of the sheep-raising areas of New South Wales and into southern Queensland (Seddon & Albiston, 1965). Anthrax was first recognized in Victoria at Warrnambool followed by other areas in the south west of the state. The disease was later identified in southern and central Victoria following shipment of diseased sheep (Seddon & Albiston, 1965). Seddon and Albiston (1965) thought it unlikely that the initial outbreak in the south west of Victoria resulted from the spread of the disease from southern New South Wales indicating that the introduction of disease into this

area came from other sources. It is noted by Seddon and Albiston (1965) that the disease was spread quickly and over long distances to new areas when stock were moved by rail. The later distribution of the disease into the north of Victoria is considered to be most probably due to stock travelling over the border from NSW (Seddon & Albiston, 1965). We hypothesise that the presence of disease incidents along the south coast of Victoria, particularly in the south west of the state, represents constant reintroductions of the disease into these areas with possibly some short term survival and local spread. The long term survival of the organism in this area is not predicted by the model and the last case in the south west of the state was reported in 1972 in Illowa where 7 animals died and the last case in the south east in 1985 in Woorayl where a single animal died (**Appendix 1**). Of the disease reports from Victoria prior to 1990 all reports from the south of the state have reported the death of fewer than 8 animals at any one time. In contrast, two large outbreaks of disease, which occurred in 1968 and 1972 on a large number (greater than 10) of properties in a defined area over a short period of time (See Appendix 1) which led to the death of greater than 30 animals, occurred in the region predicted in the ENM model. It is speculated that the large outbreaks of disease occurred through the contact of animals with spores present in the soil, as would be predicted by the ENM model, whereas it is possible that the disease incidents in the south of the state represented localised introductions and re-introductions of the disease and are therefore not expected to be predicted by the model. The lack of isolates from these occurrences of disease along the south coast of Victoria make it impossible to identify the genotype of the isolates involved. Genotyping of isolates from this area would have allowed the identification of potentially exotic genotypes and the lack of this information makes it difficult to confirm this speculation.

All disease outbreaks and disease incidents post 1990 are predicted to fall within the *B. anthracis* distribution area predicted by the model with the exception of incidents in Walpole WA in 1994 and Rockhampton in Queensland in 1993 (**Figure 16**). The origins of these two disease incidents remain unexplained (Animal Health Australia, 2012) and anthrax has not been reported in these areas before or after these events. It is possible that these events represent an introduction into these areas via an unknown source and not the presence of spores being unearthed from a local endemic site. As discussed above the genotype of the strains responsible for these two incidents are the most divergent of the strains examined in this study which also points to the possibility that these strains were introduced and not endemic.

Ecological niche models are not transferable between countries and it has been speculated that that this may be due to variation in the ecological divergence of the different genotypes present within the countries studied (Mullins *et al*., 2013). In general, ENMs developed for a country are based upon lineages defined at the canSNP level (see Van Ert *et al.*, 2007b) for a description of canSNP groups and phylogeography) and have been found to accurately describe the distribution of *B. anthracis* within a country (Mullins *et al*., 2011; Mullins *et al*., 2013). The diversity of *B. anthracis* within Australia, particularly within the *B. anthracis* endemic region (the Anthrax Belt), is very low with all isolates falling into the A3 lineage described by Keim *et al*. (2000) (Figure 1.1). While it does remain possible that further study may identify ecological diversity associated with the genotypes present within Australia it is unlikely given the very close genetic relationship of *B. anthracis* isolates from Australia that have been genotyped. It is therefore expected that over such a large landscape as Australia with such a low level of genetic diversity no signal is likely to be found which may explain individual WGST genotypes especially given the resolution of the available environmental data.

In this study, we followed the suggestion of Joyner *et al*. (Joyner *et al*., 2010a; Joyner *et al*., 2010b) and identified the best models where AUC scores are high, omission low and commission balanced. It is possible that alternative climatic variables, or combinations of variables, would refine the models built in this study.

In conclusion, the predicted distribution of *B. anthracis* within Australia, as predicted by the ENM analysis, is consistent with the historical data and the current geographic range of disease in Australia. The ENM analysis and genotyping data is also consistent with the hypothesis that the unexplained incidents of anthrax in Walpole in Western Australia and Rockhampton in Queensland

were the result of a relatively recent introduction of the disease into these regions and not the exposure of animals to spores lying dormant in the soil.

We believe that the ENM developed here is a robust prediction of the areas of Australia where *B. anthracis* spores are likely to survive and that this model will enable us to focus surveillance and vaccination strategies to target at-risk animals before and during outbreak events. However, there is a still a need for additional research focusing on the complex environmental conditions which promote outbreaks of disease to help underpin the development of further predictive tools to enable control of anthrax in the Australian environment.

8 Summary of the National Anthrax Workshop, held in Melbourne 5-6th May 2014

A National Anthrax Workshop was held at the DEPI AgriBio Centre in Bundoora on 5-6th May 2014. The purpose of the workshop was to bring together people with past experience in, or a current interest in anthrax, and to showcase the findings of this project. Given that this project team also represent the National Anthrax Reference Laboratory (NARL), it was also an opportunity to highlight the capabilities and activities of the NARL.

A 2-day program enabled participants to travel from interstate and regional Victoria.

The programme was divided into four sections:

- Epidemiology of anthrax
- Anthrax diagnostics
- Field aspects / biosecurity
- Current research

There was a range of presentations (Refer to **Appendix 8**) with a focus on current anthrax diagnostics and research. All MLA project team members gave presentations centred on the MLA project milestones to highlight the research findings of this current project.

A total of 43 people were invited to participate in the meeting, and 30 were able to attend. Those that could attend included representatives from Animal Health Australia, Dairy Australia, Department of Agriculture, University of Melbourne, CSIRO AAHL, NSW DPI, NSW Local Land Services, MLA, Livestock Biosecurity Network, DEPI Biosecurity and various State laboratory representatives. The invitations were targeted to people currently undertaking research on anthrax, and those involved in field or laboratory diagnosis of anthrax especially if using the anthrax ICT kits. **Appendix 9** provides a list of all invitees, and the contact details of those that attended the workshop.

Unfortunately, some of those invited could not attend. Roger Paskin (CVO SA), Tristan Jubb (Livestock Health Systems Australia), Tony Britt (Director, Animal Biosecurity & Welfare, DEPI Victoria) and John Allen (CSIRO AAHL) were particularly missed given their history and knowledge of anthrax. However, there was still a good representation of people with knowledge across a broad range of topics able to attend. There was a high level of engagement by participant's which was evidenced by the large numbers of questions asked at the end of each presentation and the amount of discussion this generated from the audience. The NARL staff have also already had several key participants extending offers for providing historical information to fill in current knowledge gaps. The NARL staff plan to follow up with them in the near future.

An evaluation survey was provided to all participants (with the exception of the DEPI staff on this project) and 17 were completed and returned. A summary of the responses is provided in

Appendix 10. The questions in the evaluation survey centred around the content of the workshop, how well it was organised, the role of the Reference Laboratory, the value in attending the workshop and whether they would want to attend if it was held again. The answers to all sections were overwhelmingly positive (scores of 4 or 5) and there were several comments provided in the comment/feedback section. Whilst it is not proposed to hold the workshop annually, it was encouraging that all 17 respondents commented that they would recommend to someone else or attend themselves if there was another one in 2-3 years time. All 17 respondents also replied that the workshop was well organised and that the content represented the work being conducted in Australia.

8.1 Summary of workshop presentations

8.1.1 Epidemiology

Barbara Moloney (NSW DPI) and Laura MacFarlane-Berry (DEPI Victoria) were the first presenters of the workshop and provided an overview of the history of anthrax in each of their respective states. This session opened a lot of discussion around anthrax incidence, the history of anthrax outbreaks and introduction into Australia, and the differences/similarities in vaccination policies between NSW and Victoria.

8.1.2 Anthrax diagnostics

Janine Muller (DEPI Victoria, NARL) outlined some of the activities undertaken by the NARL, and in another talk described the work conducted to produce the anthrax ICT and the evaluation and quality assurance work done as part of the validation of each batch of kits produced. Maria Hardy (NSW DPI) discussed anthrax diagnostic procedures and established processes for dealing with an organism classified as a Tier 1 Security Sensitive Biological Agent (SSBA). EMAI have a temporary licence through the Department of Health and Aging that allows them to work with *Bacillus anthracis*, but without permission to culture the organism. This session highlighted that both NSW and Victoria in particular are in a strong position to diagnose anthrax, with the NARL having a strong capability in advanced diagnostics and genotyping.

8.1.3 Field aspects / biosecurity

There were three interesting talks in this session that highlighted some of the challenges with working with a hardy, spore-forming microorganism that can survive long periods in the environment. Mark Corrigan outlined a surveillance program that was conducted in Victoria in recent years and the training of personnel that went with the introduction of the anthrax ICT. Duncan Worsfold (DEPI Victoria) reported on a project he was involved in that led to a significant infrastructure improvement to three Victorian Knackeries with a particular focus on the Stanhope knackery which collects dead animals from the “anthrax region” of Victoria, and the use of the air curtain incineration method of carcase disposal. Graham Bailey (NSW DPI) presented an assessment of the effectiveness of incineration for anthrax decontamination which posed the question around what it means to ‘burn to ash’. This session highlighted the importance of ensuring the adequate disposal of carcases after an anthrax incident, and that even the most careful and thorough decontamination methods may not be fully effective against a spore-forming bacterium such as *Bacillus anthracis.*

8.1.4 Current research

The final session had presentations covering current research work being undertaken in Australia, Indonesia and Bangladesh. The Indonesian work presented by Kim Newberry on behalf of John Allen (CSIRO AAHL) was focussed on the vaccine comparison and validation work being undertaken by the national anthrax laboratory in DIC Wates, Yogyakarta, and laboratory capacity building in Indonesia for improved anthrax diagnostics. The Bangladesh work presented by Laura Macfarlane-Berry outlined the work done in that country to trace cutaneous human cases to livestock. The DEPI Victoria group presented a total of four talks in this session. Kelly Porter presented on the project she is about to commence funded through a Department of Agriculture

Science and Innovation award which will undertake serologic surveillance of anthrax in Victorian livestock. Mark Fegan (DEPI Victoria; NARL) presented on the enhanced diagnostics and molecular epidemiology of *B. anthracis* in Australia, as well as a second presentation on the Ecological Niche Modelling of *B. anthracis* in Australia which is being conducted in a collaboration with the University of Florida. Ilhan Mohammad (DEPI Victoria; NARL) presented a summary of the development and application of methods for the detection of *B. anthracis* in soil which showed that *B. anthracis* spores are still viable in soil more than seven years after an anthrax incident.

