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SPECIFICITY OF A HAND-HELD IMMUNOCHROMATOGRAPHIC ASSAY FOR ANTHRAX IN CATTLE

A thesis presented in partial fulfilment (50%) of the requirements for the degree of

MASTER OF VETERINARY STUDIES

IN

INFECTIOUS DISEASES

At Massey University, Turitea campus,
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ABSTRACT

The purpose of this study was to determine whether the hand-held immunochromatographic assay was a reliable method for field based diagnosis of anthrax. This study was designed and conducted with an emphasis on obtaining an estimate of performance accuracy, in terms of specificity for the protective antigen (PA) component of the anthrax toxin. To examine specificity the hand-held assay had to be assessed under similar circumstances to that experienced in the field from typical animals in which anthrax may be suspected.

To achieve this, blood samples were collected post-mortem from 240 cattle at the Stanhope and Camperdown knackeries in Victoria. Blood smears were prepared, hand-held assays were performed on-site and a sample of blood transported back to the laboratory for bacterial culture. All 240 samples gave negative results in the hand-held assay and *B. anthracis* was not detected in any sample by culture or blood smear, which were considered the definitive diagnostic tests. Thus the hand-held assay was regarded as 100% specific (98.5-100%; 95% CI) for these cattle examined in Victoria.

The purpose of the second study was to determine whether the live Sterne strain $34F_2$ vaccine for anthrax would result in false positives arising in the hand-held assay in cattle recently vaccinated. Ten cattle were vaccinated with the $34F_2$ vaccine and monitored for 15 days. No PA was detected in the blood of vaccinated cattle in the hand-held assay or on culture within this time. These results show that the hand-held assay does not give false positive test

results in cattle post-vaccination with live $34F_2$ *B. anthracis* vaccine. The hand-held assay can be used with confidence on samples from recently vaccinated cattle that have died when it is necessary to know whether they had succumbed to anthrax or not.

The hand-held assay has the potential to be adopted as a routine test for the preliminary assessment of sudden death in cattle. The simplicity of the assay enables it to be used by unskilled lay people, which means that it could be used by knackery workers for surveillance in areas with a previous history of disease or by veterinarians as a preliminary routine tool in investigating sudden death in cattle. However the study described in this thesis only assesses the specificity of the assay and a further study, involving a similar number of cattle affected with anthrax, needs to be conducted to assess the sensitivity of the assay.

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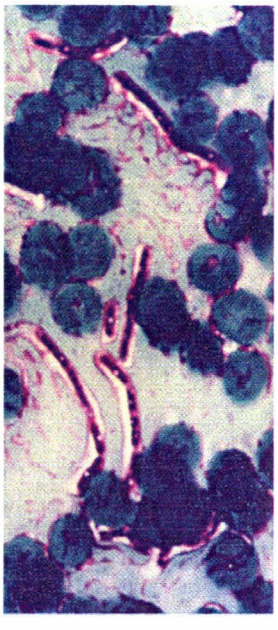
LIST OF ABBREVIATIONS

B. anthracis	*Bacillus anthracis*
B. mycoides	*Bacillus mycoides*
cfu	colony forming units
CI	confidence interval
cm	centimetre
CO_2	carbon dioxide
°C	degree celcius
D+	disease present
D-	disease absent
EDTA	ethylenediamine tetraacetic acid
EF	oedema factor
ELISA	Enzyme Linked Immunosorbent Assay
ELLA	Enzyme-Linked Lectinosorbent Assay
ET	oedema toxin
FP	false positive
g	gram
h	hour
IgG	Immunoglobulin G
IHA	indirect haemagglutination assay
kDa	kilodalton
km	kilometre
LF	lethal factor
LT	lethal toxin

mm	millimetre
mL	millilitre
ng	nanogram
NPV	Negative predictive value
P	pretest probability
PA	Protective antigen
PCR	Polymerase chain reaction
PLET	polymyxin-lysozyme-EDTA-thallous acetate
PPV	Positive predictive value
rpm	revolutions per minute
s	seconds
SBA	sheep blood agar
Se	sensitivity
Sp	specificity
T+	test positive
T-	test negative
TN	true negative
TNF	tumour necrosis factor
TP	true positive
%	percent
μg	microgram
μm	micrometre

CHAPTER ONE:
REVIEW OF THE LITERATURE



1.1Anthrax general

Bacillus anthracis is the aetiological agent of anthrax, an infectious disease of considerable economic importance worldwide that affects domestic livestock, game animals and occasionally humans (Fujikura, 1989). Although considered a disease of worldwide significance, it is most common in southern and eastern Europe, southern and central America, Africa and Asia. It is believed to have originated in Mesopotamia and northern Africa and the spread of disease has been attributed to the process of domestication of wild ungulates and later spread through the importation of infected bonemeal. Infected bonemeal was shipped to areas in Europe, Asia, the United States of America and Australia (De Vos, 1994).

Anthrax is a bacterial disease caused by the endospore forming *Bacillus anthracis*, a Gram-positive, rod-shaped, aerobic or facultatively anaerobic bacterium. Microscopically the vegetative form typically appears square ended and is approximately 4-10 µm long and 1-1.5 µm wide. In the presence of oxygen and towards the exponential phase of growth, one ellipsoidal spore is formed per cell situated centrally and not swelling the sporangium.

B. anthracis belongs to the family Bacillaceae of which all species are aerobic and spore-forming, however it is capable of causing invasive disease in humans and other mammals setting it apart from the other species within this grouping (Farchaus *et al,* 1995).

Anthrax is an historically significant disease for three main reasons. It was the first disease of man and animals to have microbial aetiology demonstrated (Klemm & Klemm, 1959). There appear to be many claims to the original detection of rod-shaped bodies in the blood of animals infected with anthrax. However Davaine was one of the first and certainly more influential scientists in the early discovery of anthrax. Although Davaine discovered "rod-shaped" microorganisms within the blood of infected animals in 1850 it was not until 1863 that he demonstrated that only the blood of sick animals contained these organisms. He gave the name "bacterides" to these rod-shaped organisms (Klemm & Klemm, 1959). Between 1863 and 1868 Davaine demonstrated that the blood of sick animals could cause disease when inoculated into healthy animals (Whitford & Hugh-Jones, 1994; Choquette & Broughton, 1981) and by 1873 that the inoculum did not produce disease when pressed through a clay filter (Klemm & Klemm, 1959). This latter work was inspired by Pasteur's work on fermentative bacteria, which led Davaine to appreciate the significance of his earlier findings in relation to the concept that microorganisms were capable of producing effects out of all proportion to their size (Choquette and Broughton, 1981). By 1868 Davaine had demonstrated that "bacterides" could transmit disease. However the true cause of the disease was established in 1876 by Koch, a German physician who cultivated the microorganisms *in vitro* and maintained infectivity. Koch cultured the anthrax bacterium, transferred it to mice and recovered it in pure culture thus satisfying "Koch's postulates" for the microbial cause of disease (Klemm & Klemm, 1959). Through these studies Koch was able to gain an understanding of the germination cycle of *B.*

anthracis; he was able to demonstrate that the rod shaped bacteria grew in size, developed spores and germinated back into rods. Pasteur devised many experiments to demonstrate the aetiological role of anthrax bacilli. He filtered cultures and found that the filtrate did not produce disease when inoculated into healthy animals. He allowed organisms to settle out of culture and demonstrated that the supernatant did not produce disease whereas the sediment or pellet of organisms did. Not only did such experiments demonstrate the cause of anthrax but also firmly established the germ theory of disease (Klemm & Klemm, 1959). Thus it was in the late 1870's that the germ theory of disease was established by Pasteur and Koch with the anthrax bacillus (Whitford & Hugh-Jones, 1994).

In 1881, Pasteur and his colleagues Chamberland and Roux reported on the efficacy of a vaccine against anthrax. It was one of the first infectious diseases against which a bacterial vaccine was shown to be an effective and practical means of prophylaxis (Choquette & Broughton, 1981). Pasteur's early anthrax vaccine was an attenuated strain of *B. anthracis* whereby the virulence had been artificially reduced. Pasteur achieved attenuation by heat shocking *B. anthracis* cultures at 42 to 43ºC for 15 to 20 days and 10 to 12 days (Turnbull, 1991). Vaccination was a two-part process consisting of "Pasteur I" and "Pasteur II". Pasteur's two-part vaccine schedule consisted of the first inoculation, Pasteur I, followed in two weeks by the less attenuated strain, Pasteur II. This method of vaccination became widely used for cattle and sheep in Europe and South America for 50 years. However it was still lethal for both guinea pigs and mice. Over years the heat-attenuated vaccines lost

potency and the virulence became difficult to stabilise; this led to occasional losses amongst vaccinated animals and the vaccines could no longer be safely administered to particular susceptible species (Sterne, 1937).

As a result between 1920 and 1930 there were improvements made to the Pasteur vaccine, such things as glycerine to improve the longevity and immunisation efficiency of the spore and 1 to 10 percent saponin to provoke an inflammatory response at the inoculation site. Saponin was supposed to reduce the virulence of attenuated vaccines, however Sterne discovered that when added to the less virulent strains of *B. anthracis* such as the Pasteur II it aided in immunisation (Turnbull, 1991).

It was Sterne's own live spore vaccine $34F_2$, which became widely used in the defence against anthrax. The avirulent live strain that Sterne produced was unable to form a capsule under any given circumstances and was therefore incapable of killing most susceptible species at the appropriate dose. It still maintained the ability to produce the same amount of toxin as a virulent strain, hence its ability to induce strong immunity when inoculated (Harris-Smith *et al,* 1958). Adjuvants such as 0.5 percent saponin and 50 percent glycerine enhanced the vaccine's effectiveness. One of the benefits of Sterne's vaccine is that avirulent but live vaccines require only a single dose to give strong immunity (Spears & Davidson, 1959).

1.2 Anthrax in Australia

Anthrax is considered uncommon in Australia with clinical cases seen sporadically in sheep, less commonly in cattle, occasionally in pigs and rarely in goats and horses (Beveridge, 1983). The first recorded outbreak of anthrax in Australia was in 1847 at Leppington, south west of Sydney. Infection rapidly spread throughout New South Wales and into southern Queensland with considerable losses experienced through the ongoing movement of livestock. In 1866 disease was recognised in Queensland and in 1876 it was reported in Victoria (Mitchell 1877 as cited in Seddon & Albiston 1965), although diagnosis was not officially accepted until 1880 (Cameron, 1906).

Initially it was known as Cumberland disease named after the county where it was first recognised. As late as 1883 Cumberland disease was still not associated with anthrax; it was attributed to plant poisoning (Hamlet, 1889). The connection between the two diseases was made initially by Willows, an English veterinary surgeon, who investigated heavy mortalities in the Lachlan pastoral district of New South Wales, where he pronounced that the animals had died from anthrax. He did this by describing typical anthrax appearance at post-mortem examination and demonstrated causative bacilli in the blood (Willows, 1883). Stanley confirmed this diagnosis in 1885; whereby extending investigations he too concluded that the disease was anthrax (Stanley, 1886). Definitive identification of Cumberland disease as anthrax came in 1888 when Loir, Germont and Hinds of the Pasteur Institute in Paris carried out extensive

bacteriological investigations concluding that Cumberland disease and anthrax were identical (Seddon & Albiston, 1965).

There are many ways in which anthrax may have entered Australia. These include; imported fodder, bonemeal, wool and hair products. However the most likely source is considered to be the importation of contaminated bonemeal, since manuring with powered bonemeal was a recommended practice for pastures and crops at this time. Indian bonemeal has been implicated in some early outbreaks in Victoria; other outbreaks in both Victoria and Tasmania were found to follow the top dressing of pasture with bonemeal (Weir, 1903). As a result of these outbreaks the importation of unsterilised bonemeal was prohibited in Victoria (Seddon & Albiston, 1965).

Anthrax was first recognised in Victoria in 1876 near Warrnambool and probably arose through the grazing of pastures and crops that had been powered with contaminated bonemeal. The disease further spread over most of the Western District and later to southern and central Victoria. The spread of anthrax into the Melbourne district was due to a shipment of diseased sheep from Warrnambool and the spread of anthrax in the north of the state as a result of livestock movement from New South Wales (Seddon & Albiston, 1965). The most recent outbreak of anthrax in Victoria occurred in cattle between 26th January and the 26th March 1997 when 83 properties in the Goulburn Valley of north central Victoria were affected (Turner *et al* 1999b). The area of the Goulburn Valley where the disease occurred is an intensive dairy farming area made up of small to large irrigation holdings with high stocking rates (Table 1.1).

