

Trends in the control of heartwater

B.A. Allsopp*

Abstract

Heartwater is an economically serious tick-borne disease of ruminants caused by the intracellular bacterium *Ehrlichia ruminantium*. The disease has traditionally been controlled by four different approaches: controlling the tick vector by dipping, establishing endemic stability, performing immunisation by infection and treatment, and preventing the disease by regular administration of prophylactic antibiotics. The first three of these methods are subject to failure for various epidemiological reasons, and serious disease outbreaks can occur. Prophylaxis is effective, but very expensive, and the logistics are daunting when large herds of animals are involved. The development of a safe, cheap and effective vaccine is the only likely way in which heartwater can be economically controlled, and over the past 15 years three new types of experimental vaccine have been developed: inactivated, attenuated, and recombinant vaccines. These new vaccines have shown varying degrees of promise, but none is as yet sufficiently successful to be marketable. We describe the experimental products, and the various technical and biological difficulties which are being encountered, and report on ways in which new technologies are being used to improve vaccine effectiveness.

Introduction

Heartwater is a lethal bacterial disease of domestic and some wild ruminants. It is caused by the intracellular rickettsia *Ehrlichia ruminantium* which is transmitted by ticks of the genus *Amblyomma*, and the disease is prevalent wherever the tick vectors occur (Provost & Bezuidenhout 1987). The endemic area includes the whole of sub-Saharan Africa, and also the French Antillean islands of Guadeloupe, Antigua and Marie Galante, to which infected *Amblyomma variegatum* ticks were introduced probably in the eighteenth century (Maillard & Maillard 1998). In the sub-Saharan region the disease is one of the major causes of stock losses, and it has been estimated that more than 150 million animals are at risk in the area (Minjauw & McLeod 2003). Typically the infection causes a high fever, nervous signs, hydropericardium, hydrothorax and oedema of the lungs and brain, and death.

The first known reference to what was subsequently thought to have been heartwater was made in 1838 by Louis Trichardt (Neitz 1947). While trekking through the Northern Province of South Africa many of his sheep died 3 weeks after they had suffered a severe tick infestation. Sixty years later Dixon (1898) and Edington (1898) showed that the disease could be passed by the transfer of blood from infected to susceptible animals, and Lounsbury (1900) confirmed that the vector in South Africa was *Amblyomma hebraeum*.

Heartwater at Onderstepoort

When the Onderstepoort Veterinary Research Institute was opened in 1908 the Director, Sir Arnold Theiler, had been

working on the disease since at least 1904 (Theiler 1904). Theiler continued to work on heartwater, and in 1925 he invited the Canadian rickettsiologist Edmund Cowdry to Onderstepoort to investigate the disease. Cowdry demonstrated the causative organism in the tissues of infected ruminants and ticks (Cowdry 1925a, b) and initially he named it *Rickettsia ruminantium*. The name was later changed to *Cowdria ruminantium* (Moshkovski 1947) and much more recently molecular phylogenetic evidence has shown that the heartwater agent is in fact a

species of *Ehrlichia* which is now known as *E. ruminantium* (Dumler, Barbet, Bekker, Dasch, Palmer, Ray, Rikihisa & Rurangirwa 2001).

Work on heartwater has continued at Onderstepoort up until the present day, and some notable advances have been made. In 1945 the introduction of antibiotics led to the development of a method of control by infection and treatment which is still in use (Neitz & Alexander 1945). In 1985 a technique for the continuous *in vitro* culture of the organism was introduced after at least 50 years of effort (Bezuidenhout, Paterson & Barnard 1985). Most recently the sequencing and annotation of the *E. ruminantium* genome has been completed (Collins *et al.* 2005), the first whole genome

to be determined in Africa. Two new experimental vaccines have also been developed during recent years, a recombinant vaccine in 2003 (Collins, Pretorius, Van Kleef, Brayton, Allsopp, Zweygarth & Allsopp 2003) and an attenuated vaccine in 2005 (Zweygarth, Josemans, Van Strijp & Steyn 2005).