8.2 Recommendations on follow-up activities to sustain workshop outcomes

Overall, the inaugural anthrax workshop was very well received. Important linkages were established between NARL staff and key people from around the country with anthrax knowledge and experience. The workshop had the dual outcome of highlighting recent anthrax research, but also highlighted the ongoing activities of the NARL. The workshop provided a forum for people to get together and discuss anthrax, and the NARL staff will continue to use these linkages to the benefit of the Australian animal health system including through the continued manufacture and distribution of the ICT kits nationwide. Given that some notable people with anthrax expertise will retire in the coming years, NARL staff are also be eager to follow up with some of these people individually to capture the tremendous amount of knowledge that has not been published in the literature.

9 Appendices

Appendix 1. Data on Anthrax incidents in Victoria 1950-2013, entries in red indicate that isolates are held in the National Anthrax Reference Collection. (Note: there is no data available for Anthrax incidents occurring prior to 1950; Data from 1950-1963 is taken from Seddon and Albiston 1965)

				Animals affected					
Date	Region	Address	Total Deaths	Bovine	Ovine	Canine	Porcine	Equine	Caprine
13/04/1950	Warrnambool	Dennington	1	1					
1/08/1950	Newfeild	NA	2	2					
1/08/1950	Nutfeild	NA	3	3					
1/02/1951	Hallam	NA	1	1					
1/05/1952	Cloverlea	NA	7	7					
1/05/1952	Cloverlea	NA	1	1					
1/07/1952	Dandenong	NA	4	4					
1/08/1952	Dandenong	NA	2	2					
1/10/1952	Cranbourne	NA	1	1					
1/12/1952	Dandenong	NA	1	1					
1/04/1953	Hallam	NA	1	1					
1/06/1953	Bacchus Marsh	NA	1	1					
1/04/1955	Cobrico	NA	1	1					
1/04/1955	Cobrico	NA	5	5					
1/04/1955	Cobrico	NA	1	1					
1/12/1955	Cloverlea	NA	2	2					
1/01/1956	Cloverlea	NA	1	1					
1/11/1956	Timboon	NA	1	1					
1/06/1957	Craigieburn	NA	4	4					
1/01/1958	Newfeild	NA	1	1					
1/01/1960	Cloverlea	NA	1	1					
1/01/1963	Garvoc	NA	3	3					
22/02/1968	Yarrawonga	Bundalong	2	2					
26/02/1968	Shepparton	Pine lodge	8	8					
26/02/1968	Shepparton	Marionvale	1	1					
26/02/1968	Yarrawonga	Bundalong	7	1					
26/02/1968	Yarrawonga	Yarrawonga	7	3					
27/02/1968	Shepparton	Pine Lodge Nth	1	1					
27/02/1968	Shepparton	Pine Lodge Nth	1	1					
28/02/1968	Shepparton	Tallygaroopna	1	1					
28/02/1968	Shepparton	Congupna	1	1					
28/02/1968	Yarrawonga	Bundalong	1	1					
28/02/1968	Yarrawonga	Yarrawonga	2	2					
29/02/1968	Shepparton	Marionvale	1	1					
29/02/1968	Shepparton	Tallygaroopna	1	1					
29/02/1968	Shepparton	Marionvale	1	1					
29/02/1968	Yarrawonga	Yarrawonga	1	1					
1/03/1968	Yarrawonga	Yarrowonga	1	1					
2/03/1968	Shepparton	Congupna	1	1					
2/03/1968	Shepparton	Congupna	1	1					

				Animals affected					
Date	Region	Address	Total Deaths	Bovine	Ovine	Canine	Porcine	Equine	Caprine
3/03/1968	Shepparton	Tallygaroopna	1	1					
3/03/1968	Shepparton	Congupna	1	1					
4/03/1968	Shepparton	Congupna	1	1					
4/03/1968	Yarrawonga	Bundalong	1	1					
5/03/1968	Shepparton	Congupna	1	1					
5/03/1968	Shepparton	Congupna	7	7					
5/03/1968	Yarrawonga	Esmond	1	1					
7/03/1968	Shepparton	Marionvale	1	1					
7/03/1968	Shepparton	Congupna	2	2					
7/03/1968	Shepparton	Congupna	1	1					
8/03/1968	Shepparton	Congupna	1	1					
8/03/1968	Shepparton	Congupna	1	1					
9/03/1968	Benalla	Stewarton	1	1					
9/03/1968	Shepparton	Congupna	1	1					
10/03/1968	Shepparton	Marionvale	1	1					
12/03/1968	Shepparton	Congupna	1	1					
13/03/1968	Yarrawonga	Yarrowonga	1	1					
17/03/1968	Shepparton	Congupna	1	1					
21/03/1968	Benalla	Stewarton	1	0	1				
27/03/1968	Shepparton	Shepp Nth	1	1					
7/04/1968	Cobram	Marion Vale	1	1					
17/08/1968	Shepparton	Pine Lodge Nth	1	1					
20/12/1968	Yarrawonga	Bundalong	1	1					
10/01/1969	Cobram	Katamatite	2	2					
17/02/1969	Cohuna	Cohuna	1	1					
5/02/1970	Corio		3	3					
11/05/1970	Corio		1	1					
26/03/1971	Shepparton	Pine Lodge	1	1					
31/07/1971	Yarrawonga	Yarrawonga	3	1					
10/10/1971	Cobram	Katamatite	1	1					
13/01/1972	Rochester	Gunbower	10	6					
14/01/1972	Rochester	Gunbower	6	1					
14/01/1972	Cohuna	Leitchville	1	1					
15/01/1972	Rochester	Gunbower	1	1					
16/01/1972	Rochester	Gunbower	2	2					
16/01/1972	Rochester	Gunbower	2	2					
17/01/1972	Cohuna	Leitchville	1	1					
18/01/1972	Deakin	Moama	2	0			1		
19/01/1972	Rochester	Gunbower	3	2					
19/01/1972	Rochester	Gunbower	1	1					
21/01/1972	Rochester	Gunbower	1	1					
26/01/1972	Rochester	Gunbower	1	1					
28/01/1972	Rochester	Gunbower	1	1					
14/02/1972	Cohuna	Cohuna	2	1					
15/02/1972	Warrnambool	Ilowa	7	2					
20/06/1972	Cohuna	Leitchville	1	1					

				Animals affected					
Date	Region	Address	Total Deaths	Bovine	Ovine	Canine	Porcine	Equine	Caprine
28/03/1973	Yarrawonga	Bundalong	2	2					
23/06/1973	Cobram	Cobram East	1	1					
1/09/1973	Rochester	Gunbower	6	6					
14/02/1974	Corio		3	1					
15/03/1974	Corio		2	2					
25/07/1974	Shepparton	Congupna	1	1					
3/10/1974	Shepparton	Congupna	1	1					
11/02/1975	Berwick	Belgrave	2	2					
18/02/1975	Berwick	Belgrave	2	2					
23/02/1975	Berwick	Belgrave	0	0					
23/02/1975	Berwick	Belgrave	0	0					
26/02/1975	Berwick	Belgrave	1	1					
21/04/1975	Berwick	Belgrave	1	1					
26/05/1975	Berwick	Belgrave	0	0					
17/05/1975	Berwick	Belgrave	1	1					
6/05/1976	Orbost	Brodribb	0	0					
21/05/1976	Rodney	Tatura	0	0		2 + 2			
25/06/1976	Orbost	Brodribb	5	2					
21/03/1979	Tungamah	Telford	5	0	1				
20/02/1980	Shepparton	Pine Lodge	4	4					
28/02/1980	Shepparton	Pine Lodge	1	1					
28/02/1980	Shepparton	Congupna	1	1					
3/03/1980	Shepparton	Pine Lodge	0	0					
22/03/1980	Tungamah	Katandra W	1	1					
14/01/1981	Tungamah	Katandra W	0	0					
17/03/1981	Corio		3	0					
7/04/1981		Stanhope	4	0			4		
9/04/1981	Shepparton	Congupna	3	3					
23/04/1981	Rodney	Stanhope	1	0		1			
23/10/1983	Corio		?	?					
7/03/1985	Woorayl	Leongatha	1	1					
9/03/1985	Woorayl	Leongatha	0	0					
26/03/1988	Yarrawonga	Bundalong	2	2					
26/01/1997	**Tatura area**		**14**	**14**					
27/01/1997	Tatura area		3	3					
3/02/1997	**Tatura area**		**12**	**12**					
4/02/1997	**Tatura area**		**8**	**8**					
6/02/1997	Tatura area		2	2					
7/02/1997	**Tatura area**		**1**	**1**					
7/02/1997	Tatura area		2	2					
7/02/1997	Tatura area		3	3					
8/02/1997	Tatura area		2	2					
8/02/1997	Tatura area		2	2					
8/02/1997	Tatura area		7	7					
8/02/1997	Tatura area		3	3					

				Animals affected					
Date	Region	Address	Total Deaths	Bovine	Ovine	Canine	Porcine	Equine	Caprine
8/02/1997	Tatura area		1	1					
8/02/1997	Tatura area		1	1					
8/02/1997	Tatura area		2	2					
8/02/1997	**Tatura area**		**13**	**13**					
9/02/1997	Tatura area		1	1					
9/02/1997	Tatura area		2	2					
9/02/1997	Tatura area		2	2					
9/02/1997	Tatura area		1	1					
9/02/1997	Tatura area		1	1					
10/02/1997	**Tatura area**		**3**	**3**					
10/02/1997	Tatura area		1	1					
10/02/1997	Tatura area		1	1					
10/02/1997	Tatura area		1	1					
11/02/1997	Tatura area		1	1					
11/02/1997	Tatura area								
11/02/1997	Tatura area		2	2					
11/02/1997	Tatura area		1	1					
11/02/1997	Tatura area		1	1					
12/02/1997	Tatura area		2	2					
12/02/1997	Tatura area		2	2					
12/02/1997	Tatura area		14	14					
13/02/1997	**Tatura area**		**3**	**1**	**2**				
14/02/1997	Tatura area		7	6	1				
14/02/1997	Tatura area		3	3					
14/02/1997	Tatura area		2	2					
14/02/1997	Tatura area		1	1					
15/02/1997	Tatura area		1	1					
15/02/1997	Tatura area		1	1					
15/02/1997	Tatura area		1	1					
15/02/1997	Tatura area		3	3					
16/02/1997	Tatura area		1	1					
16/02/1997	Tatura area		1	1					
16/02/1997	Tatura area		1	1					
16/02/1997	Tatura area		1	1					
16/02/1997	Tatura area		1	1					
16/02/1997	**Tatura area**		**2**	**2**					
16/02/1997	Tatura area		12	12					
17/02/1997	Tatura area		1	1					
17/02/1997	Tatura area		3	3					
17/02/1997	Tatura area		5	5					
18/02/1997	Tatura area		1	1					
19/02/1997	Tatura area		1	1					
20/02/1997	Tatura area		4	4					
21/02/1997	Tatura area		1	1					
21/02/1997	Tatura area		1	1					
21/02/1997	Tatura area		1		1				