Table 1.1: The stocking rates for both beef and dairy farms in the Goulburn Valley.

Cattle stocking rates in the Goulburn Valley	
2322 dairy farms	429 136 milking cattle
1742 beef farms	124 250 beef cattle

(Turner *et al*, 1999b)

Prior to this outbreak, anthrax had not been seen in Victoria for nearly ten years (Turner *et al*, 1999b). Immediately after the initial diagnosis, standard control measures were implemented. Movement of stock was prohibited, recent stock movements traced, carcases were disposed of by burning and only stock neighbouring affected properties were vaccinated, this was eventually extended to a zone of 30 km by 20 km (Turner *et al*, 1999a). Despite these actions the outbreak spread to 82 farms by the end of March 1997 with 202 confirmed cases of bovine infection and 4 ovine.

Investigations into the cause of the outbreak failed to demonstrate any single factor responsible for its temporal or geographical distribution. Earthworks to improve irrigation efficiency may have exposed the spore form of *B. anthracis,* by disturbing old anthrax carcase sites from before official records were kept, since this area was most likely a stock route from early times (Turner *et al*, 1999a). The weather was conducive to *B. anthracis* growth with an unusually prolonged period of hot and humid days, conditions which have been

associated with outbreaks in Australia and the United States of America (Turner *et al*, 1999a).

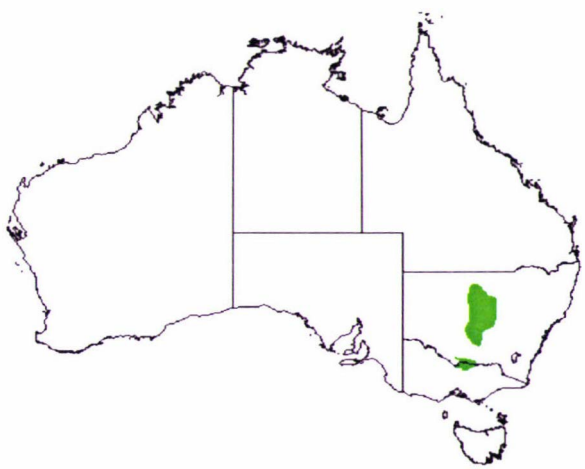
In Australia anthrax is largely confined to specific areas within the states of Victoria and New South Wales (Beveridge, 1983). The occurrence of anthrax in other parts of Australia is rare. Some states such as South Australia and Tasmania last reported outbreaks in 1914 and 1933 respectively; both states are now considered free from anthrax (Seddon & Albiston, 1965). In 1993 Queensland had anthrax confirmed in one cow on a large extensive grazing property near Rockhampton and in January 2002, 10 cases of anthrax were reported at the Collingwood station near Wandoan, 350 km north-west of Brisbane (ProMED, 2002), these being the only cases of anthrax in 50 years. In 1994 Western Australia reported their first known outbreak of anthrax. The three farms involved were in an isolated area north of Walpole on the south coast: 31 cattle died and the source of infection was not determined (Forshaw *et al*, 1996). The Northern Territory has never reported any cases of anthrax and is considered free.

1.3 Epidemiology

New South Wales and Victoria have specific anthrax zones (Figure 1.1) wherein the soil is heavily contaminated with anthrax spores; they have a neutral to alkaline soil (pH 7 to 8) and are generally associated with floodplains and waterways (Beveridge, 1983). Outbreaks in Victoria occur sporadically in the northern and north eastern parts of the state, in addition to small areas in Werribee and west Gippsland (Flynn, 1968). In New South Wales anthrax has

ceased to be a problem in the coastal regions of the state and is generally confined to an area through the centre, on the plains where the annual rainfall is between 250 and 500 mm, the wetter tablelands are considered to be free (Beveridge, 1983).

Figure 1.1: Location of the anthrax belt in Australia.



In New South Wales, sheep are most commonly affected by anthrax and in Victoria it is more commonly cattle (Wise & Kennedy, 1980). The disease has a distinctly seasonal distribution in New South Wales, with most cases occurring during the warmer months, with the peak normally between November through until February, while in Victoria the seasonal variation is much less (Seddon & Albiston, 1965). A study of the pattern of anthrax outbreaks in New South Wales by Wise and Kennedy in 1980 identified that 90 percent occurred during the hotter months of the year. Outbreaks in sheep tended to be more common in years that were drier than average and often occurred one or two weeks after a few days of high humidity. Other areas in the southern section of New

South Wales followed seasons with rainfall above average and predominantly affected cattle (Wise & Kennedy, 1980).

The initiation of an outbreak of anthrax depends on a number of interrelated factors, such as, environmental conditions, human activities, dissemination of bacteria and on specific properties of the bacterium that relate to its ability to survive outside of the host, to enter, infect and multiply successfully (De Vos, 1994). Survival of the bacterium is longer at lower temperatures. For instance, in an unopened carcase with temperatures between 25 and 30°C *B. anthracis* survives no longer than 3 days, whereas at temperatures of 5 to 10°C the bacilli can be recovered from a carcase for up to 4 weeks after death (Merchant & Packer, 1967). This can be attributed in part to the putrefactive process, since vegetative cells of *B. anthracis* within infected animal carcases die rapidly (Christie, 1987). Stein reported in 1942 that exposure to the effects of putrefactive bacteria in an unopened carcase at 25 to 30°C, for as little as 48 h destroys the vegetative anthrax bacilli to the extent that recovering them is made difficult (Stein, 1942). This is because of the fragile nature of the vegetative bacilli that can neither sporulate in the absence of oxygen nor survive and with the increase in CO_2 concentrations, after 72 h there is no chance of recovering *B. anthracis*. The only source that is left for culture are the bloody exudates if these are present (Whitford & Hugh-Jones, 1994).

Vegetative bacilli form spores when conditions are not conducive to growth and multiplication ie: in the presence of oxygen and at biological extremes of temperature and pH. The spore forms are resistant to extreme temperatures,

pH, desiccation, disinfectants, irradiation and other adverse conditions. Survival of spores within the soil depends on a variety of factors, such as initial numbers, climate, the presence of soil saprophytes, chemicals and plant material. Thus in soil that has high biological activity and a diversity of microbial life, the survival period of anthrax spores is probably limited to no more than three to four years (Whitford, 1978). Under ideal conditions spores may remain viable for fifty to potentially two hundred and fifty years (De Vos, 1990).

Spores are capable of germinating and producing vegetative bacilli outside of the host animal if the temperature is between 8 and 45°C, pH between 5 and 9, relative humidity greater than 95% and in the presence of adequate nutrients (Turnbull, 1998). The extent to which the process of germination, multiplication and re-sporulation occur in the environment is not fully known but research by Turnbull and Lindeque in 1994 suggested that the concentration of nutrients required is far greater than what would be expected in the normal environment. Should this unlikely germination occur the probable outcome is death of the emergent bacillus. Thus Lindeque and Turnbull concluded that *B. anthracis* is heavily dependant on an animal host for multiplication (Lindeque & Turnbull, 1994).

In a study conducted by Turnbull in the Etosha National Park, Namibia, the viability of vegetative cells declined rapidly after inoculation into water samples taken from various sites. Within 24 h the number of culturable bacteria dropped from over 10^5 cfu/mL to below detectable levels less than 10 cfu/mL

(Turnbull *et al*, 1989). Thus, it may be understood why the spore form is the predominant phase in existence in the environment and largely through the uptake of spores that anthrax is contracted.

The ability of these spores to remain viable for many years in animal products, soil and the environment is an important factor to consider in the epidemiology of anthrax outbreaks. Carriers of anthrax do not exist, the reservoirs of infection are spores in the soil and the hides and bones of animals that have died from anthrax (Beveridge, 1983).

Mechanical dissemination of anthrax spores by carnivores and flies, which have fed on carcases of animals dead from anthrax can occur (Turell & Knudson, 1987). As early as 1914 Dalrymple investigated the role that scavenging carnivores and biting/non-biting insects had in the spread of anthrax. He investigated a range of animals such as the turkey buzzard, carrion crow, pig, dog, cat, opossum and the common fowl. In the buzzard and crow he showed that the anthrax spores were never excreted in faeces, but could be found in the vomitus and on the beak and feet. The common fowl was the exception and it excreted anthrax spores in its faeces for up to 48 h after feeding. The feline, canine and swine were found to excrete viable spores, which were capable of killing rabbits, in faeces up to three, six and five days respectively, after feeding. This demonstrates the robust nature of the anthrax spore in that the virulence and viability was not altered after passing through the alimentary tract (Dalrymple, 1914).

A study by Turrell and Knudson in 1987 confirmed the suspicion that blood-sucking insects have the ability to transmit lethal *B. anthracis* mechanically. The transmission rates for the common stable fly and mosquitoes ranged between less than 1 h to 4 h after exposure to a bacteraemic guinea pig. The actual puncture mark or the subsequent irritation caused by this bite may provide an inlet into the cutaneous layer for the anthrax spore (Turell & Knudson, 1987). Unlike biting insects the non-biting flies carry infectious spores and bacilli on their hairy body parts and may pass virulent spores in their excreta. A single fly spot was found t o contain enough anthrax to fully cover an agar plate in culture (Dalrymple, 1914) and the infectivity was recoverable up to 20 days after exposure (Turell & Knudson, 1987).

It is therefore a possibility for all of the above animals to assist in the dissemination of anthrax spores to other susceptible species, via direct transmission or from surrounding environment.

1.4 Pathogenesis

B. anthracis spores gain entry into the body through ingestion, inhalation or skin abrasion. Abrasion is more likely to be important in sheep where grass seeds may penetrate the skin unnoticed. The inhalation of spores is fairly rare in animals (De Vos, 1994). After penetration of the natural barriers, spores germinate to produce the vegetative form of *B. anthracis,* that will, left unheeded, kill the host. The exact incubation period for *B. anthracis* under natural conditions is unknown but it is in the range of 1 to 14 days. Factors,

which affect the incubation period, are the quantity of *B. anthracis* organisms within the host, the route of infection and host resistance (De Vos, 1994).

The disease manifests in three different ways; peracute, acute and subacute to chronic. In ruminants, anthrax nearly always takes the peracute or acute form; very occasionally will it be subacute. Peracute disease progresses rapidly, in which death occurs suddenly within a few hours; most animals are found dead with little or no prior sign of illness. However, animals under close observation may be seen to have fever, dyspnoea and muscle tremors for 1 to 2 h before death (Turnbull, 1998). Shortly afterward the animal will often have terminal convulsions collapse and die, with rapid bloating and incomplete rigor mortis (Radostits *et al*, 2000). Following death blood may exude from the body openings, although this is not always observed (Merchant & Packer, 1967).

Development of acute anthrax may be seen in both cattle and horses with many clinical signs such as depression, laboured respiration, fever, anorexia, congested mucosae or oedematous swellings, which may be observed up to 48 h before death. The route of exposure will influence the extent of clinical signs. For instance, ingested spores cause enteritis and colic, accompanied by fever and depression, whereas an insect bite or skin abrasion will cause a localised oedematous subcutaneous swelling. If left untreated this localised infection may spread to the throat, thorax, abdomen, prepuce or mammary glands and be followed by death in 48 to 96 h (Turnbull, 1998). Generally the course of disease is 1 to 3 days with some animals surviving a week or more (Sterne, 1959).

Subacute anthrax is seen occasionally with prolonged illness lasting several days and is not always fatal. Localisation of infection occurs as an oedematous swelling; this may be obvious in the neck and less so in the gastrointestinal tract (Turnbull, 1998).

B. anthracis has two virulence factors; a poly-D-glutamic acid capsule and a tripartite exotoxin composed of Protective Antigen (PA, 83 kDa), Lethal Factor (LF, 87 kDa) and Oedema Factor (EF, 89 kDa) (Beall *et al*, 1961; Stanley & Smith, 1963). The bacterium contains two plasmids PX01 and PX02, which encode the genes for the virulence factors; PX01 regulates the production of the toxins (Mikesell *et al*, 1983) and PX02 regulates the synthesis of capsular material (Green *et al*, 1985; Uchida *et al*, 1985). The toxin bearing plasmid, PX01 is 184.5 kilobase pairs in size and codes for the genes that make up the secreted exotoxins. PX02 is a smaller plasmid of 95.3 kilobases and codes for 3 genes, capA, capB and capC, which are involved in the synthesis of the polyglutamyl capsule (Dixon *et al*, 1999). The three exotoxin components combine to form two binary toxins. Both these toxins are responsible for the characteristic signs and symptoms of anthrax (Turnbull, 1998).