Control methods for heartwater

The current methods used for heartwater control include

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* basil.allsopp@orange.fr

the use of acaricides to control the tick vector, antibiotic prophylaxis (Peregrine 1994), immunization by infection and treatment (Van der Merwe 1987), farming with animal breeds resistant to the disease (Camus, Maillard, Ruff, Pepin, Naves & Matheron 1996), and establishment of endemic stability (Tice, Bryson, Stewart, Du Plessis & De Waal 1998). All of these methods have serious drawbacks. Prophylactic antibiotic treatment is widely used by commercial Angora goat farmers in the Eastern Cape Province of South Africa because these animals are highly susceptible to heartwater. It is, however, a very expensive option, the logistics are daunting and there is always the likelihood that antibiotic resistance may develop. Tick control with acaricides, although also expensive, has been in use for more than a century (Dixon 1899) and it was in the past very effective. Today, however, acaricide resistance is widespread and the environmental pollution caused by the chemicals is generally deplored. Also of serious concern is the fact that animals undergoing an intensive dipping regimen lose any immunity to the disease and subsequent tick control failures can lead to catastrophic heartwater losses, as occurred in Zimbabwe in the 1970s (Norval 1979). The establishment of endemic stability, using limited dipping to control ticks to manageable levels, can be highly successful, but if herd immunity breaks down then serious disease outbreaks can occur. This can happen if new immunotypes enter an area where immunity to the previously prevalent strains offers no or limited protection, and such a situation is very likely to occur as widespread genetic exchange is known to take place in the field between different strains of *E. ruminantium* (Allsopp & Allsopp 2007).

Ultimately the only long term control method which will be cost effective is immunization, and an infection and treatment regimen has been widely used in southern Africa since its development at Onderstepoort in 1945 (Neitz & Alexander 1945). This 'vaccine' is commercially available and it consists of a cryopreserved preparation of blood taken from a sheep infected with virulent *E. ruminantium* organisms of the Ball 3 genotype (Haig 1952). The blood is injected intravenously into the animals to be immunized, their temperatures are monitored daily, and antibiotic treatment is administered when the infection becomes established, but before serious disease occurs. There are several practical disadvantages to this procedure. Firstly, *E. ruminantium* organisms rapidly lose infectivity even at 4° C (Brayton, Collins, Van Strijp & Allsopp 2003), so the infective blood must be preserved on dry ice and thawed immediately before inoculation which makes the infection and treatment method inappropriate for use in rural areas. Secondly, the procedure must be supervised by trained staff because of the need for intravenous injection and subsequent careful monitoring of the animals' condition.

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Thirdly, because live organisms are involved the procedure cannot be used in areas where heartwater is not endemic. The most serious disadvantage, however, is that the duration and effectiveness of immunity is uncertain. The Ball 3 genotype used in this procedure is known to offer only limited protection against some common virulent genotypes (Collins *et al.* 2003). However, because Ball 3 produces a temperature rise two or three days before serious disease is established it is unlikely to lead to stock deaths during the immunization procedure, whereas other common virulent genotypes like Welgevonden (Du Plessis, Van Gas, Olivier & Bezuidenhout 1989) can cause death very shortly after a rapid temperature rise and are therefore not suitable for use in infection and treatment.

Development of improved vaccines

There are three possible types of improved vaccine each of which has received extensive attention from researchers: inactivated, live attenuated, and recombinant. We will survey recent developments and future prospects for all three types.

Inactivated vaccines

The first inactivated heartwater vaccine, developed in Guadeloupe using the Gardel strain of *E. ruminantium*, was prepared from elementary bodies partially purified from tissue culture material and chemically inactivated using sodium azide (Martinez, Maillard, Coisne, Sheikboudou & Bensaid 1994). The vaccine was formulated with Freund's adjuvant and used to immunize goats, each of which received an initial and a booster inoculation. When subsequently challenged with homologous tissue culture material 50–80 % of the animals were found to be protected while 100 % of untreated controls died. The same methodology was

later used in Zimbabwe, where the vaccine was prepared using the Crystal Springs strain, inactivated with β -propiolactone, and also formulated with Freund's adjuvant. This vaccine was used to immunize sheep, each of which received five inoculations over a period of months, and in this case 50–100 % of the animals were protected against a homologous needle challenge which killed 60 % of untreated controls (Mahan, Andrew, Tebele, Burr ridge & Barbet 1995). Similar inactivated vaccines have been used over the succeeding 12 years, using different strains of *E. ruminantium* and different commercially acceptable adjuvants. When a needle challenge with a heterologous strain, or a tick challenge, is given the inactivated vaccines are less protective than in the original experiments (Mahan, Smith, Kumbula Burr ridge & Barbet 2001; Faburay, Geysen, Ceesay, Marcelino, Alves, Taoufik, Postigo, Bell-Sakyi & Jongejan 2007). Protection levels are even lower when the vaccines are tested in the field against a natural tick challenge with organisms of differing immunogenicities. A summary of all the inactivated vaccine field trials conducted

by one research group over a period of 8 years showed that overall mortality levels could be reduced, in sheep from 78 % (unvaccinated) to 54 % (vaccinated) and in cattle from 67 % (unvaccinated) to 19 % (vaccinated) (Mahan, Barbet & Burridge 2003).