				Animals affected					
Date	Region	Address	Total Deaths	Bovine	Ovine	Canine	Porcine	Equine	Caprine
21/02/1997	**Tatura area**		**3**	**3**					
21/02/1997	Tatura area		2	2					
22/02/1997	Tatura area		3	3					
22/02/1997	Tatura area		1	1					
22/02/1997	Tatura area		1	1					
24/02/1997	Tatura area		1	1					
22/04/2002	Tatura area		1	1					
10/05/2002	Tatura area		1	1					
22/11/2002	**Swan Hill**		**1**	**1**					
1/01/2003	**Tatura area**		**2**	**2**					
17/03/2004	**Tatura area**	**Harston**							
17/03/2004	**Tatura area**	**Harston**							
19/03/2004	**Tatura area**	**Harston**							
1/10/2005	**Tatura area**	**Tatura**	**1**	**1**					
31/01/2007	**Tatura area**	**Stanhope**	**5**	**5**					
1/02/2007	**Tatura area**	**Stanhope**	**3**	**3**					
6/02/2007	**Tatura area**	**Stanhope**	**1**	**1**					
7/02/2007	**Tatura area**	**Stanhope**	**2**	**2**					
7/02/2007	**Tatura area**	**Tatura**	**6**	**6**					
9/02/2007	**Tatura area**	**Stanhope**	**1**	**1**					
9/02/2007	**Tatura area**	**Stanhope**	**1**	**1**					
18/02/2007	**Tatura area**	**Wyuna**	**4**	**4**					
19/01/2007	**Tatura area**	**Stanhope**	**20**	**20**					
2/02/2007	**Tatura area**	**Stanhope**	**2**	**2**					
24/01/2008	**Tatura area**	**Stanhope**	**1**	**1**					
25/02/2008	**Tatura area**	**Harston**	**1**	**1**					
15/08/2009	**Tatura area**	**Kyabram West**	**1**	**1**					
19/08/2009	**Tatura area**	**Stanhope**	**1**	**1**					

Appendix 2. Anthrax outbreaks in NSW 2000-2013, entries in red indicate that isolates are held in the National Anthrax Reference Collection

				Animals affected					
Date	Region	Address	Total Deaths	Bovine	Ovine	Canine	Porcine	Equine	Caprine
21/02/2000	Hay	Hillston	22		22				
12/10/2000	Bourke	Bourke	60		60				
2/02/2001	Narrandera	Weethalle	17		17				
5/02/2001	Condobolin	Lake Cargelligo	138		138				
31/03/2001	Narrandera	Rankins Springs	3	3					
27/06/2002	Dubbo	Nevertire	10	10					
16/09/2002	Narrandera	Rankins Springs	9		9				
19/12/2002	Coonamble	Coonamble	3		3				
5/01/2003	**Riverina**	**Deniliquin**	**12**	**12**					
28/03/2003	Condobolin	Tottenham	11		11				
15/04/2003	Hillston	Euabalong West	6	6					
29/09/2003	Condobolin	Tullibigeal	4	4					
21/11/2003	Hay	Rankins Springs	60		60				
3/12/2003	Nyngan	Tottenham	9		9				
27/01/2004	Hillston	Condobolin	5	3	2				
30/01/2004	Condobolin	Rankins Springs	1	1					
8/02/2004	Forbes	Eugowra	18	11	7				
17/04/2004	Condobolin	Lake Cargelligo	10	10					
22/09/2004	Narrandera	Weethalle	4		4				
23/09/2004	Condobolin	Lake Cargelligo	5		5				
18/10/2004	Condobolin	Lake Cargelligo	5		5				
25/10/2004	Narrandera	Rankins Springs	5		5				
8/11/2004	Nyngan	Girilambone	14	14					
3/12/2004	Condobolin	Condobolin	20		20				
6/12/2004	Nyngan	Hermidale	2	2					
10/12/2004	**Narrandera**	**Rankins Springs**	**5**		**5**				
21/01/2005	Condobolin	Rankins Springs	3		3				
24/01/2005	**Murray**	**Berrigan**	**10**	**10**					
30/03/2005	**Narrandera**	**Yenda**	**10**		**10**				
6/10/2005	Northern Slopes	Gravesend	8	8					
2/12/2005	Condobolin	Burgooney	30		30				
5/12/2005	Murray	Mayrung	4	2	2				
13/12/2005	**Riverina**	**Mayrung**	**5**		**5**				
15/12/2005	Murray	Mayrung	1	1					
20/12/2005	Hillston	Condobolin	40		40				
14/01/2006	**Narrandera**	**Tabbita**	**4**	**4**					
17/03/2006	Narrandera	Nericon	7	6					1
17/03/2006	Narrandera	Nericon	6	6					
17/04/2006	Hillston	Lake Cargelligo	4	4					
1/08/2006	Forbes	Forbes	1	1					
12/10/2006	Murray	Finley	4	4					
17/10/2006	Hillston	Lake Cargelligo	5		5				
24/11/2006	Condobolin	Albert	33		33				

				Animals affected					
Date	Region	Address	Total Deaths	Bovine	Ovine	Canine	Porcine	Equine	Caprine
27/11/2006	Nyngan	Bobadah	31		30	1			
11/12/2006	Condobolin	Condobolin	50		50				
12/12/2006	Murray	Berrigan	4	4					
4/01/2007	Condobolin	Lake Cargelligo	4		4				
19/01/2007	Condobolin	Gubbata	14	14					
25/01/2007	Murray	Berrigan	8	8					
31/01/2007	**Narrandera**	**Yenda**	**200**		**200**				
6/02/2007	Murray	Berrigan	8	8					
24/02/2007	Condobolin	Naradhan	4	4					
8/03/2007	Condobolin	Tullibigeal	5	5					
15/03/2007	Nyngan	Hermidale	2	2					
2/04/2007	Condobolin	Tullibigeal	3	3					
26/11/2007	Condobolin	Condobolin	38		38				
30/11/2007	Condobolin	Tullibigeal	2	2					
18/12/2007	Forbes	Parkes	3	3					
20/12/2007	**Hunter**	**Upper Rouchel**	**8**	**8**					
20/12/2007	**Hunter**	**Upper Rouchel**	**23**	**23**					
23/12/2007	Hunter	Upper Rouchel	2	2					
23/12/2007	Hunter	Upper Rouchel	4	3				1	
23/12/2007	Hunter	Rouchel	1	1					
23/12/2007	Hunter	Upper Rouchel	2	2					
24/12/2007	**Hunter**	**Rouchel**	**2**	**2**					
24/12/2007	Hunter	Rouchel	1	1					
24/12/2007	Hunter	Aberdeen	4	4					
28/12/2007	Narrandera	Matong	59	59					
30/12/2007	Hunter	Rouchel	2	2					
3/01/2008	Hunter	Aberdeen	1	1					
7/02/2008	**Dubbo**	**Cumnock**	**12**	**12**					
27/02/2008	Forbes	Trundle	5	5					
28/04/2008	Nyngan	Hermidale	2	2					
13/06/2008	Hunter	Upper Rouchel	1	1					
5/08/2008	**Dubbo**	**Cumnock**	**6**		**6**				
7/10/2008	Bourke	Bourke	15		15				
20/10/2008	Nyngan	Tottenham	60		60				
21/10/2008	Hay	Rankins Springs	58		58				
21/09/2009	Riverina	Yenda	17		17				
13/11/2009	Narrandera	Rankins Springs	386		386				
16/11/2009	Condobolin	Tullibigeal	14	1	13				
25/01/2010	**Narrandera**	**Rankins Springs**	**20**		**20**				
2/02/2010	**Narrandera**	**Rankins Springs**	**4**		**4**				
4/11/2010	Nyngan	Hermidale	30		30				
9/01/2012	Murray	Deniliquin	30		30				
23/11/2012	Forbes	Parkes	100		100				
18/12/2012	Molong	Cumnock	3	3					
23/12/2012	Condobolin	Albert	1	1					
22/02/2013	Moree	Moree	30	30					

				Animals affected					
Date	Region	Address	Total Deaths	Bovine	Ovine	Canine	Porcine	Equine	Caprine
27/02/2013	Moree	Moree	30	30					