PA binds to specific cell receptors in the host and is subsequently activated by a cell surface protease (Leppla *et al*, 1987), which cleaves a 20 kDa fragment thereby exposing a secondary cell bound receptor site for which LF and EF must compete for binding (Little *et al*, 1988). Five regions of homology were identified at the PA binding domain on both LF and EF (Bragg & Robertson, 1989), suggesting why they compete for the PA receptor (Little & Ivins, 1999).

Once bound, the complex of both LF+PA or EF+PA is then internalised by endocytosis to the cell cytosol and the EF or LF released into the host cell (Gordon *et al*, 1988).

PA has many roles. It mediates the attachment and entry into the cytosol of LF and EF; it contains regions involved in binding to the cell receptor, binding the LF and EF and membrane insertion and translocation of the anthrax toxins (Leppla, 1991). PA is also the major immunogen present in anthrax vaccines (Little & Ivins, 1999).

Oedema Toxin (ET) consists of EF, which is an adenylate cyclase whose catalytic activity is dependent on the presence of calmodulin and calcium ions and PA which is a binding moiety that permits entry of the toxin into the host cell (Leppla, 1982). The conversion of host cell ATP to cyclic AMP by the EF is responsible for the effects of the ET (Hanna, 1998). Proper water homeostasis within the eukaryotic cell is altered with increased levels of cyclic AMP hence the massive oedema seen in cutaneous anthrax (Dixon *et al*, 1999). In general, bacterial toxins that increase cyclic AMP decrease innate immune responses of phagocytes, thus contributing to establishment of the infection (Confer & Eaton, 1982).

Lethal Toxin (LT) consists of LF, which is a zinc metalloprotease and PA (Dixon *et al*, 1999). Shock-inducing cytokines like Tumour Necrosis Factor (TNF) and interleukin-1B play an important role in the sudden death seen in anthrax (Hanna *et al*, 1993). Sublytic concentrations of LT stimulate the expression of these two factors (Hanna *et al*, 1993) and whereas the TNF is secreted into the

extracellular environment the interleukin-1B remains intracellular until lysis of the macrophage in which the bacilli are present. It is the sudden bursting of macrophages with simultaneous release of the shock mediators that is suspected to be the cause of sudden death seen in anthrax (Hanna, 1999). Support for the active function of cytokines in systemic shock was provided through the findings that mice that were passively immunised against TNF and interleukin-1B were protected against the lethal effects of anthrax (Hanna, 1998).

Lethal toxin is the dominant virulence factor produced by *B. anthracis* and is the major cause of death in infected animals. This was shown by Pezard, Berche and Mock (1991), who demonstrated the relative virulence of toxins in infection by using strains of *B. anthracis* that had either LF or EF but not both present. Mice that were challenged with a strain expressing the components of either LT or ET illustrated that LT was the more important virulence factor in this animal model and that the two toxins may act synergistically in infection (Pezard *et al*, 1991).

Lethal toxin cytotoxicity appears to be limited to macrophages, unlike oedema toxin which can affect all cell types. Evidence that macrophages are important *in vivo* was demonstrated by Hanna in 1992 with his experiments in mice. Mice became resistant to LT after depletion of their macrophages. Toxicity was restored by injecting into them sensitive macrophage cell lines. These results lead to the suggestion that LT is responsible for the sequence of biochemical

and physiological events that lead to death of the macrophages and the host animal (Hanna *et al*, 1992).

In conjunction with LT and ET, whose target cells include macrophages and neutrophils, the capsule allows *B. anthracis* to grow unimpeded in the infected host (Ezzell Jr & Welkos, 1999). The exotoxins are thought to inhibit the immune response against infection, whereas the capsule inhibits phagocytosis of the vegetative bacilli (Dixon *et al*, 1999).

In the absence of either plasmid the bacterium is non-virulent and in the absence of either bicarbonate or PX02, *B. anthracis* does not produce a capsule (Fouet *et al*, 1999). Regulation of the expression of toxin and capsule genes is mediated by the transcriptional activator AtxA, whose functions are affected by environmental conditions such as high temperature, concentration of CO_2 and the presence of serum components (Koehler *et al*, 1994).

B. anthracis multiplies in the lymph nodes from where it can pass via the lymph and blood to the reticuloendothelial system, which leads to further proliferation and dissemination into the bloodstream. Initially, during the incubation period, the bacteria are filtered out of the bloodstream via the spleen and other parts of the reticuloendothelial system (Turnbull, 1998). The actions of *B. anthracis* toxins within the host result in necrosis of the lymphatic tissue and breakdown of blood vessels, resulting in the release of large numbers of organisms into the circulation, internal bleeding and rapid fatal septicaemia. These events lead to the characteristic haemorrhage to the exterior, which is an essential part in the infection cycle of *B. anthracis* (Radostits *et al*, 2000), although not always

observed. At death in most susceptible species the blood contains 10^7 to 10^8 bacilli per mL (Watson & Keir, 1994).

Humans appear to be moderately resistant to anthrax. Human cases of anthrax occur as a result of exposure to infected animals or their products. They are commonly cutaneous, responding favourably to penicillin, and fewer than 5 percent of cases are respiratory or gastrointestinal, which tend to be fatal (Watson & Keir, 1994).

1.5 Diagnostic Tests

A variety of methods for the identification of *B. anthracis* exist. These tests differ in complexity, sensitivity and specificity and their value for either diagnostic or research purposes. A number of the more frequently used assays include traditional culture and colonial morphology, blood smear, ELISA and rapid tests utilising PCR and lectin interactions (Ezzell *et al*, 1990).

1.5.1 Culture

Isolation of *B. anthracis* is possible from blood or tissues of a recently dead animal and in relatively high numbers and pure culture. However, as the carcase decomposes the putrefactive bacteria tend to overgrow and eventually eliminate the infective organism. Thus in old or decomposed animals or environmental samples, detection is likely to involve a search for relatively few *B. anthracis* organisms within a background of putrefactive bacteria, many of which may be other *Bacillus* spp, so selective techniques are required. The most successful selective media for *B. anthracis* is polymyxin-lysozyme-EDTA-

thallous acetate (PLET) agar (Knisley, 1966). *B. anthracis* grows readily in nutrient broth or on nutrient agar incubated aerobically at 37°C. When virulent strains of *B. anthracis* are grown on media containing either serum, bicarbonate or both and incubated in a CO_2 enriched atmosphere they produce capsules and the colonies appear mucoid. In the absence of these conditions the capsule is not produced and the colony appears like ground glass (Green *et al*, 1985).

1.5.2 Blood Smear

The reference standard for the diagnosis of *B. anthracis* is microscopic examination of a suitably stained blood smear in which large numbers of encapsulated bacilli can be visualised. The clearly defined capsules and occurrence in long chains are the distinguishing features for *B. anthracis*. Spores are not found in blood smears due to the high concentration of CO_2 present in dead animals (Turnbull, 1998).

The blood smear is regarded as a stand-alone diagnostic test, however it does require the skills and expertise of an experienced microbiologist and veterinary pathologist to interpret results. Smears made within a couple of hours from animals that have died from anthrax are prepared from the peripheral blood and stained with aged Polychrome methylene blue. This staining method is designed to show up the encapsulated *B. anthracis* bacilli for definitive diagnosis. This stain is a mixture of methylene blue, ethanol and potassium hydroxide. The mixture is ripened and matured for one year to achieve oxidisation and the resulting azure A and azure B, produced form the active constituents in the staining process (Turnbull, 1998).

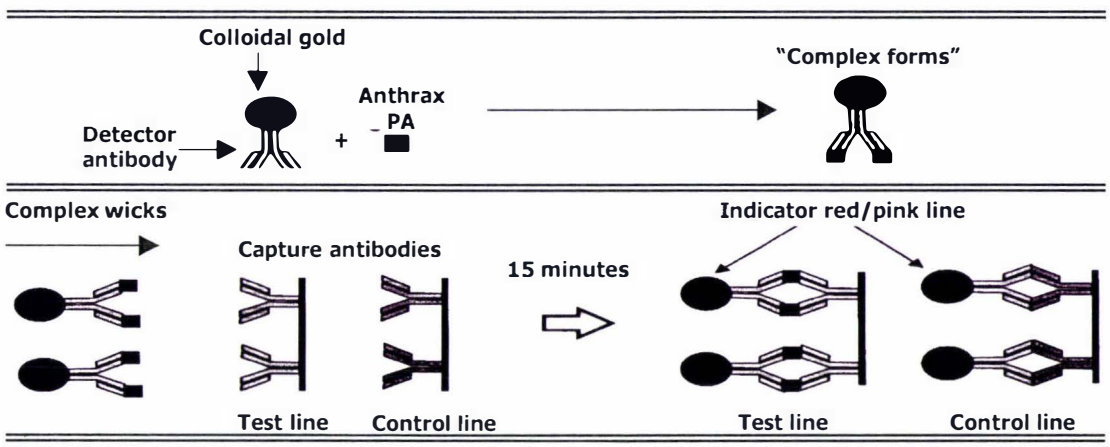
1.5.3 Antigen Detection tests

Over the years there has been much research into identifying specific anthrax antigens, and to design detection systems for these. The most readily isolated and purified antigens are the exotoxins PA, LF and EF, since capsular antigen is considered specific but poorly immunogenic (Turnbull, 1998).

A rapid diagnostic test developed by researchers at the United States Naval Medical Research Centre is an immunochromatographic assay. This assay is designed for the rapid detection of *B. anthracis* in the field and is specifically designed to detect the PA in the blood, serum or other tissue fluids.

The immunochromatographic hand-held assay of interest in this thesis utilises a monoclonal capture antibody to the anthrax PA which is bound to a nitrocellulose membrane. A second monoclonal antibody specific for a different epitope of PA is bound to colloidal gold particles, which become visible when they accumulate at these capture sites (Burans *et al*, 1995) (Figure 1.2). From hereafter the immunochromatographic hand-held assay will be referred to as the hand-held assay.

Figure1.2: Schematic diagram of the intricate details of the hand-held assay.



(Burans *et al*, 1995)

The assay is quoted to detect as little as 25 ng/mL of PA and is able to be read within fifteen minutes of reaction (Burans *et al*, 1995). This system is used for direct diagnosis of cases of anthrax by detection of PA in the blood or tissue fluids.

The Ascoli precipitin test dates back to 1911, when it was designed for the purpose of detecting *B. anthracis* antigens in the tissues of animals that had died from anthrax. This test is not highly specific for *B. anthracis,* as the targeted thermostable antigens are commonly present in other *Bacillus* spp. This means that this test is heavily dependent on the probability that only *B. anthracis* is capable of proliferating throughout the animal and depositing sufficient antigen within the tissue to give a positive reaction. It is therefore not a recommended test for the detection of *B. anthracis* in environmental samples where the likelihood of encountering other *Bacillus* spp is high. This test is still widely used in eastern Europe (OIE, 2000a).

1.5.4 Serology

Historically there has been no real need for serological identification of anthrax as the traditional methods of culture and smear have been adequate. As a result the interest in this has come from the human aspect of this disease, to monitor antibody titres in the vaccine recipients and for epidemiological interests in seroconversion to detect prior exposure in both humans and animals (Turnbull *et al*, 1992).

The original serological test used in the 1960's was gel diffusion, using culture filtrates of the Sterne vaccine as the antigen, however this was too insensitive for direct detection of antibodies in the sera of vaccinated humans. In 1971 Buchanan and colleagues developed an indirect haemagglutination assay (IHA) utilising PA, which had an improved sensitivity and was more rapid (Buchanan *et al*, 1971). However, this method was not without its drawbacks; it lacked reproducibility, the preparation time was still time consuming and reagents were difficult to acquire and had a short shelf life (Johnson-Winegar, 1984). The ELISA provides a more rapid and simplified assay for measuring antibody titres, it is sensitive and the reagents are easily acquired and used. Both systems require 50 μg of purified PA, however the ELISA is able to process one third more samples than the IHA (Johnson-Winegar, 1984). The ELISA is a 7 h process and is therefore manageable within a working day. On comparison between ELISA and IHA, it can be stated that the ELISA is the test system of choice. However, both systems are not optimal for detecting low levels of antibody (Johnson-Winegar, 1984).

Immunologic detection of the anthrax exotoxins in the blood of an animal suffering systemic infection is possible if the antibodies to the toxins are available, however these remain unreliable for diagnosis. Hence, although serological tests are of epidemiological value, they pose little value with acute illness (Harrison & Ezzell, 1989). During systemic infections, antibodies to the anthrax toxin cannot be detected until final stages of disease and therefore too late to initiate treatment (Dixon *et al*, 1999).