One early difficulty with these inactivated vaccines was that large amounts of material had to be used, in multiple inoculations, and the cost of production from tissue culture was unacceptably high. There have, however, been marked improvements in culture technology for *E. ruminantium* in recent years, which have put commercial vaccine preparation within reach (Marcelino, Sousa, Verissimo, Cunha, Carrondo & Alves 2006; Marcelino, Vachieri, Amaral, Roldao 2007). The problem that remains to be addressed is the disappointing levels of protection achieved against natural tick challenge in the field, especially in Africa. The high mortalities of vaccinated animals in this situation are likely to be the result of infection with non-cross protecting immunotypes. It is therefore of great significance for vaccine development to note that there is extensive polymorphism between *E. ruminantium* strains, particularly in southern Africa (Allsopp, Van Heerden, Steyn & Allsopp 2003; Allsopp & Allsopp 2007), and moreover there is large-scale recombination between the different genotypes (Allsopp, Van Strijp, Faber, Josemans & Allsopp 2007). So while inactivated vaccines could be useful in the Caribbean, where there is limited genetic variability within the *E. ruminantium* population, it may be difficult to achieve the improvements in protectivity in the African situation which would be required to make these vaccines commercially viable.

Recombinant vaccines

The observation that inactivated heartwater vaccines can stimulate protective immunity indicates that the development of a successful recombinant vaccine, using selected *E. ruminantium* genes, is theoretically possible. A recombinant vaccine should be cheaper to manufacture, and easier to store than an inactivated vaccine, and recombinant heartwater vaccine development is a very active area of research. The first attempts to develop such a vaccine used the map1 gene of *E. ruminantium*, cloned into an expression vector together with a viral enhancer/promoter, which was tested for its ability to protect mice against a lethal homologous challenge (Nyika, Mahan, Burridge, McGuire, Rurangirwa & Barbet 1998). Protection levels ranging from 23–88 % were obtained in several different experiments using this naked DNA vector alone, and subsequently attempts were made to improve the protection levels by boosting with recombinant MAP1 protein (Nyika, Barbet, Burridge & Mahan 2002). This procedure enhanced protection levels in mice from a range of 13–27 % without boosting to a range of 53–67 % with boosting.

There are some experimental indications that the map1 gene might not be the best choice for recombinant vaccine development. For example, purified MAP1 protein inoculated into goats and a sheep induced high specific antibody titres but conferred no protection against homologous challenge

(Van Kleef, Neitz & De Waal 1993). Then it must be remembered that *E. ruminantium* map1 genes are highly polymorphic (Allsopp, Dorfling, Maillard, Bensaid, Haydon, Van Heerden & Allsopp 2001) so any vaccine using this gene would have to include variants of all important virulent strains. The complete *E. ruminantium* genome contains 888 annotated genes (Collins *et al.* 2005), any of which are potentially available for use in experimental vaccines, however identifying those which code for antigens which stimulate the required protective T-cell response is not a trivial exercise (Esteves *et al.* 2004). One research group has attempted to identify suitable genes by screening *E. ruminantium* expression libraries (Barbet, Whitmire, Kamper, Simbi, Ganta, Moreland, Mwangi, McGuire & Mahan 2001). Clones were selected whose expression products were both recognized by anti-*E. ruminantium* antibodies and also stimulated proliferation of peripheral blood mononuclear cells (PBMC) from cattle immunised against *E. ruminantium* by infection and treatment. The selected clones were expressed in recombinant bacteria and pools of bacterial lysates containing the *E. ruminantium* gene products were used to immunize mice. Some pools of recombinants stimulated protective immunity against homologous challenge in 58–89 % of the immunized mice, levels of protection which were no improvement on those obtained using the map1 gene.

A striking feature of all the *E. ruminantium* immunization trials in mice discussed above is the highly variable levels of immunity which were reported. Even more problematic is the observation that some genes confer immunity in the mouse model but this protection is not reproducible in ruminants (Louw, Brayton, Collins, Pretorius, Van Strijp & Allsopp 2002; Collins *et al.* 2003). These observations led one laboratory to begin conducting all *E. ruminantium* vaccination trials in sheep, and the results have been much more reproducible. In some of the most successful experiments a cocktail of four *E. ruminantium* genes, cloned in a DNA vaccine vector and used to immunize sheep, was found to stimulate complete protection against a virulent needle challenge with both homologous, and five different heterologous, *E. ruminantium* strains (Collins *et al.* 2003). This experimental vaccination was, however, not very effective when used in the field against a natural heartwater-infected tick challenge, when levels of protection only reached 20 %.