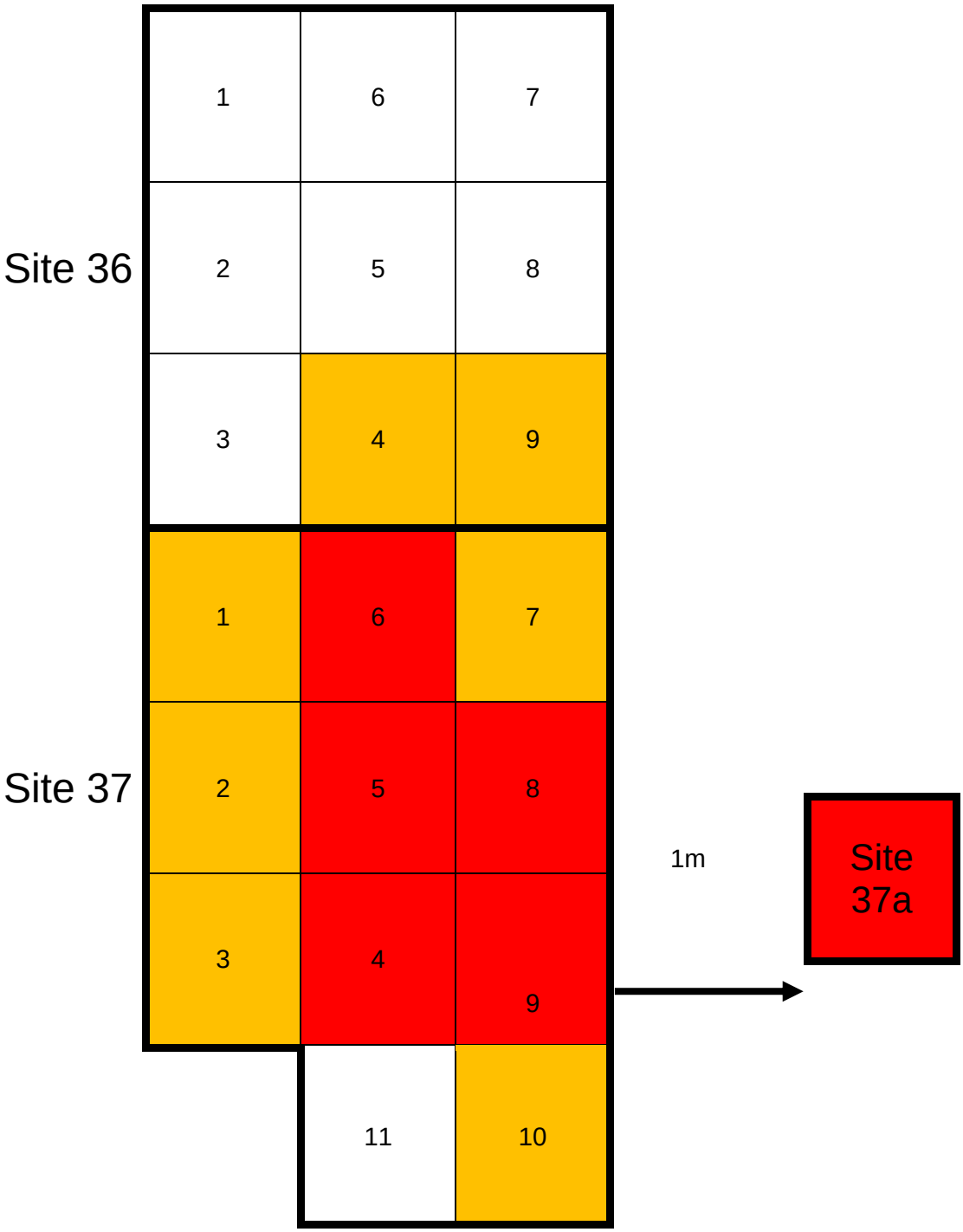
Appendix 3. Anthrax outbreaks in Queensland after 1933, entries in red indicate that isolates are held in the National Anthrax Reference Collection

				Animals affected					
Date	Region	Address	Total Deaths	Bovine	Ovine	Canine	Porcine	Equine	Caprine
01/01/2003		**Wandoan**	**12**	**12**					
1993	**Rockhampton**	**Yaamba**	**1**	**1**					

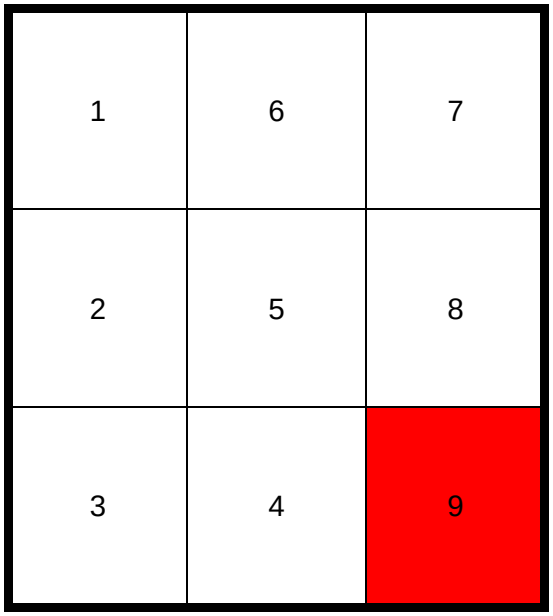
Appendix 4. Anthrax outbreaks in Western Australia, entries in red indicate that isolates are held in the National Anthrax Reference Collection

				Animals affected					
Date	Region	Address	Total Deaths	Bovine	Ovine	Canine	Porcine	Equine	Caprine
?/01/1994 – 25/03/1994		**Walpole**	**31**	**31**					

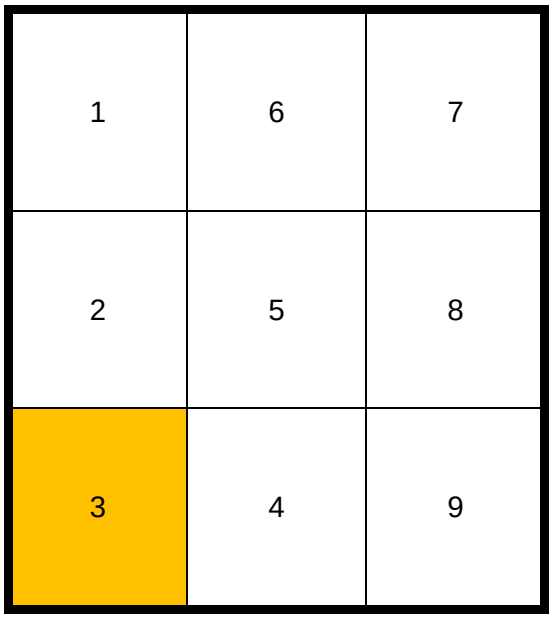
Appendix 5. Pictorial representation of results for isolation and Bio-PCR of *B. anthracis* from positive sites (squares coloured orange indicate Bio-PCR positive only, squares coloured red indicate Bio-PCR and culture positive)