1.5.5 Immunofluorescence

Immunofluorescence has been used to observe the capsule in a research situation but does not lend itself to routine diagnosis (Ezzell Jr & Abshire, 1996) because the standard stained smear is simpler and faster to perform, requires no special equipment other than a light microscope and is just as sensitive.

1.5.6 Polymerase Chain Reaction (PCR)

Full confirmation of virulence of an isolate of *B. anthracis* can be carried out using the PCR with primers that are specific for the toxin genes. PCR is becoming more widely available as a means of confirming the presence of virulence factors such as capsule and toxin genes. For routine purposes primers to one of the toxin genes, usually the PA gene due to its presence in all toxin produced and to one of the enzymes mediating the formation of capsule, are generally used (Turnbull, 1998).

1.5.7 MIDI automated bacterial identification system

The United States Army Medical Research Institute of Infectious Diseases in

Fort Detrick, Maryland, serves as the reference laboratory for the Department of Defence and has established a database for the identification of *B. anthracis* using the automated bacterial identification system known as the MIDI. The MIDI system is based on chromatographic analysis of methyl-ester fatty acids derived from the bacterial membranes; this method is used to distinguish *B. anthracis* from closely related *Bacillus* spp (Teska *et al*, 2001).

1.5.8 Differentiation using Lectins

Cole, Ezzell Keller and Doyle recognised that lectins are convenient reagents for the study of cell surfaces of bacilli. This work is based on the principles of rapid slide agglutination reactions. The rationale for these tests being that the agglutination is specific for each bacterial species, in that different lectins have differing sugar specificities and can therefore selectively agglutinate certain bacteria. *B. anthracis* has a galactose containing polysaccharide on its cellular envelope so it was reasoned that the galactose-binding lectins may be agents which could selectively agglutinate the bacterium. Experiments with *Bacillus* spp demonstrated that purified lectins of differing specificities could agglutinate *B. anthracis* and strains of a closely related *Bacillus* sp, *B. mycoides*. Not all lectins bound these two species; one in particular that bound both was, *Glycine max,* a non-toxic Soy Bean Agglutinin and one that was able to agglutinate *B. mycoides* but not *B. anthracis* was *Helix pomatia.* Thereby affording a means to differentiate between the two. Spores could also be identified using this method. This work represents a rapid tool for identifying *B. anthracis* (Cole *et al*, 1984).

Another study demonstrated the use of enzyme-linked lectins that were first conjugated with horseradish peroxidase so that when an enzyme substrate is added a measurable colour reaction takes place. This Enzyme-Linked Lectinosorbent Assay (ELLA) was found to be highly specific and sensitive for *B. anthracis*. This is because of its ability to detect the presence of relatively small numbers of bacteria (Graham *et al*, 1984).

1.6 Evaluation of Diagnostic Tests

The process of validating a particular test is to evaluate and assess its performance for a particular use. A test that has been validated is able to detect evidence of exposure to a particular organism or the presence of the organism directly and allows predictions to be made about the test subject. In the Veterinary/Medical setting diagnostic tests have an important role to play, because results form the basis on which many important decisions are made. For instance, to initiate or withhold treatment, initiate control programs and quarantine practices, vaccinate herds at risk, accreditation for export or for breeding, as a process of selection, to identify the cause of a disease process or to measure the frequency of disease within a herd or population of animals. Such decisions may only be made with confidence if the diagnostic testing provided is valuable and informative. This requires knowledge about the performance characteristics such as sensitivity and specificity of the test.

To validate a particular test it is important to identify the purpose for which the test is to be used so as to consider the variables that will affect its performance. The variables that affect such tests can be broadly grouped into three

categories; the sample, the actual test system and the test result. Sample quality can be affected by numerous factors such as host/organism interactions, sample contamination and sample deterioration, all of which affect the concentration or composition of the organism of interest. The test system may be affected by a number of environmental factors such as temperature, pH, other related organisms and potential technician error. Factors affecting the capacity of a test result to accurately predict the status of the host are the most critical and are based around the sensitivity and specificity of the test of interest.

Each individual diagnostic test has its own set of parameters, which help define the significance of the results provided. In order to establish these diagnostic parameters the test must first be evaluated and the performance assessed in the relevant population of animals.

An assay can only be considered valid when the test results fall within an acceptable range, determined by statistically defined limits, and provide accurate information on exposure or infection. There are three basic ways in which a result may be interpreted; nominal which is either a positive or negative result, ordinal which is strong or weak positive and interval where the response is measured on a scale such as antibody titres (Smith, 1995). For the hand-held assay that has been described it is considered to be nominal, either the animal has anthrax or it does not.

A crucial part of evaluating a diagnostic test is to have an accepted reference standard against which to compare the results of the test in question. The

reference standard's function is to determine the accuracy and therefore quality of the device in question (OIE, 2000b).

The process of evaluating a test is to define accuracy, which is comprised of two properties of the test, sensitivity and specificity. The sensitivity of a test is defined as the probability of a positive test result given the presence of disease whereas the specificity of a test is defined as the probability of a negative result given the absence of disease (Kraemer, 1992; Smith, 1995).

Calculation of diagnostic sensitivity and specificity can be determined by associating the positive and negative categorical data with the known infection status for each animal using the two-way contingency table (Table 1.2).

Table 1.2: Two-way contingency table demonstrating the possible diagnostic test outcomes and definitions.

	Disease Present (D+)	Disease Absent (D-)
Test Positive (T+)	**(TP)** True Positive	**(FP)** False Positive
Test Negative (T-)	**(FN)** False Negative	**(TN)** True Negative

$$Sensitivity = \frac{TP}{(FN + TP)}$$

$$Specificity = \frac{TN}{(TN + FP)}$$

(Smith, 1995)

There are four possible test outcomes: two correct and two incorrect. The values for all four outcomes are used to estimate test sensitivity, specificity, predictive value and accuracy and the prevalence of disease (Smith, 1995). Individuals are categorised into these four sections depending on how the results of the test of interest compare to the reference standard. Hence those in agreement with the standard would be either TP or TN (OIE, 2000b; Smith, 1995)

An accurate prediction of a positive or negative test result relies not only on diagnostic sensitivity or diagnostic specificity; it also depends heavily on the prevalence of disease in a given population. Generally the prevalence will not affect the sensitivity or specificity but will alter the predictive values for both

positive and negative disease status (Greiner & Gardner, 2000). The predictive value is the probability of a test result reflecting what is truly occurring in the population (Kraemer, 1992). A positive predictive value is the probability of disease in an animal with a positive test result and a negative predictive value is the probability that an animal does not have the disease when the test result is negative. The predictive values are relative; they will vary with the prevalence of disease in the population from which the animal came (Smith, 1995; Gardner & Greiner, 1999). As the likelihood of disease is increased so too the negative predictive value decreases.

The predictive values can therefore be improved if the determined sensitivity and specificity are at the highest rate achievable. High sensitivity and high specificity result in high predictive values, thus minimising the chance of false positives and negatives (Smith, 1995; Gardner & Greiner, 1999). However the prevalence of disease in a population has an over-riding effect on predictive value and even highly sensitive and specific tests can have low predictive values if the prevalence of disease is close to 100% or close to 0%.

The validation of diagnostic tests does not end with a time limited set of experiments, rather it must be considered a process that requires constant monitoring and reassessment of performance characters for the populations to which it is applied.

1.7 Thesis outline

The optimisation and standardisation of reagents and protocol has been established in the designed hand-held assay and so the purpose of this study was to evaluate the assay performance in terms of its specificity and sensitivity with appropriate samples collected from Australian animals. From the outset obtaining an estimate of specificity appeared highly likely due to the sufficient number of naive animals from which samples could be collected. However, the assessment of diagnostic sensitivity depended on naturally occurring cases of anthrax during the time frame of the study. This was always going to be difficult because of the infrequent and unpredictable occurrence of outbreaks in Australia. Nevertheless, even if only an estimate of specificity could be obtained this was considered sufficient justification for the project and in the Australian context, an estimate of diagnostic specificity is of most importance to avoid wrongly declaring the presence of anthrax. The ramifications of an incorrect diagnosis are considerable both within Australia and internationally due, amongst other things, to trade effects. Estimates of specificity need to be made with samples collected in the same geographic area, with the same species of animals and under the same conditions that tests for anthrax would be conducted. This ensures that any substances, microbial or otherwise, present in that environment that might give rise to false positive reactions, are detected. This study was therefore conducted with an emphasis on obtaining information on specificity of the test while at the same time being alert to capitalise on any situation that might arise, which would provide samples for estimating sensitivity.

CHAPTER TWO: SPECIFICITY OF THE HAND-HELD IMMUNOCHROMATOGRAPHIC ASSAY

2.1 Introduction

Identifying animals that have died from anthrax is reasonably straightforward. The traditional blood smear stained with aged methylene blue and bacterial culture are the recommended standards for detecting the bacterium (OIE, 2000b). However there are two main disadvantages with these methods. First, the availability of a microscope and the skills required to make and interpret a blood smear on site are not always practicable resulting in significant time delays. Bacterial culture must be performed in a registered veterinary laboratory with an overnight incubation. Secondly, if the unopened carcase is in an advanced stage of decomposition the likelihood of collecting a meaningful sample for either of these methods is remote; the longer the exposure to putrefactive bacteria the less likely you are to detect *B. anthracis* (Stein, 1942). There are also considerations of occupational health and safety related to the collection and transport of samples to a laboratory and the propagation in the laboratory of the bacterium and the possibility of exposure of laboratory technicians.

The immunochromatographic assay is a rapid, hand-held device capable of detecting *B. anthracis* in the body fluids of an animal that has died from anthrax; it is comparable to a sandwich immunoassay (page 23 Figure 1.2, page 38 Figure 2.1). Antibody is coated onto pink/red-coloured colloidal gold particles. If PA is present in a sample it will bind to this antibody-gold complex and pass along an absorbent wick. Once this complex reaches the bound immobilised capture antibody on the test strip it will bind and produce an

indicative red line. The positive control well has capture antibody reactive with the antibody coated onto the colloidal gold particles as well and must therefore indicate a red line in each assay. If there is no detectable PA in the sample only the positive control will form a pink/red line indicating that the test has worked even though the sample has yielded a negative result (Burans *et al*, 1995). It is both robust and specific for detection of the PA component of the *B. anthracis* toxin and is still able to perform when the tissues are decomposed. This can be demonstrated by work performed in the Etosha National Park by Tubbesing 1997, where it was found that the assay was able to identify anthrax cases even in advanced stages of putrefaction, where bacterial culture was not possible, suggesting a high degree of sensitivity (Tubbesing *et al*, 1997). Analytical assessment of the hand-held assay detects as little as 25 ng/mL of PA in blood or tissue (Burans *et al*, 1995).

Classically anthrax runs a peracute course in ruminants resulting in death before the expression of clinical signs (Turnbull, 1998). The textbook description of blood exuding from orifices (Merchant & Packer, 1967) is not always observed. On-site diagnosis is often not possible, as veterinary practitioners do not have the equipment or the ability to perform and interpret results of a stained blood smear. It would therefore be valuable, in the absence of clinical signs, to have a rapid, "cow-side" test to preclude on-site necropsy in the case of sudden death when anthrax is suspected.

An important attribute of any diagnostic test is its specificity. This is particularly true with a test for anthrax because a positive result has serious implications for

animal and public health and can impinge on local and international trade. In the large outbreak that occurred in the Goulburn Valley in 1997, beef exports to Asia were at risk, Indonesia for example imposed a ban on beef and live cattle imports (Lovett, 1997). A test that gave false positive reactions could have severe financial implications as well as decrease the credibility of veterinary authorities, unless the test results were confirmed by definitive diagnostic tests such as blood smear or culture, before an official diagnosis was reported. Hence the importance of estimating the diagnostic specificity of this rapid on-site test for anthrax. This study was conducted to provide an estimate of specificity by testing animals not infected by anthrax but presented at knackeries under circumstances and in conditions similar to those under which anthrax infected carcases would be presented.

2.2 Materials and methods

Blood samples were collected post-mortem from 240 cattle at the Stanhope and Camperdown knackeries in Victoria. Assays were performed on site, blood smears prepared and stained and a sample of blood transported back to the laboratory for bacterial culture. Details of the sex, breed, location and any information known about the cause of death of the animal were recorded.