The four genes in the protective cocktail are thought to be components of an ABC transporter system but it was not known whether the protection which they offered was specific to their function. The map1 gene had been shown to provide some protection when presented as a DNA vaccine, even though there were reasons to believe that it might not be a good choice for recombinant vaccine development, so further work was carried out to determine how the protection stimulated by the putative ABC transporter genes compared with that stimulated by other cocktails of *E. ruminantium* genes (Pretorius, Collins, Steyn, Van Strijp, Van Kleef & Allsopp 2007). The protection was shown to be specific to the ABC

transporter genes, since cocktails of other *E. ruminantium* ORFs presented in the same vaccine system failed to provide any protection. Furthermore, the levels of protection stimulated by each of the putative ABC transporter genes presented individually was similar to that stimulated by the four-gene cocktail. The protection against field challenge remained poor, however, and this appeared to be a result of markedly enhanced virulence of *E. ruminantium* organisms injected by the tick, as compared to those which are present in infected mammalian blood (Pretorius *et al.* 2007).

Heterologous prime-boosting is a well tried strategy for improving DNA vaccine protection levels, it involves boosting the initial immunity with recombinant protein or a recombinant viral vaccine vector (Vordermeier, Lowrie & Hewinson 2003; Gilbert *et al.* 2006) Enhancing the protection levels of the experimental *E. ruminantium* recombinant vaccine has been attempted by boosting with either recombinant protein or recombinant lumpy skin disease virus (LSDV) (Pretorius, Van Kleef, Collins, Tshikudo, Louw, Faber, Van Strijp & Allsopp 2008). Recombinant protein boosting appeared to be more efficient than boosting with rLSDV, since only lymphocytes isolated from animals which received the protein boost showed specific proliferation and increased interferon gamma expression when exposed to the recombinant *E. ruminantium* proteins. Complete protection against an experimental needle challenge was retained, but the levels of protection against an infected tick challenge in the field were not improved at all.

In summary, the development of a recombinant vaccine shows great promise, especially when using a prime-boost configuration, but there are problems to be resolved. The algorithms used for selecting vaccine candidate genes *in silico* are currently unreliable, and *in vivo* stimulation of cytokine secretion or T-cell responses does not correlate with the protective ability of genes. It is also evident that protection against a needle challenge is not a reliable predictor of immunity against a natural tick challenge, so a way must be found to implement a laboratory based tick challenge to simulate the natural situation in a controlled environment. It is also important to note that candidate vaccines must be tested in ruminants, since tests in the mouse model do not give reproducible results nor can the findings be reliably extrapolated to predict how the vaccine will behave in ruminants.

Attenuated vaccines

Attenuated vaccines are the oldest form of vaccine, and as they are based on pathogens which cause no clinical disease, but which retain their immunogenic properties, they can stimulate solid long-lasting immunity. The main difficulty is that traditional methods for attenuating virulent organisms are imprecise processes which take indeterminate lengths of time. The first strain of *E. ruminantium* to be attenuated was the Senegal strain, which attenuated after culture in endothelial cells over 11 passages and 239 days (Jongejan 1991). When inoculated into sheep and goats the attenuated organism caused no clinical reaction and the animals were completely protected against a subsequent homologous needle challenge. When field trials were conducted, however, the protection levels were lower, with reductions in mortality, as compared to unvaccinated controls, from 70–43 % in one study (Gueye, Jongejan, Mbengue, Diouf & Uilenberg 1994), and from 100–25 % in another (Faburay *et al.* 2007).

Immunity to the Senegal strain provides limited protection against other *E. ruminantium* strains (Jongejan, Thielemans, Briere & Uilenberg 1991) while the Welgevonden strain, in contrast, provides good cross-protection against a range of other virulent strains (Collins *et al.* 2003). In principle, therefore, the Welgevonden strain would be a much better candidate from which to develop an attenuated vaccine, but unfortunately it failed to attenuate after culture through hundreds of passages over several years (Gueye *et al.* 1994; Zweygarth, Vogel, Josemans & Horn 1997).