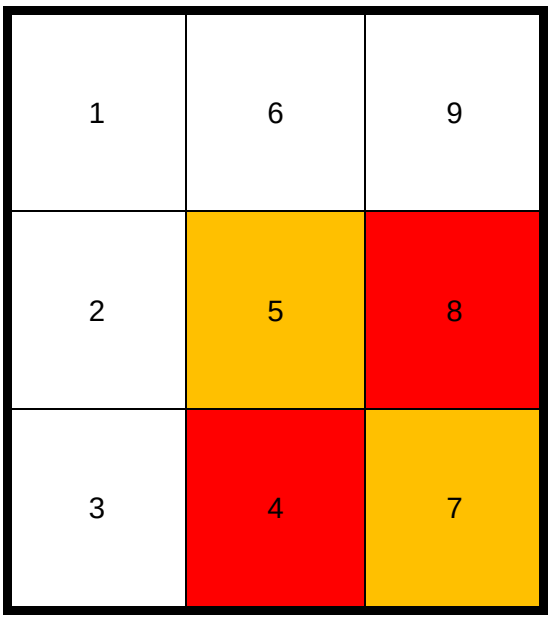
Site 48



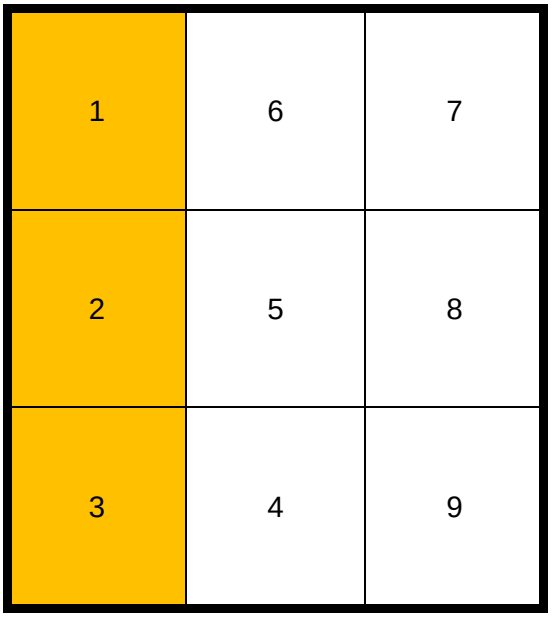
Site 26



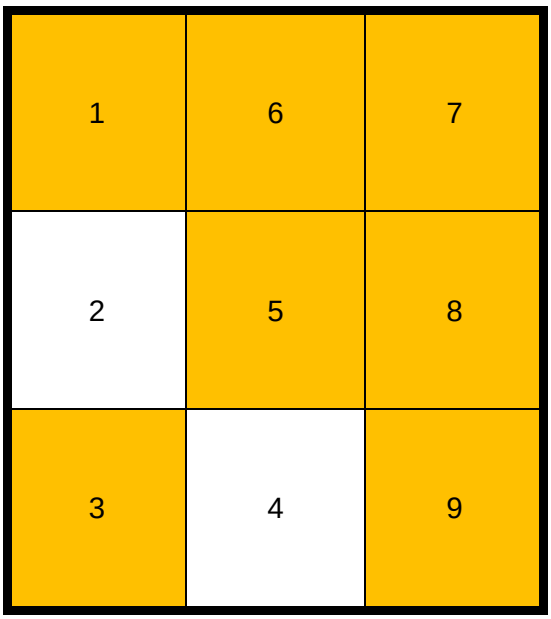
Site 30



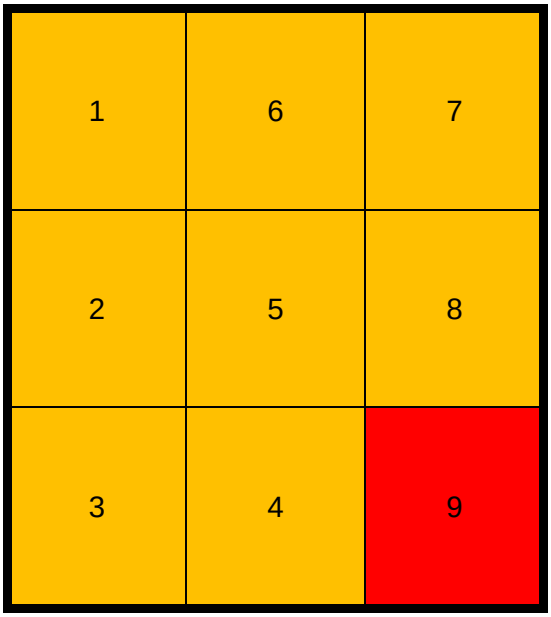
Site 21



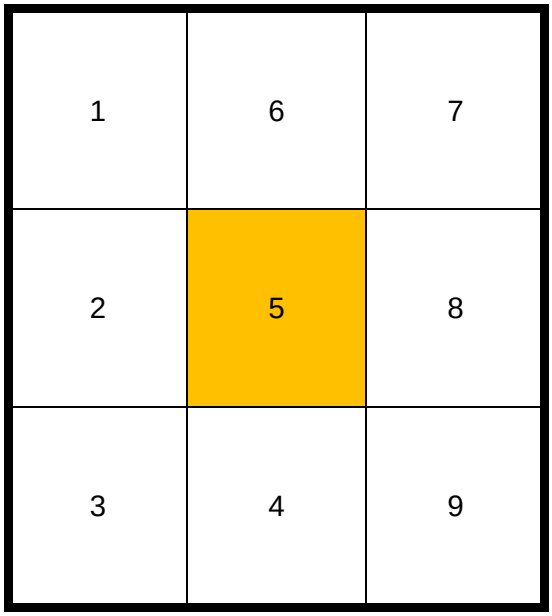
Site 5



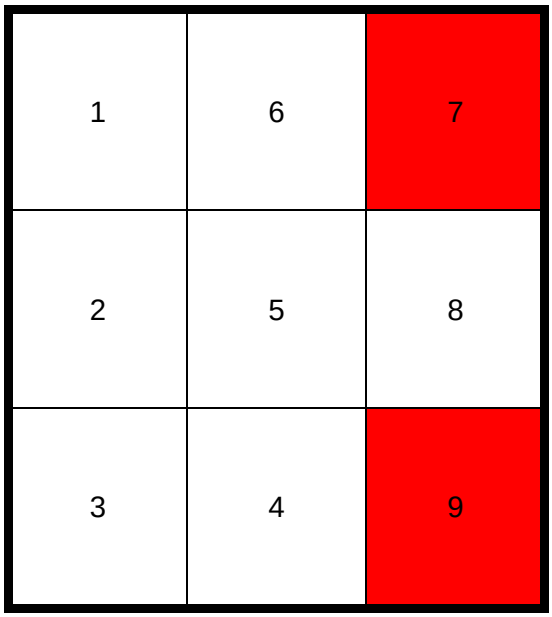
Site 6



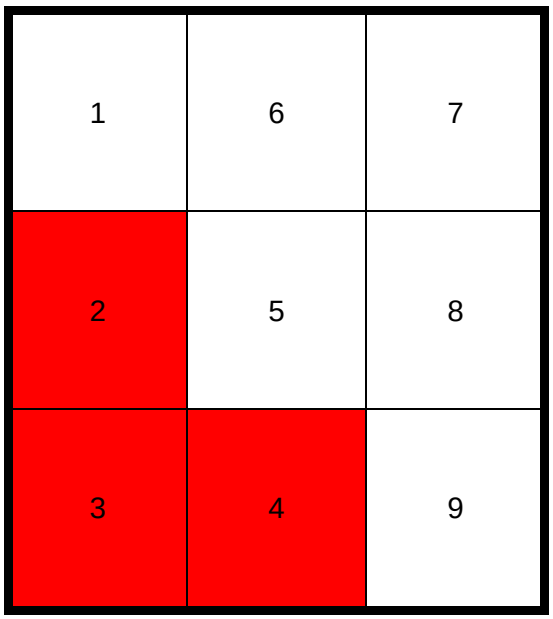
Site 10



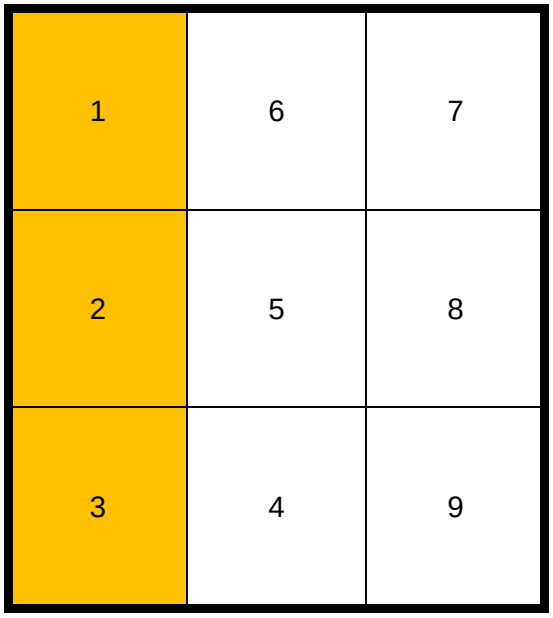
Site 14



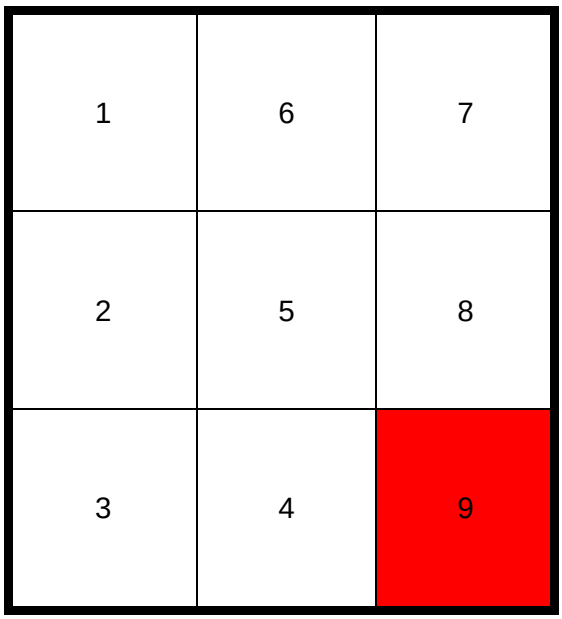
Site 18



Site 26



Site 52a



Appendix 6. Results of modifications trialled for improvement of isolation of *B. anthracis* using the GABRI method of Fasanella *et al*. (2013)

Treatment[a]	Plate	Growth (TMSP no SBA)	PCR Results (C_T values)		
			pX02	pX01	PL3
1	1	+++++	-	-	16.07
	2	+++++	-	13.77	14.48
	3	+++++	-	15.33	14.83
	4	+++++	-	15.54	15.16
2	1	++++	-	-	16.96
	2	++++	-	16.39	16.01
	3	++++	-	13.85	14.57
	4	++++	-	-	20.63
3	1	++	-	12.56	12.94
	2	++	-	12.64	13.32
	3	++	-	13.72	13.48
	4	++	-	12.55	13.02
4	1	++	-	13.83	14.34
	2	++	-	13.88	14.68
	3	++	-	14.07	14.65
	4	++	-	14.11	14.59

[a] Treatment 1 = Original GABRI method + growth on TMSP without sheep blood; Treatment 2 = Modified GABRI method (75°C heat step) + growth on TMSP without sheep blood; Treatment 3 = Modified GABRI method (Ethanol wash no heat step) + spin & re-suspension of pellet in water + growth on TMSP without sheep blood; Treatment 4 = Modified GABRI method (Ethanol wash no heat step) + growth on TMSP without sheep blood

Appendix 7. Usage of ICT kits from 2012-2014

Table 1: Usage of ICT kits over the 2012-2013 Summer/Autumn period

Region	No of kits	ICT Result	Confirmatory test	Overall Result	Species
Victoria					
South West	1	Neg	Pathology neg	Neg	Bovine
	2	Neg	Pathology neg	Neg	Bovine
	3	Neg	Not required	Neg	Bovine
North East	4	Neg	Pathology neg	Neg	Bovine
	5	Neg	Culture Neg	Neg	Bovine
	6	Neg	Not required	Neg	Bovine
	7	Neg	Not required	Neg	Bovine
	8	Neg	Not required	Neg	Bovine
	9	Neg	Not required	Neg	Bovine
	10	Neg	Not required	Neg	Bovine
	11	Neg	Culture Neg	Neg	Bovine
	12	Neg	Culture Neg	Neg	Bovine
	13	Neg	Not required	Neg	Bovine
	14	Neg	Not required	Neg	Bovine
	15	Neg	Culture Neg	Neg	Bovine
	16	Neg	Not required	Neg	Bovine
	17	Neg	Not required	Neg	Bovine
	18	Neg	Not required	Neg	Bovine
	19	Neg	Not required	Neg	Bovine
	20	Neg	Not required	Neg	Bovine
	21	Neg	Not required	Neg	Bovine
	22	Neg	Culture Neg	Neg	Bovine
	23	Neg	Not required	Neg	Bovine
	24	Neg	Culture Neg	Neg	Bovine
	25	Neg	Culture Neg	Neg	Bovine

Region	No of kits	ICT Result	Confirmatory test	Overall Result	Species
Gippsland	26	Neg	Not required	Neg	Bovine
	27	Neg	Not required	Neg	Bovine
	28	Neg	Not required	Neg	Bovine
	29	Neg	Path neg	Neg	Equine
North West	No Kits Used				
New South Wales					
Central North	1	Neg	PCMB Smear & PCR Neg	Neg	
	2	Neg	Not required	Neg	
Central West	3	Neg	PCMB Smear & PCR Neg	Neg	
	4	Neg	PCMB Smear & PCR Neg	Neg	
	5	Neg	PCMB Smear & PCR Neg	Neg	
	6	Neg	PCMB Smear & PCR Neg	Neg	
	7	Neg	PCMB Smear & PCR Neg	Neg	
	8	Neg	Not required	Neg	
	9	Neg	Not required	Neg	
	10	Neg	Not required	Neg	
	11	Neg	Not required	Neg	
	12	Neg	Not required	Neg	
	13	Neg	Not required	Neg	
	14	Pos	PCMB Smear & PCR Pos	Pos	Ovine
Hume	15	Neg	Not required	Neg	
	16	Neg	Not required	Neg	
	17	Neg	Not required	Neg	
Lachlan	18	Pos	PCMB Smear & PCR Pos	Pos	Bovine
North Coast	19	Neg	Not required	Neg	