2.2.1 *Collection of blood samples*

Blood was collected into a sterile 10 mL tube (Sarstedt, Australia) from the jugular vein of each animal sampled. Each sample was given a unique number that would correlate with the record sheet and all tests performed.

2.2.2 *Blood smear*

A small drop of blood was placed at one end of a glass microscope slide. A smear was prepared by positioning another slide upright over this drop to spread the blood across the slide in one smooth movement. The smear was heat fixed by flaming and placed into aged polychrome methylene blue stain (Appendix 1.1) for 2 to 5 minutes, washed in fresh water and then placed onto a heat box to dry. The stained slides were transported back to the laboratory for microscopic examination. One drop of Biomount mounting fluid (Biocorp, Australia) was placed at the centre of the stained film and a coverslip gently lowered onto the drop and centred. The mounting fluid was given a day to harden before examining. Each smear was given an individual number corresponding to the culture and the hand-held assay and identifying the knackery and the date of sample collection.

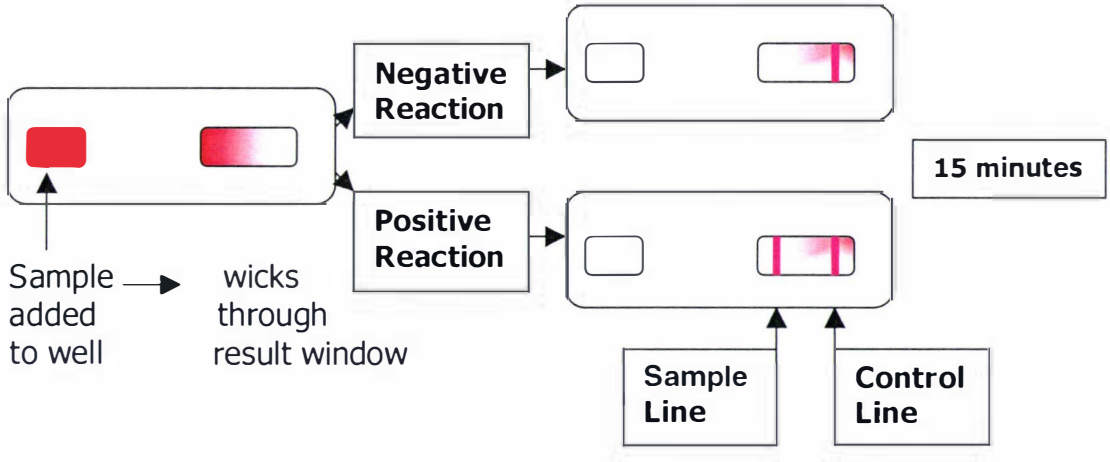
Blood smears were examined, independently of culture or hand-held assay results, under oil immersion. The presence of large, square ended, pink to red, encapsulated bacilli present in long chains was classed as positive for anthrax. The absence of these characteristic bacilli was recorded as negative. All organisms observed were noted.

2.2.3 *Hand-held immunochromatographic assay*

A sterile dacron swab (Crown Scientific, Australia) was dipped in the tube of blood and placed into a 5 mL tube (Biocorp, Australia) containing 3 mL of sample dilution buffer (Appendix 1.2). Using a transfer pipette (Biocorp, Australia) 5 drops, approximating 200 μL, of the sample preparation were

placed into the sample well. The sample was allowed 15 minutes to wick through the assay and the result was then read.

Figure 2.1: Schematic diagram of the interpretation of results in the hand-held assay.



A negative test result for a sample indicates that the sample does not contain anthrax PA or that PA is present but the concentration is below that detectable in this assay. A positive result indicates that the test sample contains anthrax PA at a level greater than 25 ng/mL. Hand-held assays were kept cool on ice and brought to room temperature just prior to use.

2.2.4 *Culture*

The remaining blood samples were transported back to the laboratory on ice and bacterial culture performed. Each blood sample was cultured on two sheep blood agar (SBA) plates (Appendix 1.3), one aerobically and one anaerobically, and aerobically on a PLET (Knisley, 1966) (Appendix 1.4) and a MacConkey agar plate (Appendix 1.5). Each agar plate was labelled with the date and

identification number. A loop of blood was streaked onto each agar plate using aseptic techniques in a Class II biological hazard cabinet. Once the samples were added to each plate they were placed within a sealed container and incubated at 37°C for 24 h. The second SBA plate was placed within an air proof chamber with an anaerotest strip (Merck, Australia) and a single anaero-pack bag (bioMerieux, Australia) to create the anaerobic conditions and incubated at 37°C for 24 h.

Each plate was examined for growth within the Class II cabinet and results recorded. The anaerotest strip was examined for each chamber to determine whether the anaerobic conditions had been met. Each culture result was recorded in a spreadsheet and anything that looked similar to *B. anthracis,* such as irregular shape, ground glass appearance and a dry texture (Parry *et al*, 1983), was further investigated. The colony of interest was subcultured onto a fresh SBA plate to check for haemolysis, onto MacConkey as *B. anthracis* will not grow on this agar and onto PLET as this is considered to be the most selective medium for *B. anthracis*. Gram stain (Appendix 1.6), catalase tests (Appendix 1.7) were also performed. Motility tests (Appendix 1.8) were performed as *B. anthracis* is non-motile, which is an unusual characteristic among *Bacillus* spp. Isolated organisms were classified non-anthrax *Bacillus* spp but not taken out to full identification, *Clostridium* spp was based only upon morphology and Gram stain.

Whole blood and serum have been stored at -80°C for potential further testing.

2.2.5 *Preparation of protective antigen*

PA was extracted from the Sterne vaccine to be utilised as a positive control for the imported tests as no field cases of anthrax occurred during the study. Sterne vaccine strain $34F_2$ serial number 270 was used to extract PA. A small volume (0.1 mL) of vaccine was streaked onto a SBA culture plate within a Class II Biological Hazard Cabinet using aseptic techniques, placed within a sealed container and incubated aerobically at 37°C for 24 h. After 24 h a loop of colonies was transferred to an 80 mL conical flask containing 3 mL of germination medium (Appendix 1.9). The flask was sealed with parafilm and placed on a shaking platform at 37°C for 17 h. After the incubation period 1.5 mL of the flask contents was transferred to a 2 mL eppendorf tube (Selby Biolab, Australia), sealed with parafilm and spun in a microcentrifuge (Beckman MicrofugeE, United Kingdom) at 16,000 rpm for 2 minutes. Once centrifuged, the cellular debris was pelleted and discarded as the PA was contained within the supernatant. The supernatant was diluted 1:2 with sample dilution buffer and then 200 µL transferred to the sample well of the hand-held assay and allowed 15 minutes to wick through the assay. The extracted PA was used as a positive control to test the two batches of imported tests once they were received in the laboratory, but was not used in the field alongside each sample.

2.3 Results

A positive result with the prepared PA antigen was obtained with each of the two batches of hand-held assays confirming that they were able to detect PA.

Blood samples from 240 cattle were tested in the hand-held assay and compared with the results of culture and blood smear. All samples gave negative results in the hand-held assay and *B. anthracis* was not detected in any sample by culture or by direct microscopic examination (Table 2.1).

Table 2.1: Results of examination of blood from cattle at two knackeries for *B. anthracis* by hand-held assay, stained blood smear and culture.

Location	Number of samples	Number of Positives on hand-held assay	Number of Culture positive	Number of Smear positive
Stanhope Knackery	82	0	0	0
Camperdown Knackery	158	0	0	0

There were no anthrax positive cases detected using either the hand-held assay or blood smear and culture. Assuming that there are no false negatives, which is a reasonable assumption on the basis that anthrax was not diagnosed in Victoria at any time during sample collection and by comparison with the definitive diagnostic tests, the hand-held assay had a specificity of 100% (98.5-100% 95% CI Fisher's exact) for Victorian cattle (Gahlinger & Abramson, 1995).

The following tables provide information on the types of bacterial flora detected in the cattle sampled from Stanhope (Table 2.2) and Camperdown knackeries (Table 2.3).

Table 2.2: Results of isolation from the blood of 82 cattle at Stanhope knackery.

	CULTURE TYPE			
	PLET	**MacConkey**	**SBA aerobic**	**SBA anaerobic**
No growth	71	50	38	25
***Bacillus* spp**	0	0	1	0
***Bacillus* spp & others**	2	0	7	1
***Clostridium* spp**	0	0	0	2
***Clostridium* spp & others**	0	0	0	4
Others	9	32	36	19
Totals	82	82	82	51*

*Note: The difference in total is due to anaerobic conditions not being met in 31 culture plates out of the 82.

Table 2.3: Results of isolation from the blood of 158 cattle at Camperdown knackery.

	CULTURE TYPE			
	PLET	**MacConkey**	**SBA aerobic**	**SBA anaerobic**
No growth	131	83	61	62
***Bacillus* spp**	1	0	3	0
***Bacillus* spp & others**	1	0	12	1
***Clostridium* spp**	0	0	0	0
***Clostridium* spp & others**	0	0	0	2
Others	25	75	82	88
Totals	158	158	158	153*

*Note: The difference in total is due to anaerobic conditions not being met in 5 culture plates out of the 158.

2.4 Discussion

The purpose of this study was to determine the specificity of the hand-held assay for anthrax. A total of 240 cattle were sampled from two areas within Victoria. The samples were collected from animals that were either found dead on farm or euthanased and were typical of animals from which anthrax may be

suspected in the field. All assays performed were negative and in agreement with the definitive diagnostic tests; culture and blood smear. Thus the hand-held assay was regarded as 100% specific (98.5-100%; 95%CI) for these cattle examined in Victoria.

The hand-held assay is based on specific Anti-PA monoclonal antibodies. Monoclonal antibodies are directed towards a single antigenic determinant, which makes them highly specific for a particular epitope of the bacterium of interest and therefore less likely to produce cross-reactions with other bacterial antigens (Brock *et al*, 1994). The two monoclonal antibodies chosen for this assay were shown to have high epitopic specificity and affinity for PA. Monoclonal antibodies 14-B7 and 3D-2 were shown to be directed at two different epitopes on the PA molecule and that 14-B7 was the most suitable as a capture antibody and 3D-2 was most suitable as a detector antibody (Burans *et al*, 1995). The hand-held assay was designed to be a simple rapid and robust field assay, so the positive control is internal.

Extracted PA was used to demonstrate that the two batches of tests that arrived from the United States Naval Medical Research Center were capable of detecting the anthrax PA, beyond this, samples were tested in the field with only the internal control. The absence of an indicative pink/red line in the test control window was to be interpreted as a failed test; this was never experienced during this study.

The tripartite exotoxin (PA, EF, LF) is widely distributed at μg/mL concentrations throughout the body at death from anthrax (Tubbesing, 1997; Burans *et al*, 1995). Since the hand-held assay has an analytical sensitivity of 100% at 25 ng/mL of PA (Burans *et al*, 1995) it is highly unlikely that an animal having died from anthrax would go undetected in this system.

As this study was designed to evaluate the specificity of the hand-held assay, sites for sampling were chosen to represent two different geographic areas with either recent history of anthrax (Turner *et al,* 1999b) or an area where anthrax has not been detected for more than fifty years (Seddon & Albiston, 1965). The cattle sampled in this study were taken from two knackeries. They were chosen largely for convenience of obtaining large numbers of dead cattle that ranged from immediate sampling post-euthanasia to those that died on farm ranging from hours to days old.

Stanhope knackery situated in the north of Victoria receives carcases from all over the Goulburn Valley, an area which experienced a large scale anthrax outbreak in 1997. Since 1999 there has been no anthrax reported throughout this area (Murray, 1997) and it was therefore regarded as a representative area to sample. Cattle are routinely vaccinated for anthrax in this region, however the vaccination status of the animals sampled was unknown. The presence of vaccinated animals was not expected to be a problem for the analysis of the hand-held assay because the monoclonal antibodies in the assay detect PA not antibody titres. No cattle in this region were vaccinated for anthrax in 2001. Camperdown knackery in the south west of Victoria is considered to draw

carcases from an area that has not had anthrax diagnosed since 1963 (Seddon & Albiston, 1965). However the history of the cattle from both knackeries is unknown and it is conceivable that some may have recently arrived in the district from other areas.