A common technique to induce attenuation of a pathogen is to culture it in an abnormal host cell, and the Welgevonden strain was induced to infect a canine macrophage-monocyte cell line (DH82) by adding cycloheximide to the culture medium in order to slow the metabolism of the host cells. After continuous propagation in DH82 cells the organism was found to have lost all virulence for mice between passages 25 and 50. Since DH82 cells are cancerous and not suitable for vaccine use the attenuated strain was re-adapted to bovine endothelial cells after 61 passages in DH82 cells (Zweygarth *et al.* 2005). When the re-adapted attenuated Welgevonden strain was used to infect sheep or goats there was a brief rise in body temperature, without any other adverse symptoms, and the animals were subsequently found to be fully protected against

TABLE 1 Immunising ability of attenuated *E. ruminantium* Welgevonden (after Zweygarth *et al.* 2008)

Species	Dose of elementary bodies	Route	Challenge	Protection (%)
Merino sheep	1.1 x 10 ⁵	iv	Homologous	100
Merino sheep	Various	iv	Heterologous (five different strains)	100
Merino sheep	6.7 x 10 ⁵	sc	Homologous	83
Merino sheep	2.6 x 10 ⁵	im	Homologous	80
Boer goats	Various	iv	Homologous	100
Angora goats	0.9 x 10 ⁴ –1.3 x 10 ⁵	iv	Homologous	90
Friesian cattle	1.7 x 10 ⁵ –2.2 x 10 ⁶	iv iv	Heterologous (Gardel)	83

Note: All animals were needle challenged with blood stabilates of virulent *E. ruminantium*

a lethal needle challenge with both the homologous, and four different heterologous, *E. ruminantium* strains (Zweygarth et al. 2005).

Further testing of this promising attenuated vaccine has been undertaken (Zweygarth, Josemans, Van Strijp & Steyn 2008) and a summary of the results in different ruminant species, administered by different routes, is shown in Table 1. The intravenous route was the most effective, providing 100 % protection in Merino sheep and Boer goats, and while the protection in Angora goats was only 90 % these animals are known to be exceptionally sensitive to *E. ruminantium* (Du Plessis, Jansen & Prozesky 1983). The protection rate in cattle was also less than ideal, at 83 %, but it should be noted that the cattle were challenged with the Gardel strain, to which they are very sensitive, because they are not particularly sensitive to the Welgevonden strain.

Attempts to use an intramuscular or subcutaneous route of inoculation led to less effective protection than when using the intravenous route. Ideally a vaccine would provide lifelong immunity, and some experiments have been conducted to determine the duration of the immunity which this attenuated vaccine provides. So far these have only been carried out in Merino sheep, and the immunity remains at 100 % for at least 6 months, but then falls to 80 % after one year (Zweygarth et al. 2008). It is likely, however, that under normal field conditions the natural heartwater challenge would prevent early loss of immunity.

Estimates made of the possible production costs of this attenuated Welgevonden vaccine suggest that the culture costs for 60 sheep vaccine doses would be around US \$1. This compares very favourably with the current cost of \$27 for 60 doses of Ball 3 infected blood for infection and treatment. Further development of this promising attenuated vaccine is needed, and the first priority is to carry out tick challenge experiments on immunized animals. Several commercial vaccine development criteria must also be addressed, such as possible formulation of the vaccine for intramuscular or subcutaneous inoculation, which would be much more convenient than the intravenous route, and testing of retention of effectiveness after lyophilization, since a lyophilized vaccine would not need a cold chain for distribution.

The perennial concern with attenuated vaccines is the possibility that they may revert to virulence, perhaps as the result of recombination in the field with wild types of the pathogen. This, however, is not of major consequence in the endemic area, since new genes would not be added to the existing environment and widespread recombination between strains is already known to occur. Obviously this attenuated vaccine would not be useable in non-endemic areas, but

it would be an excellent stopgap vaccine for use in Africa pending the development of a recombinant vaccine.

Summary

The last ten years have seen the most significant developments in *E. ruminantium* vaccination since the introduction of the infection and treatment procedure in 1945, with exciting advances in both recombinant and attenuated vaccines. A recombinant vaccine would be the ideal solution to heartwater control, especially for export, since it would be useable anywhere in the world. This would be essential in the event of any spread of the disease outside the current endemic area, such as the long feared possibility of a cattle egret mediated spread of heartwater infected ticks from the Lesser Antilles to the American mainland (Deem 1998). Another advantage of a recombinant vaccine would be the relative ease with which the evolution of vaccine genes in the wild could be followed, allowing the vaccine formulation to be changed to keep up with pathogen changes. The research needed to develop a recombinant vaccine is, however, likely to be relatively time-consuming and expensive, and in the interim the new attenuated Welgevonden vaccine shows great promise. The development of this vaccine to the level of commercialization should be undertaken as a matter of urgency.

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