Region	No of kits	ICT Result	Confirmatory test	Overall Result	Species
North West	20	Neg	PCMB Smear & PCR Neg	Neg	
	21	Neg	Not required	Neg	
	22	Neg	Not required	Neg	
	23	Neg	Not required	Neg	
	24	Neg	Not required	Neg	
	25	Neg	Not required	Neg	
	26	Pos	PCMB Smear & PCR Neg	*	Bovine
	27	Pos	PCMB Smear & PCR Pos	Pos	Bovine
	28	Pos	PCMB Smear & PCR Pos	Pos	Bovine
Riverina	29	Neg	Not required	Neg	
	30	Neg	Not required	Neg	
Western Australia					
South Perth	1	Neg	Culture Neg	Neg	Bovine
	2	Neg	Culture Neg	Neg	Bovine
	3	Neg	Culture Neg	Neg	Bovine
Albany	4	Neg	Culture Neg	Neg	Equine
	5	Neg	Culture Neg	Neg	Equine
	6	Neg	Culture Neg	Neg	Equine
	7	Neg	Culture Neg	Neg	Equine
	8	Neg	Culture Neg	Neg	Equine
Tasmania					
Southern Tasmania	1	Neg	Culture & Smear Neg	Neg	Caprine
Northern Tasmania	2	Neg	Culture & Smear Neg	Neg	Bovine

Table 2: Usage of ICT kits over the 2013-2014 Summer/Autumn period

Region	No of Kits	ICT Result	Confirmatory test	Overall Result	Species
Victoria					
South West	1	Neg	Not required	Neg	Bovine
	2	Neg	Not required	Neg	Bovine
	3	Neg	Not required	Neg	Bovine
	4	Neg	Not required	Neg	Bovine
	5	Neg	Not required	Neg	Bovine
	6	Neg	Not required	Neg	Ovine
	7	Neg	Not required	Neg	Ovine
	8	Neg	Not required	Neg	Bovine
	9	Neg	Not required	Neg	Bovine
	10	Neg	Not required	Neg	Bovine
	11	Neg	Not required	Neg	Bovine
	12	Neg	Not required	Neg	Ovine
	13	Neg	Not required	Neg	Bovine
	14	Neg	Not required	Neg	Bovine
	15	Neg	Not required	Neg	Bovine
North East	16	Neg	Not required	Neg	Bovine
	17	Neg	Not required	Neg	Bovine
	18	Neg	Culture Neg	Neg	Bovine
	19	Neg	Not required	Neg	Bovine
	20	Neg	Not required	Neg	Ovine
	21	Neg	Not required	Neg	Bovine
	22	Neg	Not required	Neg	Bovine
	23	Neg	Culture Neg	Neg	Bovine
	24	Neg	Not required	Neg	Bovine
	25	Neg	Not required	Neg	Bovine

Region	No of Kits	ICT Result	Confirmatory test	Overall Result	Species
	26	Neg	Not required	Neg	Bovine
	27	Neg	Culture Neg	Neg	Bovine
	28	Neg	Not required	Neg	Bovine
	29	Neg	Culture Neg	Neg	Bovine
	30	Neg	Not required	Neg	Bovine
	31	Neg	Not required	Neg	Bovine
	32	Neg	Culture Neg	Neg	Bovine
	33	Neg	Not required	Neg	Bovine
	34	Neg	Not required	Neg	Bovine
	35	Neg	Not required	Neg	Bovine
	36	Neg	Not required	Neg	Bovine
	37	Neg	Not required	Neg	Bovine
	38	Neg	Not required	Neg	Bovine
	39	Neg	Not required	Neg	Bovine
	40	Neg	Not required	Neg	Bovine
	41	Neg	Not required	Neg	Bovine
	42	Neg	Not required	Neg	Bovine
	43	Neg	Culture Neg	Neg	Bovine
	44	Neg	Not required	Neg	Bovine
	45	Neg	Not required	Neg	Bovine
	46	Neg	Not required	Neg	Bovine
	47	Neg	Not required	Neg	Bovine
	48	Neg	Not required	Neg	Bovine
	49	Neg	Culture Neg	Neg	Bovine
	50	Neg	Not required	Neg	Bovine
	51	Neg	Not required	Neg	Ovine
	52	Neg	Not required	Neg	Bovine
	53	Neg	Not required	Neg	Bovine

Region	No of Kits	ICT Result	Confirmatory test	Overall Result	Species
	54	Neg	Not required	Neg	Bovine
	55	Neg	Not required	Neg	Bovine
	56	Neg	Not required	Neg	Bovine
	57	Neg	Not required	Neg	Bovine
	58	Neg	Not required	Neg	Bovine
	59	Neg	Not required	Neg	Bovine
	60	Neg	Not required	Neg	Bovine
	61	Neg	Not required	Neg	Bovine
Gippsland	62	Neg	Not required	Neg	Bovine
	63	Neg	Not required	Neg	Bovine
	64	Neg	Not required	Neg	Bovine
North West	No kits used				
New South Wales					
Central Tablelands	1	Neg	PCMB Smear & PCR Neg	Neg	Bovine
	2	Neg	Not tested	Neg	Bovine
Central West	3	Pos	PCMB Smear & PCR Neg	Inc*[1]	Bovine
	4	Pos			
	5	Pos			
	6	Neg	PCMB Smear & PCR Neg	Neg	Caprine
	7	Neg	PCMB Smear & PCR Neg	Neg	Ovine
	8	Neg	PCMB Smear & PCR Neg	Neg	Ovine
	9	Neg	PCMB Smear & PCR Neg	Neg	Ovine
	10	Neg	PCMB Smear & PCR Neg	Neg	Ovine
	11	Neg	PCMB Smear & PCR Neg	Neg	Ovine
	12	Neg	PCMB Smear & PCR Neg	Neg	Ovine
	13	Pos	PCMB Smear & PCR Pos	Pos	Bovine
	14	Pos	PCMB Smear & PCR Pos	Pos	Ovine

Region	No of Kits	ICT Result	Confirmatory test	Overall Result	Species
	15	Neg	Not tested	Neg	Camelid*[2]
	16	Neg	Not tested	Neg	Ovine*[3]
	17	Neg	Not tested	Neg	Bovine
	18	Neg	Not tested	Neg	Bovine
	19	Neg	Not tested	Neg	Bovine
	20	Neg	Not tested	Neg	Bovine
	21	Neg	Not tested	Neg	Bovine
	22	Neg	Not tested	Neg	Bovine
	23	Neg	Not tested	Neg	Bovine
	24	Neg	PCMB Smear & PCR Neg	Neg	Ovine
Hunter	25	Neg	PCMB Smear & PCR Neg	Neg	Ovine
Murray	26	Neg	PCMB Smear & PCR Neg	Neg	Ovine
North Coast	27	Neg	PCMB Smear & PCR Neg	Neg	Bovine
	28	Neg	PCMB Smear & PCR Neg	Neg	Bovine
	29	Neg	PCMB Smear & PCR Neg	Neg	Bovine
North West	30	Neg	PCMB Smear & PCR Neg	Neg	Bovine
	31	Neg	PCMB Smear & PCR Neg	Neg	Bovine
	32	Neg	PCMB Smear & PCR Neg	Neg	Bovine
	33	Neg	PCMB Smear & PCR Neg	Neg	Bovine
	34	Neg	PCMB Smear & PCR Neg	Neg	Bovine
	35	Neg	PCMB Smear & PCR Neg	Neg	Bovine
	36	Neg	Not tested	Neg	Bovine
	37	Neg	Not tested	Neg	Bovine
	38	Neg	Not tested	Neg	Bovine
	39	Neg	Not tested	Neg	Bovine
	40	Neg	Not tested	Neg	Bovine
	41	Neg	Not tested	Neg	Bovine

Region	No of Kits	ICT Result	Confirmatory test	Overall Result	Species
	42	Neg	Not tested	Neg	Equine
	43	Neg	Not tested	Neg	Ovine
Northern Tablelands	44	Neg	Not tested	Neg	Bovine
	45	Neg	Not tested	Neg	Bovine
Riverina	46	Neg	PCMB Smear & PCR Neg	Neg	Bovine
	47	Neg	PCMB Smear & PCR Neg	Neg	Bovine
	48	Neg	PCMB Smear & PCR Neg	Neg	Bovine
	49	Neg	Not tested	Neg	Ovine
Western	50	Neg	PCMB Smear & PCR Neg	Neg	Ovine
	51	Neg	PCMB Smear & PCR Neg	Neg	Ovine
South Australia					
Glenside	1	Neg	PCMB Smear Neg	Neg	Ovine
	2	Neg	PCMB Smear Neg	Neg	Porcine*[4]
	3	Neg	PCMB Smear Neg	Neg	Ovine
Tasmania					
Northern	No kits used				
Southern	No kits used				
Western Australia					
South Perth	1	Neg	Not Tested	Neg	Ovine
	2	Neg	Culture Neg	Neg	Bovine

*[1] = Inconclusive result due to inappropriate samples supplied for follow up testing. given the advanced state of decomposition. Comment from the DV “Carcass badly decomposing with maggots everywhere. Animal laying on its left hand side. Left ear missing. Right ear with NLIS tag was crispy, ear removed for further lab testing. Maggots inside ear and no blood present in ear. Photograph taken of NLIS ear tag before removing ear. Rear leg missing and located several feet away from carcass. Unable to find obvious blood since carcass badly decomposed. No eye balls present. Nasal swabs faint positive on ICT test kit. Swab of leg and neck incision also faint positive on ICT test kit."

*[2] =South American Camelid – Alpaca

*[3]= Barbary sheep

*[4]= not recommended for pigs

Appendix 8. National Anthrax Workshop programme, 5-6 May 2014, Melbourne

Monday 5th May		
Opening and Welcome		
10.30am	Morning tea on arrival	
11am	Catherine Ainsworth	Welcome to AgriBio
	Simone Warner	Workshop opening address
Session 1: Epidemiology of anthrax		
11.30am – 12pm	Barbara Moloney	Anthrax in NSW - does historical occurrence influence current risks?
12pm – 12.30pm	Laura MacFarlane-Berry	A brief history of anthrax in Victoria
12.30pm – 1.30pm	Lunch	
Session 2: Anthrax Diagnostics		
1.30pm – 1.50pm	Janine Muller	Activities of the National Anthrax Reference Laboratory
1.50pm – 2.10pm	Maria Hardy	Anthrax diagnostic capability and activities at EMAI
2.10pm – 2.30pm	Janine Muller	Evaluation of the anthrax ICT
2.30pm – 3.30pm	Tour of the AgriBio facility	
3.30pm – 4pm	Afternoon tea	
Session 3: Field aspects / Biosecurity		
4pm – 4.20pm	Mark Corrigan	Anthrax Surveillance and ICT testing since 2007
4.20pm – 4.40pm	Duncan Worsfold	Biosecurity Improvements for Anthrax Control
4.40pm – 5pm	Graham Bailey	Assessment of the effectiveness of incineration for anthrax decontamination
5pm	Brief Wrap up	
	Transfer to Rydges Bell City	
6.45pm -	Dinner at Moors Head High Street, Thornbury	
Tuesday 6th May		
Session 4: Current Anthrax Research		
9am – 9.30am	Mark Fegan	Molecular epidemiology and enhanced diagnostics of *B. anthracis* in Australia
9.30am – 9.50am	Ilhan Mohammad	Development and application of methods for the detection of *B. anthracis* in soil
9.50am – 10.10am	Kelly Porter	Serologic surveillance of anthrax in Victorian livestock
10.15am – 10.45am	Morning tea	
10.45am – 11.05am	Mark Fegan	Ecological Niche Modelling of *B. anthracis* in Australia
11.05am – 11.25am	Kim Newberry	Laboratory Capacity Building in Indonesia for improved Anthrax Diagnostics and Control
11.25am – 11.45pm	Laura MacFarlane-Berry	Anthrax outbreak investigations in Bangladesh
11.45am – 12.45pm	Open session with all workshop participants / future directions Workshop close	
Lunch 12.45pm -		

Appendix 9. List of people invited to attend the National Anthrax Workshop. A total of 30 people attended of the 43 people invited.