Culture was used to demonstrate the presence of all bacteria in the blood samples collected. SBA is an enriched medium that supports the growth of most bacteria, so you would expect most bacterial isolates on this medium. MacConkey agar contains bile salts selective for enteric organisms. PLET is a medium that is designed to exclude the growth of bacteria other than *B. anthracis* and is therefore unlikely to have anything else grow. *Bacillus* spp other than *B. anthracis*, *Clostridium* spp and other general putrefactive bacteria were all cultured from samples collected in this study. Putrefactive bacteria or bacterial flora in the environment can alter the specificity of a test through cross-reactions that lead to false positive results or mask the presence of *B. anthracis* in smears or on culture plates. It was for this reason that it was important to take note of all species of bacteria that were cultured. From the results of culture it can be stated that there was a high rate of animals that had no bacteria present in culture; 46% for Stanhope and 39% at Camperdown based on the standard SBA culture. It is probable that the negative cultures were common with those animals presented at the knackery within a couple of hours of euthanasia, as opposed to those found dead from a variety of causes.

Bacterial flora in a carcase will vary with geographical location and this may interfere with the specificity of a diagnostic test. A good example of

geographical differences altering the specificity of a test is the difference seen between northern and southern Australian cattle in an absorbed ELISA for Johne's disease. The Johne's disease absorbed ELISA was found not to be as specific in northern Australian cattle with 1.2 and 3% rate of false positives compared to 0.7% for southern Australian cattle (Pitt *et al*, 2002). The major difference observed between the northern Australian animals and those of southern Australian animals was geography, thus suggesting that differences in environmental factors such as presence of other cross-reacting bacterial antigens, perhaps from environmental bacteria, could cause the higher rate of false positives (Pitt *et al*, 2002). This study emphasises the differences that can exist with tests performed in the same country, for the same disease, in the same species. This is particularly so in a country like Australia where vast distances may cover a range of physical and climatic types from mild Mediterranean, through hot and arid desert to wet tropics. It is important when estimating the specificity of a diagnostic test that the samples should represent the population for which the test is going to be used (Noordhuizen *et al*, 1997). This will ensure that any cross-reacting bacterial antigens in the environment of the population of interest are present in the samples from which estimates of specificity may be derived.

Different species of animals will also influence the outcome of the epidemiological determinants of sensitivity and specificity, though this may be more of an issue when the test is detecting antibodies in serum rather than testing for the presence of specific antigen. For example in a study conducted by Turnbull in the Etosha National Park in 1995 using the hand-held assay for

detection of PA in various animals it was found that 40% of hyena sera gave rise to false positives. This was attributed to a component, thought to be anti-mouse IgG antibody in the hyena sera and was overcome by incorporating normal mouse or rabbit IgG into the specimen (Turnbull personal communication). This illustrates problems that may occur when extending the limits of a test specified for a particular species and the differences that can exist between species of animals.

In Australia anthrax occurs in belts throughout Victoria and New South Wales (Beveridge, 1983); much less so in Victoria where it is regarded as occurring in sporadic outbreaks rather than as an endemic disease. Within these anthrax belts, sheep tend to be more commonly affected in New South Wales and cattle in Victoria (Wise & Kennedy, 1980). The specificity of the hand-held assay has now been determined for use in cattle within Victoria. It cannot be stated with certainty how specific the assay would be when used in other parts of Australia, internationally or in other animal species since the test sensitivity and specificity may vary according to population, age and geography. However since the test relies on detecting antigen and not antibody, it is suggested that this potential variation would be unlikely to be significant.

Although the diagnosis of anthrax can often be made through blood smear stained with aged methylene blue and bacterial culture, the availability of these methods and time involved makes them less useful to a field-based veterinarian. The hand-held assay is a simple test that can be performed on-site. There are no requirements for specialised equipment, such as the

microscope, microbiological knowledge or skills beyond very basic training in its use. The hand-held assay provides a rapid and simplified assay for determining the presence of anthrax in animals that have died suddenly, offering an on-site diagnosis rather than a retrospective diagnosis. Time to disease diagnosis is important in the event of an outbreak. The hand-held assay offers a result in 15 minutes; this allows for immediate carcase disposal and area disinfection, which limits the amount of area contamination. A rapid result will also allow for timely commencement of vaccination of in-contact stock as deemed appropriate by the responsible veterinary authorities. Importantly, in this study no false positive reactions were detected with the assay. On the basis of the number of animals tested, one can have 95% confidence that, if a much larger population had been tested, then the number of false positives would not have been greater than 1.5%.

CHAPTER THREE: EVALUATION OF THE HAND-HELD ASSAY IN CATTLE IMMEDIATELY AFTER VACCINATION FOR ANTHRAX

3.1 Introduction

The importance of vaccination as a means of controlling anthrax is well established. Sterne's early trials with the $34F_2$ vaccine in South Africa between 1925 and 1941 highlighted its effectiveness, with the number of deaths of cattle in areas that had previously suffered major losses being reduced from about 7500 in 1925 to less than 700 per year in 1941 (Sterne *et al*, 1942). This number is possibly more significant as there is now active surveillance to identify cases of anthrax whereas previously it was considered less important. After the dramatic reduction in deaths Sterne deduced that vaccination could be an effective means of limiting further spread in the event of an outbreak (Turnbull, 1991). Upon introduction of a specific vaccination program into a population, the incidence of anthrax drops markedly, since disease spreads poorly through a population in which large proportions of individuals are immune. However the organism does persist in the environment for many years so vaccination programs may need to be continued in endemic areas long after the last case of anthrax has been detected.

The anthrax spore vaccine $34F_2$ (Colorado Serum Company, Colorado) is prepared from a non-pathogenic, non-encapsulated ($PXO1^+$, $PXO2^-$), toxigenic variant strain of *B. anthracis*, originally developed by Sterne in 1937. The animal vaccines that are still in use today are essentially as originally formulated (Sterne & Robinson, 1939), with approximately 10^7 spores per mL suspended in 0.5% saponin in 50% glycerine-saline. For susceptible species such as sheep and cattle, anthrax is probably invariably fatal. Since there is no cure in these

economically important species, preventative methods such as vaccination are the only defence against spread of disease. In the event of an outbreak ring-vaccination, a process of vaccinating in-contact animals and those on surrounding farms, is employed to prevent further spread of disease. This approach to control was used in the Goulburn Valley outbreak in 1997, where 78,649 cattle within 457 herds were vaccinated in an area approximately 30 km east-west and 20 km north-south surrounding the initially detected cases. The ring vaccination ceased when there were no longer any new infected properties and all of the infected properties were inside the vaccination zone. Of the 83 properties infected with anthrax 50 had one confirmed case, 25 had two to four cases and 8 had five or more cases confirmed (Turner *et al*, 1999a). Vaccine manufacturers claim that animals will have developed protection 10 to 14 days post vaccination (Turner *et al*, 1999a). In the Louisiana outbreak, United States of America, in 1971 the vaccine consistently reduced mortalities in susceptible species in 8 days or less (Kaufmann *et al*, 1973) and in small limited outbreaks in Australia it has been shown to be effective at 7 days (Turner *et al*, 1999a). Once a vaccination program was started in the Goulburn Valley outbreak the number of deaths was 132 after 10 days, 12 in the following 5 days, 13 in the following 20 days and one individual 57 days post vaccination (Turner *et al*, 1999a). Thus emphasising the value of a vaccination program as an effective means to limit the spread of disease.

Vaccines are made of immunogens which are substances that, when administered to animals in the appropriate manner induce an immune response (Brock *et al*, 1994). The PA component of the toxin is considered to be highly

immunogenic (Hambleton *et al*, 1984; Stanley & Smith, 1963) and due to its presence in both oedema and lethal toxins produced it is the ideal immunogen for the anthrax vaccine (Little & Ivins, 1999). As such protection against anthrax in the susceptible host is dependent almost entirely on its immune response to one antigen, the PA component of the anthrax toxin (Ivins & Welkos, 1988). The presence of PA by itself in a chemical vaccine (generally produced for human vaccines), or its production by a live strain (current animal vaccine), is both essential and sufficient to induce protective immunity to anthrax (Little & Ivins, 1999). Immunity to the LF and EF components are believed to have negligible contributions to protection and as yet no other antigens have been identified as contributory (Turnbull, 1998). The vaccine strain of anthrax is still capable of producing toxin equivalent to that of a virulent strain but generally without the fatal effects (Harris-Smith *et al*, 1958). The vaccine thereby retains a high antigenicity capable of inducing strong immunity when inoculated into animals yet without causing disease (Brock *et al*, 1994).

The protective effects of a single dose of $34F_2$ strain of *B. anthracis* vaccine is estimated to last one year (Sterne, 1939). Experimentally it has been shown that sheep are fully resistant to experimental challenge in less than one week after vaccination and that immunity lasts 14 months. This may vary according to the animal; for example, a horse can take as long as 4 to 6 weeks to become fully immune. The safety margin for the vaccine is therefore set at 10 to 14 days for cattle and sheep to be classed as immune and annual boosters are recommended in endemic anthrax areas (Spears & Davidson, 1959).

The hand-held assay detects PA in the bloodstream or tissue fluids of an animal that has died from anthrax. The PA is present in combination with both EF and LF and this may be cleaved to produce active ET and LT respectively (Little & Ivins, 1999). In an animal that has died from anthrax, PA is always present in blood and in the tissue fluids (Burans *et al*, 1995). During infection with *B. anthracis* there is a septicaemic phase when PA is released in the bloodstream. However following vaccination with the live Sterne vaccine, PA is also produced and there is at least a theoretical possibility that this might be present in blood at a sufficient concentration to yield a positive result, particularly in the first few days post-vaccination. In the event of an anthrax outbreak it is common practice to ring-vaccinate to prevent further spread. If the assay detected PA in the blood of vaccinated animals in this early post-vaccination period, false positive diagnoses of anthrax could be obtained with animals that died for unrelated reasons.

Anthrax is a List B disease in the World Organisation for Animal Health, which means that it is a transmissible disease considered to be of socio-economic and/or public health importance within countries that are significant in the international trade of animals and animal products (OIE, 2000a). Properties are placed under quarantine when anthrax has occurred and when ring-vaccination has commenced. Both require that movement of animals and animal products are restricted for a withholding period of 42 days on the sale of any animal (Murray, 1997). The repercussions for a property with a positive test for anthrax are more severe affecting the overall status of the property; this status may have lasting effects for future management. All milk from the infected

farm, including milk from any cow dying due to anthrax up to eight hours after milking, is destroyed and all equipment sanitised. Vaccinated dairy cattle have a nil withholding period on their milk (Murray, 1997). This will have lasting economic penalties on a property owner. The risk of false positive results caused by a common procedure requires investigation. This study was therefore conducted to determine whether positive reactions were detected with the blood of cattle immediately after vaccination and, if so, how long they persisted.

3.2 Materials and methods

3.2.1 Calculation of concentration of spores in vaccine

Into 99 mL of glycerol saline (Appendix 2.1) was added 1 mL of Sterne $34F_2$ serial number 270 vaccine. This 1:100 dilution approximates 10,000 spores/mL. After vigorous mixing, the 1:100 vaccine dilution was diluted one hundred fold in glycerol saline to achieve a 1:10,000 dilution which approximates 100 spores/mL. After mixing thoroughly, the 1:10,000 vaccine dilution was diluted ten fold in glycerol saline to achieve a 1:100,000 dilution, which approximates 10 spores/mL. The 1:10,000 and 1:100,000 dilutions were used to estimate by quantitative culture the number of spores in the vaccine used on the cattle. One mL of each dilution was added in triplicate to sterile Petri dishes (Crown Scientific, Australia) within the Class II Biological hazard cabinet. To each dish was added 20 mL of pre-melted tryptose agar (Appendix 2.2) cooled to 53°C, the dish was then swirled in a figure eight pattern to gently mix the agar and diluted vaccine together. The plates were allowed to cool,

inverted and placed into a sealed container and incubated at 37°C for 24 h. After 24 h the viable colonies were counted on each plate and the number of spores calculated.

Fort Dodge Australia (Penrith, New South Wales) anthrax vaccine strain $34F_2$ serial number 282 expiry 25th August 2003 was used as a reference standard.

3.2.2 Cattle vaccination

Permission to conduct this study with the $34F_2$ anthrax vaccine was obtained from the Chief Veterinary Officer for Victoria, Dr. Hugh Millar.

A group of six female and four male 18-month-old Angus and Angus/Hereford cross cattle, at pasture in a ten-acre paddock at the Victorian Institute of Animal Science, Werribee site, was used for this study. Each individual had a day zero blood sample taken and tested in the hand-held assay before vaccination. The recommended dose of 1 mL Sterne $34F_2$ vaccine (Serial Number 270) was administered subcutaneously in an area immediately cranial to the scapula by a veterinarian.