	Invited attendees	Attended	Organisation Representing
1	Tony Britt	No	Director, Animal Biosecurity and Welfare
2	Leanna Dries	Yes	Biosecurity Victoria, DEPI Vic
3	Malcolm Ramsay	No	Biosecurity Victoria, DEPI Vic
4	Cameron Bell	Yes	Acting CVO, DEPI Vic
5	Laura McFarlance-Berry	Yes	Biosecurity Victoria, DEPI Vic
6	Kelly Porter	Yes	Biosecurity Victoria, DEPI Vic
7	Catherine Ainsworth	Yes	BRD, DEPI Vic
8	Mark Corrigan	Yes	Biosecurity Victoria, DEPI Vic
9	Duncan Worsfold	Yes	Biosecurity Victoria, DEPI Vic
10	Chris Werner	No	Biosecurity Victoria, DEPI Vic
11	Dwane O'Brien	Yes	Biosecurity Victoria, DEPI Vic
12	Joanne Wall	Yes	Biosecurity Victoria, DEPI Vic
13	Mark Fegan	Yes	BRD, DEPI Vic
14	Janine Muller	Yes	BRD, DEPI Vic
15	Ilhan Mohammad	Yes	BRD, DEPI Vic
16	Simone Warner	Yes	BRD, DEPI Vic
17	Johann Schroder	Yes	Program Manager, MLA
18	John Allen	No	CSIRO AAHL
19	Roger Paskin	No	Chief Veterinary Officer, SA
20	Kevin de Witte	Yes	Animal Health Australia
21	Barbara Moloney	Yes	NSW DPI, Orange
22	Tristan Jubb	No	Livestock Health Systems Australia
23	Richard Rubira	Yes	Australian Department of Agriculture
24	Robin Condron	Yes	Dairy Australia
25	Dan Salmon	Yes	NSW Local Land Services
26	Vin Delahunty	No	Victorian Farmers Federation
27	Geoff Fisken	No	Chair, Sheep & Goat compensation Fund
28	Chris Wallace Smith	No	Chair, Cattle Compensation Fund
29	Patrick Kluver	Yes	Livestock Biosecurity Network
30	Simon Firestone	Yes	University of Melbourne
31	Graham Bailey	Yes	NSW DPI, Orange
32	Jef Hammond	No	EMAI, NSW DPI
33	Maria Hardy	Yes	EMAI, NSW DPI
34	Kathy Pike	Yes	DPIPWE, Tasmania
35	Linda Donachie	No	DPIPWE, Tasmania
36	Natasha Baker	Yes	Gribbles SA
37	Nicky Buller	No	Animal Health Laboratory, DAFWA
38	Siobhan Gleeson	Yes	NSW Local Land Services
39	Sam Allan	No	NSW DPI Biosecurity, Tamworth
40	Kim Newberry	Yes	CSIRO AAHL
41	Mark Stevenson	Yes	University of Melbourne
42	Angus Campbell	Yes	Mackinnon Project, Uni Melbourne
43	Colin Wilks	Yes	University of Melbourne
	TOTAL	30	

Appendix 10. Summary of the responses to the National Anthrax Workshop Evaluation questionnaire. A total of 17 responses were submitted, excluding any from the project team members. The numbers in the table represent the total number of responses obtained for each of the scores available.

Please rate the following:	Negative/Low 1	2	3	4	Positive/High 5
How well was the workshop organised?	0	0	0	11	6
How well did the content represent work being conducted in Australia in anthrax?	0	0	0	12	5
Do you understand the role of the Anthrax Reference Lab better as a result of this workshop?	0	0	0	11	6
Did you find attending the workshop to be valuable for you?	0	0	0	8	9
Would you recommend the workshop to someone else if it was held again?	0	0	0	9	8
Would you come again if another workshop was held in 2-3 years time?	0	1	1	6	9
To what level would you now rate anthrax as a disease of national significance?	0	1	7	8	1
Do you have any comments on the content of the workshop?	• Good mix of information from a few states, and a good balance of field and research work • Valuable sharing of information and better understanding of evidence • Content very interesting • Trade/market access reputation • Production effects are limited • More presentations on field aspects of anthrax epidemiology and control • As someone outside the inner circle of anthrax clinical work or research, it was great to get a holistic overview of anthrax in Australia • Good to establish contacts • Need to produce a report of the workshop • Need to capture local data before officers involved are no longer available				
Any other feedback?	• Very good • Access to power points please • Final session could have been a bit more structured • Need powerpoints and report • Perhaps could have expanded guest list • Thank you to the organisers • Would be good to repeat every year/few years as required to keep everyone updated with current/recent work • Well organised and a great setting. Relaxed approach gave plenty of time for informal discussions. More work needed for all of us.				

10 Bibliography

Achtman, M. (2008). Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. *Annu Rev Microbiol* 62, 53-70.

Adjemian, J. C. Z., Girvetz, E. H., Beckett, L. & Foley, J. E. (2006). Analysis of Genetic Algorithm for Rule-Set Production (GARP) modeling approach for predicting distributions of fleas implicated as vectors of plague, *Yersinia pestis*, in California. *J Med Entomol* 43, 93-103.

Anderson, R. P., Gómez-Laverde, M. & Peterson, A. T. (2002). Geographical distributions of spiny pocket mice in South America: insights from predictive models. *Glob Ecol Biogeogr* 11, 131-141.

Anderson, R. P., Lew, D. & Peterson, A. T. (2003). Evaluating predictive models of species' distributions: Criteria for selecting optimal models. *Ecol Model* 162, 211-232.

Animal Health Australia (2012).Australian Veterinary Emergency Plan (AUSVETPLAN). Disease strategy: anthrax. Edition 3.3. Canberra, Australia: Primary Industries Ministerial Council.

Antwerpen, M. H., Zimmermann, P., Bewley, K., Frangoulidis, D. & Meyer, H. (2008). Real-time PCR system targeting a chromosomal marker specific for *Bacillus anthracis*. *Mol Cell Probes* 22, 313-315.

Berg, T., Suddes, H., Morrice, G. & Hornitzky, M. (2006). Comparison of PCR, culture and microscopy of blood smears for the diagnosis of anthrax in sheep and cattle. *Lett Appl Microbiol* 43, 181-186.

Beveridge, W. I. B. (1983). Anthrax. In *Animal Health in Australia Bacterial diseases of cattle, sheep and goats*, pp. 14-19. Canberra, Australia: Australian Government Publishing Service.

Beyer, W. & Turnbull, P. C. B. (2009). Anthrax in animals. *Mol Asp Med* 30, 481-489.

Beyer, W., Bellan, S., Eberle, G. & other authors (2012). Distribution and molecular evolution of *Bacillus anthracis* genotypes in Namibia. *PLoS Negl Trop Dis* 6, e1534.

Biek, R., O'Hare, A., Wright, D. & other authors (2012). Whole genome sequencing reveals local transmission patterns of *Mycobacterium bovis* in sympatric cattle and badger populations. *PLoS Pathog* 8.

Blackburn, J. K., McNyset, K. M., Curtis, A. & Hugh-Jones, M. E. (2007). Modeling the geographic distribution of *Bacillus anthracis*, the causative agent of anthrax disease, for the contiguous United States using predictive ecologic niche modeling. *Am J Trop Med Hyg* 77, 1103-1110.

Burans, J., Keleher, A., O'Brien, T., Hager, J., Plummer, A. & Morgan, C. (1995). Rapid method for the diagnosis of *Bacillus anthracis* infection in clinical samples using a hand-held assay. *Salisbury medical bulletin* 87, 36-37.

Costa, J., Peterson, A. T. & Beard, C. B. (2002). Ecologic niche modeling and differentiation of populations of *Triatoma brasiliensis* neiva, 1911, the most important Chagas' disease vector in Northeastern Brazil (hemiptera, reduviidae, triatominae). *Am J Trop Med Hyg* 67, 516-520.

Dauphin, L. A. & Bowen, M. D. (2009). A simple method for the rapid removal of *Bacillus anthracis* spores from DNA preparations. *J Microbiol Methods* 76, 212-214.

Dragon, D. C. & Rennie, R. P. (2001). Evaluation of spore extraction and purification methods for selective recovery of viable *Bacillus anthracis* spores. *Lett Appl Microbiol* 33, 100-105.

Durrheim, D. N., Freeman, P., Roth, I. & Hornitzky, M. (2009). Epidemiologic questions from anthrax outbreak, Hunter Valley, Australia. *Emerg Infect Dis* 15, 840-842.

Fasanella, A., Di Taranto, P., Garofolo, G., Colao, V., Marino, L., Buonavoglia, D., Pedarra, C., Adone, R. & Jones, M. H. (2013). Ground Anthrax Bacillus Refined Isolation (GABRI) method for analyzing environmental samples with low levels of *Bacillus anthracis* contamination. *BMC Microbiol* 13, 167.

Fielding, A. H. & Bell, J. F. (1997). A review of methods for the assessment of prediction errors in conservation presence/absence models. *Environ Conserv* 24, 38-49.

Forshaw, D., Higgs, A. R., Moir, D. C., Ellis, T. M. & Links, I. J. (1996). Anthrax in cattle in southern Western Australia. *Aust Vet J* 74, 391-393.

Garofolo, G., Ciammaruconi, A., Fasanella, A., Scasciamacchia, S., Adone, R., Pittiglio, V. & Lista, F. (2010). SNR analysis: molecular investigation of an anthrax epidemic. *BMC Vet Res* 6.

Gulledge, J. S., Luna, V. A., Luna, A. J., Zartman, R. & Cannons, A. C. (2010). Detection of low numbers of *Bacillus anthracis* spores in three soils using five commercial DNA extraction methods with and without an enrichment step. *J Appl Microbiol* 109, 1509-1520.

Hanley, J. A. & McNeil, B. J. (1982). The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* 143, 29-36.

Hay, S. I., Tatem, A. J., Graham, A. J., Goetz, S. J. & Rogers, D. J. (2006). Global environmental data for mapping infectious disease distribution. *Adv Parasitol* 62, 37-77.

Hugh-Jones, M. & Blackburn, J. (2009). The ecology of *Bacillus anthracis*. *Mol Asp Med* 30, 356-367.

Joyner, T. A., Lukhnova, L., Pazilov, Y., Temiralyeva, G., Hugh-Jones, M. E., Aikimbayev, A. & Blackburn, J. K. (2010a). Modeling the potential distribution of *Bacillus anthracis* under multiple climate change scenarios for Kazakhstan. *PLoS ONE* 5.

Joyner, T. A., Lukhnova, L., Pazilov, Y., Temiralyeva, G., Hugh-Jones, M. E., Aikimbayev, A. & Blackburn, J. K. (2010b). Modeling the potential distribution of Bacillus anthracis under multiple climate change scenarios for Kazakhstan. *PLoS ONE* 5, e9596.

Keim, P., Price, L. B., Klevytska, A. M., Smith, K. L., Schupp, J. M., Okinaka, R., Jackson, P. J. & Hugh-Jones, M. E. (2000). Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis*. *J Bacteriol* 182, 2928-2936.

Keim, P., Van Ert, M. N., Pearson, T., Vogler, A. J., Huynh, L. Y. & Wagner, D. M. (2004). Anthrax molecular epidemiology and forensics: using the appropriate marker for different evolutionary scales. *Infect Genet Evol* 4, 205-213.

Kenefic, L. J., Beaudry, J., Trim, C. & other authors (2008). High resolution genotyping of *Bacillus anthracis* outbreak strains using four highly mutable single nucleotide repeat markers. *Lett Appl Microbiol* 46, 600-603.

Kenefic, L. J., Pearson, T., Okinaka, R. T. & other authors (2009). Pre-Columbian origins for North American anthrax. *PLoS ONE* 4.

Knisely, R. F. (1966). Selective medium for *Bacillus anthracis*. *J Bacteriol* 92, 784-786.

Köser, C. U., Holden, M. T. G., Ellington, M. J. & other authors (2012). Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. *N Engl J Med* 366, 2267-2275.

Kostelnick, J. C., Peterson, D. L., Egbert, S. L., McNyset, K. M. & Cully, J. F. (2007). Ecological niche modeling of black-tailed prairie dog habitats in Kansas. *Trans Kans Acad Sci* 110, 187-200.

Lewis, T., Loman, N. J., Bingle, L., Jumaa, P., Weinstock, G. M., Mortiboy, D. & Pallen, M. J. (2010). High-throughput whole-genome sequencing to dissect the epidemiology of *Acinetobacter baumannii* isolates from a hospital outbreak. *J Hosp Infect* 75, 37-41.

Lim, B. K., Townsend Peterson, A. & Engstrom, M. D. (2002). Robustness of ecological niche modeling algorithms for mammals in Guyana. *Biodivers Conserv* 11, 1237-1246.

Lindeque, P. M. & Turnbull, P. C. (1994). Ecology and epidemiology of anthrax in the Etosha National Park, Namibia. *Onderstepoort J Vet Res* 61, 71-83.

Lista, F., Faggioni, G., Valjevac, S. & other authors (2006). Genotyping of *Bacillus anthracis* strains based on automated capillary 25-loci multiple locus variable-number tandem repeats analysis. *BMC Microbiol* 6, 33.

Marston, C. K., Beesley, C., Helsel, L. & Hoffmaster, A. R. (2008). Evaluation of two selective media for the isolation of *Bacillus anthracis*. *Lett Appl Microbiol* 47, 25-30.

McNyset, K. (2005). Use of ecological niche modelling to predict distributions of freshwater fish species in Kansas. *Ecol Freshw Fish* 14, 243-255.

Muller, J. D., Wilks, C. R., O'Riley, K. J., Condron, R. J. C., Bull, R. & Mateczun, A. (2004). Specificity of an immunodiagnostic test for anthrax. *Aust Vet J* 82, 220-222.

Mullins, J., Lukhnova, L., Aikimbayev, A., Pazilov, Y., Van Ert, M. & Blackburn, J. K. (2011). Ecological niche modelling of the *Bacillus anthracis* A1.a sub-lineage in Kazakhstan. *BMC Ecol* 11, 32.

Mullins, J. C., Garofolo, G., Van Ert, M., Fasanella, A., Lukhnova, L., Hugh-Jones, M. E. & Blackburn, J. K. (2013). Ecological niche modeling of *Bacillus anthracis* on three continents: evidence for genetic-ecological divergence? *PLoS ONE* 8, e72451.

Nakazawa, Y., Williams, R. A. J., Peterson, A. T., Mead, P. S., Kugeler, K. J. & Petersen, J. M. (2010). Ecological niche modeling of *Francisella tularensis* subspecies and clades in the United States. *Am J Trop Med Hyg* 82, 912-918.

Office International des Epizooties (2008). Anthrax, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals OIE. Paris, France: OIE.

Okutani, A., Sekizuka, T., Boldbaatar, B., Yamada, A., Kuroda, M. & Inoue, S. (2010). Phylogenetic typing of *Bacillus anthracis* isolated in Japan by multiple locus variable-number tandem repeats and the comprehensive single nucleotide polymorphism. *J Vet Med Sci* 72, 93-97.

Pearson, T., Busch, J. D., Ravel, J. & other authors (2004). Phylogenetic discovery bias in *Bacillus anthracis* using single-nucleotide polymorphisms from whole-genome sequencing. *Proc Natl Acad Sci U S A* 101, 13536-13541.

Peterson, A. T., Sánchez-Cordero, V., Martínez-Meyer, E. & Navarro-Sigüenza, A. G. (2006). Tracking population extirpations via melding ecological niche modeling with land-cover information. *Ecol Model* 195, 229-236.

Rasko, D. A., Altherr, M. R., Han, C. S. & Ravel, J. (2005). Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiol Rev* 29, 303-329.

Rasko, D. A., Worsham, P. L., Abshire, T. G. & other authors (2011). *Bacillus anthracis* comparative genome analysis in support of the Amerithrax investigation. *Proc Natl Acad Sci U S A* 108, 5027-5032.

Robinson, S. & Maloney, B. (2006). Anthrax: Primefact 114. *NSW Department of Primary Industries [cited 2013 Dec 20] Available from* http://wwwdpinswgovau/aboutus/resources/factsheets/primefacts/?a=52312.

Schürch, A. C., Kremer, K., Hendriks, A. C. A. & other authors (2011). SNP/RD typing of *Mycobacterium tuberculosis* Beijing strains reveals local and worldwide disseminated clonal complexes. *PLoS ONE* 6.

Seddon, H. R. (1953). Anthrax. In *Diseases of Domestic Animals in Australia Part 5 Vol 1*, pp. 8-40. Canberra, ACT, Australia: Australian Department of Health.

Seddon, H. R. & Albiston, H. E. (1965). Bacterial Diseases. In *Diseases of Domestic Animals in Australia*, pp. 12-26: Australian Department of Health.

Smith, K. L., DeVos, V., Bryden, H., Price, L. B., Hugh-Jones, M. E. & Keim, P. (2000). *Bacillus anthracis* diversity in Kruger National Park. *J Clin Microbiol* 38, 3780-3784.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28, 2731-2739.

Tourasse, N. J., Helgason, E., Økstad, O. A., Hegna, I. K. & Kolstø, A. B. (2006). The *Bacillus cereus* group: Novel aspects of population structure and genome dynamics. *J Appl Microbiol* 101, 579-593.

Townsend Peterson, A., Sánchez-Cordero, V., Ben Beard, C. & Ramsey, J. M. (2002). Ecologic niche modeling and potential reservoirs for Chagas disease, Mexico. *Emerg Infect Dis* 8, 662-667.

Townsend Peterson, A., Papeş, M. & Eaton, M. (2007). Transferability and model evaluation in ecological niche modeling: A comparison of GARP and Maxent. *Ecography* 30, 550-560.

Turnbull, P. C. B., Hutson, R. A., Ward, M. J., Jones, M. N., Quinn, C. P., Finnie, N. J., Duggleby, C. J., Kramer, J. M. & Melling, J. (1992). *Bacillus anthracis* but not always anthrax. *J Appl Bacteriol* 72, 21-28.

Turnbull, P. C. B. (1999). Definitive identification of *Bacillus anthracis*-a review. *J Appl Microbiol* 87, 237-240.

Turner, A. J., Galvin, J. W., Rubira, R. J., Condron, R. J. & Bradley, T. (1999a). Experiences with vaccination and epidemiological investigations on an anthrax outbreak in Australia in 1997. *J Appl Microbiol* 87, 294-297.

Turner, A. J., Galvin, J. W., Rubira, R. J. & Miller, G. T. (1999b). Anthrax explodes in an Australian summer. *J Appl Microbiol* 87, 196-199.

Van Ert, M. N., Easterday, W. R., Huynh, L. Y. & other authors (2007a). Global Genetic Population Structure of *Bacillus anthracis*. *PLoS ONE* 2, e461.

Van Ert, M. N., Easterday, W. R., Simonson, T. S. & other authors (2007b). Strain-specific single-nucleotide polymorphism assays for the *Bacillus anthracis* Ames strain. *J Clin Microbiol* 45, 47-53.

Wielinga, P. R., Hamidjaja, R. A., Ågren, J. & other authors (2011). A multiplex real-time PCR for identifying and differentiating *B. anthracis* virulent types. *Int J Food Microbiol* 145, S137-S144.

Wiley, E. O., McNyset, K. M., Peterson, A. T., Robins, C. R. & Stewart, A. M. (2003). Niche modeling and geographic range predictions in the marine environment using a machine learning algorithm. *Oceanography* 16, 120-126.

World Health Organization (2008). *Anthrax in humans and animals*, 4th edn. Geneva, Switzerland: World Health Organization.