3.2.3 Post-vaccination sampling and testing

Each animal was weighed and daily blood samples were collected for six days after vaccination and then on day 8 and day 15. The blood sample was taken from the coccygeal vein of the tail using a plain vacutainer (McFarlane Medical Scientific, Australia) and 18g needle (McFarlane Medical Scientific, Australia).

On day 2, 5 mL of blood from each of the 10 cattle was transferred into a culture bottle containing 20 mL Tryptose agar slopes with 5 to 8 mL germination medium. The cultures were placed at 37°C to incubate aerobically for 24 h. On removal each culture was swirled so that the blood and germination medium was drawn over the tryptose agar slope, then placed back at 37°C for a further 24 h incubation. After the total 48 h incubation, cultures were examined for bacterial growth.

In addition to the blood cultures from all 10 animals at day 2, any individual that experienced a temperature increase of 1°C or greater was to be cultured.

Hand-held assays were performed on-site immediately after the blood was collected, according to the methods described in 2.2.3. The inoculation site was examined for inflammation and the rectal temperature recorded at each bleed.

3.3 Results

The vaccine administered in this study was Serial No: 270, with a revised expiry date 15th November 2003 (Appendix 3.0) and had an anthrax spore count of 9.5×10^6 per mL (Table 3.1).

Table 3.1: The calculation of viable spore numbers in one mL of $34F_2$ anthrax vaccine.

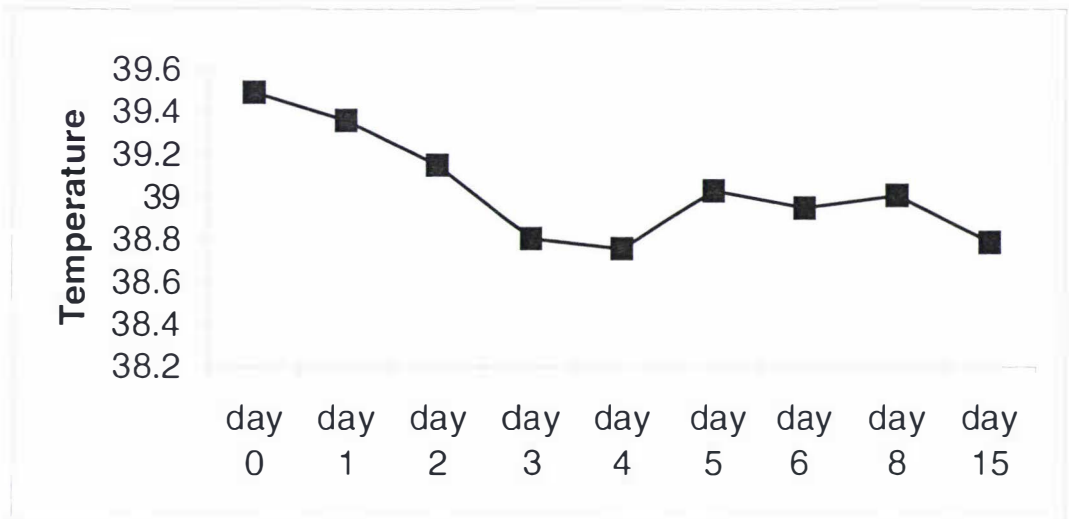
	Spore count in triplicate at a dilution of 10^5 /mL			Average Spore count 10^5 /mL
Vaccine administered	90	95	100	95
Fort Dodge Control	64	64	71	66

All hand-held assays performed on-site with the blood from cattle vaccinated for anthrax were negative for anthrax PA and all bacterial cultures performed were negative.

All cattle had localised inflammation at the inoculation site although the extent of reaction varied with each individual with a range of response from approximately 3 x 5 cm in diameter to 10 x 8 cm in diameter. Each individual had a hot swelling for the first 3 days. At 15 days the swelling around the inoculation site had reduced in all individuals and at day 44 there was no apparent swelling in any but one individual that had a 1 cm nodule under the skin.

Rectal temperatures had a gradual decline over the first three days and became stable thereafter (Figure 3.1).

Figure 3.1: Mean daily rectal temperatures for the ten cattle vaccinated in this study.



One individual experienced a 1°C rise in temperature but it was only on one occasion, day 8. All other temperatures were within the range of 38.1 to 40.7°C.

3.4 Discussion

The purpose of this study was to investigate whether vaccination with the Sterne $34F_2$ vaccine gave rise to false positive results in the hand-held assay. The ten Angus and Angus/Hereford cross cattle were blood sampled for a period of 15 days to cover the time that PA theoretically would be most likely to be present in blood after vaccination. Within this timeframe no PA was detected in the blood of vaccinated cattle. The immune status, in terms of antibody levels or protection was not monitored because there is no antibody ELISA currently available within Australia and it was not possible to challenge the individuals with virulent *B. anthracis,* nor was this the purpose of the study.

However this procedure for vaccination is equivalent to that performed in an anthrax outbreak situation and prophylactically in areas where anthrax is endemic.

Rectal temperature was monitored in each individual for the duration of the experiment to ascertain whether a febrile reaction, defined as a rise of 1°C from the baseline measurement, occurred. The baseline rectal temperature may have been affected by the excitement of the cattle on the first day and then the drop in average temperature over the next three days may be attributed to the cattle acclimatising to daily contact in the crush. There was no detectable febrile reaction. One individual on day 8 had a rise in rectal temperature of 1°C but it was confirmed to be in oestrus. No *B. anthracis* organisms were isolated in culture of the blood samples taken, which indicates that there was no detectable bacteraemia with vaccination. This is also consistent with the negative findings in all hand-held assays performed and indicates that, if PA was present in blood, the concentration was below that detectable in this assay.

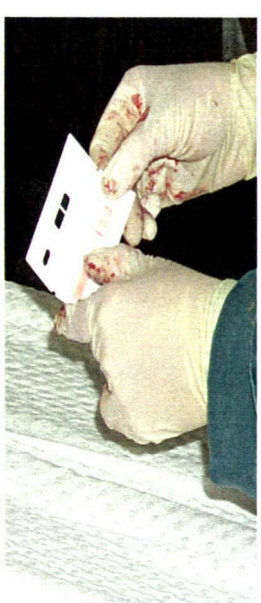
The $34F_2$ anthrax vaccine contains saponin and glycerine-saline (Turnbull, 1991). Saponin is used as a potent adjuvant to stimulate immune complexes in some killed vaccines, however the $34F_2$ anthrax vaccine is a live vaccine and is therefore not in need of an adjuvant. A toxic saponin mixture is used in the anthrax vaccine, where it destroys the tissues at the site of inoculation so that the anthrax spores may germinate (Tizard, 1996). This causes localised tissue necrosis and an anaerobic focus. An inflammatory response was detected in all ten individuals in the study and varied quite considerably in size and

persistence. The swelling started to decrease between day 8 and day 15 and was completely resolved at day 44 in all but one individual.

The role of the capsule in anthrax pathogenesis is crucial and can be demonstrated clearly through the live $34F_2$ vaccine. Antitoxic immunity is slow to develop so in the case of *B. anthracis,* toxin production tends to be prolonged because the organism is encapsulated and phagocytic cells are unable to eliminate the source. As a result death of the individual is usually inevitable in unvaccinated animals. The vaccine is nonencapsulated but is a toxigenic strain of *B. anthracis*, which allows for antitoxic immunity to develop because the nonencapsulated bacteria can be removed by phagocytosis before lethal levels of toxin can be synthesised but not before antitoxic immunity is stimulated (Tizard, 1996).

In the event that a recently vaccinated animal has succumbed to anthrax before protection has developed, it would be highly likely that PA, produced by the infecting organism, would be present in blood at sufficient concentration to be detected in the hand-held assay. This situation was not tested in the current study but would require an experiment in which animals were tested after they were vaccinated and then challenged with virulent *B. anthracis* in the first day or two after vaccination.

This present study clearly shows that the $34F_2$ vaccine when administered to cattle does not give false positive test results in the hand-held assay. It can therefore be used with confidence on samples from recently vaccinated cattle that have died, when determining whether they have succumbed to anthrax or not.



CHAPTER FOUR:
GENERAL DISCUSSION

The purpose of this study was to determine whether the hand-held assay was an accurate tool for the diagnosis of anthrax in the field. An in-field assay for anthrax is required for the investigation of sudden death in cattle, as the lack of clinical signs with acute anthrax makes it a difficult disease to diagnose in the field and it can be overlooked as the cause until the results of laboratory tests are available. Being a zoonotic disease it is important for occupational health and safety, to be able to rule out anthrax as a potential cause of death, before necropsy of the carcase and the consequent area contamination that may occur. In the last two notable anthrax outbreaks in Australia the index case was not diagnosed immediately. Subsequently, the carcase contaminated the immediate area, vegetative bacilli were exposed to oxygen allowing the formation of spores, the herd was exposed and there was a potential for blowflies and birds to spread infection. The veterinarian performing the necropsy, the microbiologists and technicians that handled the anthrax infected samples, all would have been at risk of exposure. As a result the outbreak experienced in the Goulburn Valley of Victoria in January 1997 resulted in the death of 206 animals (Turner *et al,* 1999b) and during the most recent outbreak at Collingwood station near Wandoan, Queensland in January 2002, 10 cattle died (ProMED, 2002). This demonstrates that the availability of a "cow-side" diagnostic tool and suspicion by the veterinarian when investigating cases of sudden death in animals could rule out anthrax as the cause of death, thus permitting appropriate action to ensure minimal exposure.

It was therefore important to determine the operating characteristics of the hand-held assay, in terms of its specificity and sensitivity. Due to the lack of

cases in the period of the study it was only possible to determine the specificity and make some assumptions about the relative sensitivity of the test. The specificity of the assay was regarded as 100% for Victoria, derived from the 240 cattle assessed. Sensitivity was unable to be determined, however based on the analytical sensitivity of 25 ng/mL it was reasoned that it would have high sensitivity because it has been shown in other studies that the blood of an infected animal contains μg/mL of PA (Burans *et al*, 1995). Some comparison may be made with Turnbull's study performed on 6 springbok, 18 zebra, 12 wildebeest and 3 other unidentified species of animals that died during an anthrax outbreak in the Etosha National Park (Turnbull personal communication) (Table 4.1).

Table 4.1: Hand-held assay results to all positive anthrax cases identified in either blood smear or culture.

		Disease Present
		Smear or Culture Positive
Hand-held assay	**Positive**	**14**
	Negative	**8**
	Total	**22**

$$Sensitivity = \frac{14}{(8+14)} = 0.63$$

An animal was considered to be positive for anthrax if it was positive for the reference standard of comparison, smear or culture. Fourteen out of the 22 positive cases of anthrax identified by smear or culture were detected in the hand-held assay. If you assume truly infected on either culture or smear then the hand-held assay has a sensitivity of 63%. This is a surprisingly low sensitivity with respect to the determined analytical sensitivity. However it is difficult to draw any definitive conclusions on the sensitivity of this assay based on 22 samples, the species sampled and the small sample size, which is based on convenient samples rather than a structured study.

Determination of sensitivity and specificity of a diagnostic test requires the identification of clearly defined groups of diseased and non-diseased animals. A study examining the accuracy of the hand-held assay with respect to its sensitivity still needs to be undertaken. Determination of sensitivity requires appropriate samples to be collected during a natural outbreak of disease and with clear definitions of what constitutes a "true" case of anthrax so that there is objective comparison.

The interpretation and the way a test is used will depend upon the situation and the consequences involved with false diagnosis. The predictive values of a test are conditional probabilities, which describe the performance of a diagnostic test with reference to a true result. Positive predictive value (PPV) is the proportion of test positive animals that have the disease and the negative predictive value (NPV) is the proportion of test negative animals that are not infected. Prevalence or pretest probability will determine the predictive value of

a test and can be calculated for both negative test results, NPV and positive test results, PPV (Kraemer, 1992; Smith, 1995).

The NPV is calculated using the following formula;

$$NPV = \frac{(1-P)Sp}{(1-P)Sp + P(1-Se)}$$

(Sackett *et al,* 1985)

Where P is the pretest probability of disease and is representative of prevalence in a random sampled population. *Sp* is the specificity and *Se* is the sensitivity of the test under examination.

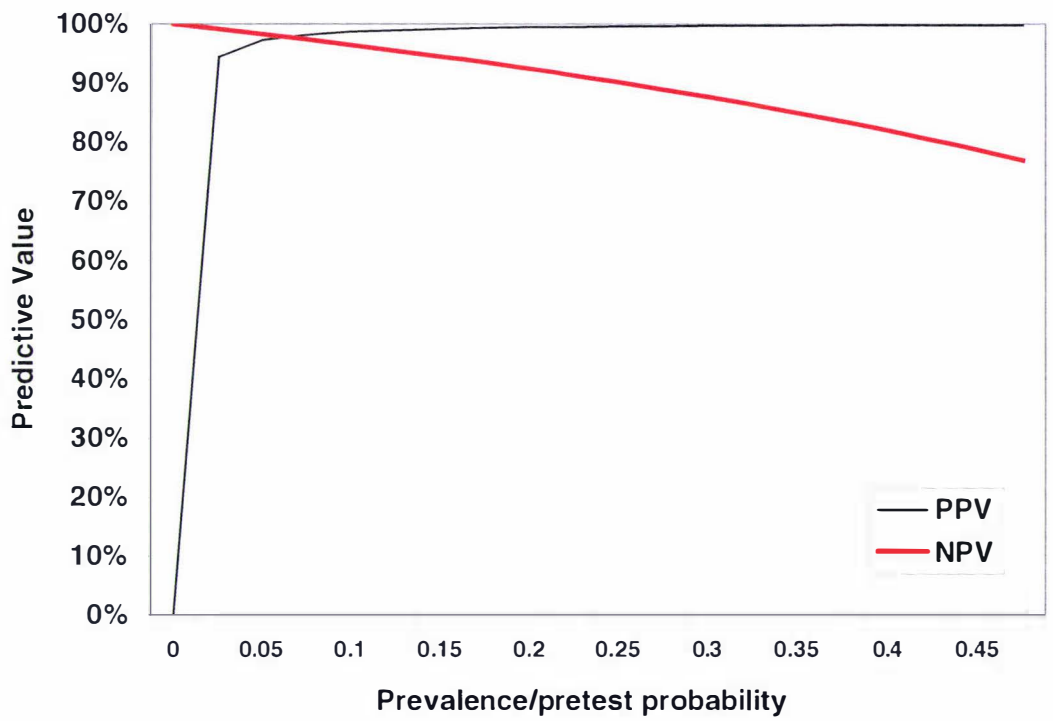
The PPV is calculated using the following formula;

$$PPV = \frac{PSe}{PSe + (1-P)(1-Sp)}$$

(Sackett *et al,* 1985)

The NPV and the PPV can be graphically represented for a test of 100% specificity and 63% sensitivity (Figure 4.1). The NPV for any test decreases as the pretest probability of disease increases.

Figure 4.1: The predictive values of a diagnostic test with a specificity of 100% and sensitivity of 63%.



The hand-held assay has a NPV of greater than 99% when the pretest probability or prevalence is less than 2.5%. This makes it a practical test to use for elimination of suspicion of disease in areas where anthrax is unlikely. In situations where the prevalence maybe higher such as during an outbreak a follow up smear and/or culture maybe required before the disease could be ruled out. For instance where the pretest probability is high i.e.: greater than 12.5%, the predictive value of a negative test is less than 95%, a rate of 5% false negatives might be expected.

Where testing is not random, as in the case of sampling dead animals in a disease outbreak, the pretest probability is a function of prevalence, risk

factors, clinical history and clinical findings. A cow that dies suddenly with no prior clinical signs and a recent history of anthrax on farm will have a high pretest probability of disease.

The PPV for a test will decrease as the prevalence or pretest probability decreases. For tests approaching 100% specificity the PPV will always be high, except at very low prevalences. In situations where the disease prevalence is low, it is unlikely that you would depend upon an indirect test alone for diagnosis of an index case in an area that may have had no previous history of disease. But a positive test result would almost certainly preclude a field post-mortem, which in the case of a truly infected animal would prevent further contamination of the environment and prevent human exposure.

In the situation of an outbreak or previous history of exposure, a positive test is very likely to be truly positive and could be relied upon as a stand-alone test. Animals, which gave positive test results in such situations, would then be rapidly disposed of in an appropriate manner minimising potential environmental exposure and contamination.

A large proportion of cattle that die on farm in Australia, have no formal post-mortem examination. Often a diagnosis of cause of death is not known or a diagnosis from a knackery worker is relied upon. Knackery workers are untrained and although many have had extensive experience their diagnostic and pathology skills should be considered limited because of the lack of any formal training. Passive surveillance of disease in Victoria is limited due to the withdrawal of regional government laboratories and the reluctance of many

farmers to pay for a post-mortem examination. As a result the knackery is the most likely place for detection of cases of anthrax. Thus any surveillance for anthrax should be conducted through sampling animals at knackeries. In the anthrax outbreak in the Goulburn Valley in 1997 a veterinary pathologist was employed to prepare blood smears of every animal processed at the Stanhope knackery and this was repeated the following year. This type of surveillance is prohibitive, as it is expensive for ongoing surveillance.

The hand-held assay has certain advantages over the traditional diagnostic methods. It is a simple test that requires very basic training in the performance and interpretation, with results available in 15 minutes. There are no requirements for specialised equipment. The hand-held assay is therefore able to be performed by an operator with very little training, such as knackery staff, and could potentially be used for active surveillance of sudden death animals.

The specificity is approaching 100 percent and the analytical sensitivity indicates that diagnostic sensitivity should be high. However a comprehensive study, similar to that which has been performed for specificity needs to be conducted before any recommendations can be made about the validity of this test for the diagnosis of anthrax in the field.

APPENDICES

Appendix 1.1

Aged Polychrome methylene blue stain

Loefflers Methylene Blue.....8 g

95 % Ethanol.....300 mL

Distilled water .....1300 mL

Potassium hydroxide.....0.13 g

1. Dissolve Loefflers methylene blue in the ethanol.
2. Add distilled water and potassium hydroxide, mix thoroughly.
3. Filter through Whatman No. 1 filter paper.
4. Store at room temperature in cupboard with cap loosened to allow for oxidation.
5. Polychrome methylene blue must oxidase for one year.

Appendix 1.2

Preparation of sample dilution buffer.

1. Add 1 packet of Phosphate Buffered Saline (Sigma Catalogue No 1000-3), to 900 mL of deionised water.
2. Add 1 mL of Triton X-100 (Sigma catalogue No T9284) to PBS to make a 0.1% solution.
3. Add 10 mL of 10% sodium azide stock solution in the PBS to make a 0.1% solution.
4. Add deionised water to make a final volume of 1000 mL.
5. Adjust pH to 7.4.
6. Sterile filter using a 0.22 μm filter into 3 mL aliquot's.
7. Store buffer at 4°C.

Appendix 1.3

Sheep Blood Agar

Oxoid Columbia.....195 g

Oxoid Special Peptone L72.....10 g

Oxoid Agar No.1 L11.....5 g

Distilled Water.....5 L

IMVS Sheep Blood.....250 mL

Appendix 1.4

PLET

EDTA .....0.3 g/L

Thallous acetate.....0.04 g/L

1. Difco Heart Infusion Agar is made up according to the manufacturer's instructions.
2. Add EDTA and Thallous acetate to Heart Infusion Agar, mix thoroughly.
3. Autoclave and cool to 50°C and polymyxin (30 000 units/L) and lysozyme (300 000 units/L) are added.
4. After swirling to ensure even suspension of the ingredients, the agar is poured into Petri dishes.

Appendix 1.5

MacConkey agar

Distilled water.....1 L

Peptone .....20 g

Lactose.....10 g

Bile salts.....5 g

Neutral red.....0.075 g

Agar.....12 g

1. Dissolve ingredients
2. Adjust pH to 7.4.
3. Sterilise by autoclaving at 121°C for 20 minutes.
4. Cool and mix well before pouring plates.

Appendix 1.6

Gram stain

1. With a sterile loop remove a small amount of the bacterial colony of interest.
2. Place this bacterial culture onto a glass slide with one drop of sterile water.
3. With the sterile loop combine the bacterial culture and sterile water.
4. When mixed dry on a heat block.
5. Place the slide preparation on a rack within a sink.
6. Cover the slide in 1 % crystal violet for 30 s, then wash with running tap water.
7. Cover the slide in Jensons iodine for 30 s, then wash with running tap water.
8. Decolourise with acetone/alcohol for 2 s, wash with running tap water for 5 s.
9. Cover with dilute carbol fuchsin for 30 s, rinse briefly.
10. Place slide on heat block to dry.
11. Examine under oil immersion

    Gram negative = red

    Gram positive = purple

Appendix 1.7

Catalase test

1. Using a sterile flamed loop, remove a colony of the bacteria of interest and place onto a glass slide.
2. Place one drop of 6% H_2O_2 onto the bacteria and swirl around.
3. Read the test immediately.

   Positive = gas bubbles

   Negative = no bubbles

Appendix 1.8

Motility test

1. Using a sterile flamed straight wire, remove an isolated suspicious colony of growth from the agar plate.
2. Inoculate the motility tube by carefully stabbing the wire into the medium and then drawing directly back out along the same line of inoculum.
3. Incubate the tube aerobically at 37°C overnight.
4. Result to be interpreted as non-motile when organisms form a line of growth down the inoculum stab and motile when the organisms form a diffuse growth zone around the inoculum stab.

Appendix 1.9

Germination medium

Difco Heart Infusion Broth (10%).....25.0 mL

40X Amino acid stock.....2.5 mL

Heat inactivated Horse serum.....50.0 mL

MilliQ water.....22.5 mL

Add together all ingredients and filter sterilise components using a 0.45 μm low protein binding filter unit. Store at –20°C.

10% heart infusion broth (Difco)

Dissolve 10 g of heartbroth in 100 mL of MilliQ water. Filter sterilise and freeze at –20°C.

40X Amino acid stock

To prepare 40x stock solution of amino acids, salts and nucleotide bases, add the indicated amounts of the components listed below to approximately 400 mL of MilliQ water while stirring. When all of the ingredients have been added make up to a volume of 500 mL with MilliQ water. Make 25 mL aliquot's and store frozen.

L-leucine.....4.60 g

L-histidine.....1.08 g

L-proline.....0.86 g

L-tryptophan.....0.72 g

L-phenylalanine.....2.52 g

Glycine.....1.30 g

L-lysine.....4.60 g

L-arginine.....2.52 g

L-methionine.....1.44 g

L-isoleucine.....3.40 g

L-threonine.....2.40 g

L-serine.....4.68 g

L-valine.....3.46 g

$MnSO_4$ ..... 0.017 g

$CaCl_2 2H_20$.....0.147 g

$MgSO_4 7\ H_20$ .....0.197 g

Adenine sulphate.....0.042 g

Uracil.....0.028 g

Thiamine HCL.....0.020 g

L-Na glutamate.....12.24 g

L-aspartic acid .....3.68 g

Appendix 2.1

50% Glycerol diluent

1. Mix equal parts of glycerol and 0.85% NaCl solution.
2. Place 300 mL of diluent into 500 mL flask.
3. Autoclave at 121°C for 25 to 30 minutes.
4. Store at room temperature for up to 1 year.

Appendix 2.2

Tryptose Agar deeps

Difco Tryptose .....20 g/L

NaCl..... 5 g/L

Difco Dextose.....1 g/L

Difco Agar.....15 g/L

Distilled water.....1 L

Appendix 3.1

DEPARTMENT OF NATURAL RESOURCES & ENVIRONMENT

TO: **DR. J. GALVIN, MAHO** Ref: VT/01/0136

FROM: DR. H. MILLAR, CVO 16 November 2001

SUBJECT: **ANTHRAX VACCINE EXPIRY DATE**

PURPOSE

1. To advise you of an extension of the expiry date on currently held anthrax vaccine stocks.

BACKGROUND

2. Attwood Laboratory recently titrated samples of currently held anthrax vaccine stocks (Lab Ref 01-004801-RJC, dated 15/11/01).
3. All batches exceeded the current WHO recommended standard of $1x10^6$ spores/ml.
4. Dr. Condron has consulted with the manufacturer, Fort Dodge, and on the basis of these results it has been recommended that the expiry date be extended to 15/11/03.

COMMENT

5. This vaccine is only to be used in Victoria under the approval of the CVO.
6. I hereby authorise a revised expiry of two years from the date of assay.
7. Accordingly, stocks of batches 271A, 270 and 265B are to be relabelled (vials and boxes) with an expiry date of 15/11/03.

RECOMMENDATION

8. That you note the above and arrange to relabel the vaccine stocks accordingly.

HUGH W MILLAR
Chief Veterinary Officer (Victoria)

copy – Dr RJ Condron / Dr RJ Rubira

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Anthrax vacc expiry 19-oov-01.doc

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