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# THE PRODUCTION OF HEARTWATER VACCINE

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ABSTRACT

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The events leading to the production of an effective vaccine against heartwater are summarized. The production techniques used at Onderstepoort to produce and control the quality of the infected blood and nymph suspension vaccines, and covering their respective advantages and disadvantages, are compared. The many problems of these vaccines and how they may best be overcome are discussed.

## HISTORY

It has long been recognized that animals which recover from heartwater acquire an immunity to the disease. Based on this, numerous attempts at producing a vaccine have been made. The early trials were generally unsuccessful and are adquately described by Alexander (1931). It was in the early years of this century that Hutcheon (1902) and Spreull (1904) considered the use of blood of animals suffering from heartwater as an inoculum. It was, however, only in 1931 that Alexander used the correct combination of inoculum (infected blood) and route of administration (intravenous). Neitz (1940), through the discovery that heartwater could be treated with sulphonamides, and Neitz & Alexander (1941) through their observations that young calves and lambs are relatively insusceptible to heartwater, developed a feasible, although cumbersome, method of vaccination. Later, Bezuidenhout (1981) introduced the use of homogenized *Cowdria ruminantium* infected *Amblyomma hebraeum* nymphs as inoculum in an attempt to reduce the production costs of the vaccine. This did not, however, solve the problems caused by these both being live, virulent vaccines.

Although infected brain material has been found to be infective if administered subcutaneously (Ilemobade & Blotkamp, 1978), only blood and nymph suspension have ever been produced and used commercially. The method of production of the blood vaccines has been described in relative detail (Anon., 1984; Arnold & Kanhai, 1979). Uilenberg (1983) briefly reviewed both methods of production and their limitations.

## PRESENT-DAY TECHNIQUES

The techniques used to produce the blood and nymph vaccines are essentially the same. In fact, it is only in the production of the working antigen that the 2 techniques differ. For this reason the production techniques will be dealt with simultaneously and only the differences emphasized.

### Stock antigen

The production of the stock antigen involves 3 distinct steps:

(a) *The isolation of strains or isolate*

A number of ways by which field strains can be isolated have been listed (Anon, 1984). These include the collection of blood from suspected cases of heartwater, pooling of blood from animals in endemic areas and the use of brain emulsions from animals which have died of heartwater, as described by Ilemobade & Blotkamp (1978). Other sources which have been successfully employed for this purpose are lymph nodes (Du Plessis & Kümm, 1971); ticks (Barré, Camus, Birnie, Burridge, Uilenberg & Provost, 1984; Burridge, Barré, Birnie, Camus & Uilenberg, 1984; Du Plessis, 1985) and kidneys (Jongejan, Van Winkelhoff & Uilenberg, 1980).

(b) *The selection of a strain or isolates*

Despite many attempts (Alexander, 1931; Neitz, Alexander & Adelaar, 1947; Ilemobade, 1976; Van Winkelhoff & Uilenberg, 1981; Uilenberg, Zivkovic, Dwinger, Ter Huurne & Perie, 1983) until very recently only Du Plessis & Kümm (1971) and Mackenzie & Van Rooyen (1981) have demonstrated any immunological differences between isolates of *Cowdria*. L. L. Logan (personal communication, 1986) has shown conclusively that such differences are not limited to mouse-adapted isolates and, during provisional studies, some indications were obtained that the Ball 3 isolate may not protect sheep against a challenge with the Welgevonden isolate (J. A. Olivier, personal communication, 1986).

There are, however, considerable isolate differences as regards virulence (Neitz *et al.*, 1947; Haig, 1952; Camus & Barré, 1982). Bearing these 2 facts in mind, and also that immunity does not depend on the virulence of the isolate (Anon., 1984), it follows that as mild an isolate as possible, which must be able to protect against the desired wide range of other isolates, should be chosen.

In South Africa the natural Ball 3 isolate (Haig, 1952) is used. It was probably not chosen for low virulence but because it generally causes a marked febrile response a few days prior to the onset of other clinical signs.

(c) *Production and storage of stock antigen*

Stock antigen is produced in sheep at Onderstepoort. The sheep are obtained from the Barkley East/Molteno area of the Republic where bluetongue and heartwater do not occur due to the very high altitude and extremely cold winters. The sheep are infected using blood of an animal suffering from heartwater. Once the sheep have developed a temperature of 40 °C for 2 days (usually 9 to 10 days post-inoculation) they are bled directly into cold (4 °C) sterile buffered lactose-peptone-citrate (BLP) with 10 % dimethylsulphoxide (DMSO) as cryoprotectant until a 50 % blood BLP ratio is reached. The BLP diluent is prepared as follows: 2,75 g $KH_2PO_4$; 20 g sodium citrate, 100 g lactose, 20 g protease peptone, 2,4 m$\ell$ 10 N sodium hydroxide per litre distilled water. Penicillin G at 240 mg/$\ell$ and dihydrostreptomycin at 200 mg/$\ell$ are also added. Approximately 1$\ell$ of blood is drawn from each sheep. The flasks containing the diluent, into which the animals are bled, are packed in ice immediately to lower the temperature of the blood to between 4 °C and 8 °C. This blood is then bottled in 10 m$\ell$ containers and stored as stock antigen. Alexander (1951, cited in Haig, 1952), originally described deep-freezing of the antigen to preserve it. The stock antigen used for the Onderstepoort vaccines is snap frozen and stored at −196 °C in liquid

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nitrogen. While DMSO is used at Onderstepoort by Bezuidenhout (1981) and Ramisse & Uilenberg (1970) as a cryoprotectant, Ilemobade, Blotkamp & Synge, (1975) showed that the addition of DMSO to infected blood did not improve the viability of the organisms during freezing. This is in keeping with the findings of Bezuidenhout & Oberem (unpublished data, 1983), who worked with nymph homogenates. It must be mentioned at this point that glycerol is toxic to *Cowdria* (Alexander, 1931; MacKenzie & Van Rooyen, 1981) and other rickettsiae (Foggie, Lumsden & McNeillage, 1966) and should therefore be avoided as cryoprotectant for heartwater vaccines.

The stock antigen is then tested for freedom from bacterial and fungal contamination, absence of adventitious agents, inocuity and minimum infectivity, employing the same methods used for the quality control of the final vaccine.

**Working antigen**

(a) *Infected sheep-blood vaccine*

Ten heartwater-susceptible Merino sheep are each inoculated with 5 mℓ of stock antigen intravenously. These sheep are also obtained from the Barkley East/Molteno District of the Republic. At the height of the subsequant febrile reaction the sheep are bled into BLP-citrate-DMSO as described above.

(b) *Infected-nymph vaccine*

The method for the preparation of an infected nymph homogenate is described by Bezuidenhout (1981). Many more steps are required before the nymph suspension reaches this stage.

The first is the infection of the *Amblyomma hebraeum* larvae. One sheep is usuallysufficient. A heartwater-susceptible Dorper sheep is usually used as its back is broader and smoother than that of a Merino sheep. It is infected intravenously with 5 mℓ of the stock antigen. Five days after inoculation its back is shaved and 6 to 8 bags are glued in place on the shaved area (Heyne, Elliot & Bezuidenhout, 1987). During the following 3 days up to 6 000 larvae are placed in 2 of the bags daily. The rectal temperature of the sheep is then recorded daily. The replete larvae are collected daily and kept separately. Only those larvae which have engorged and fallen during the temperature reaction are used. They are then stored at 27 °C, at a relative humidity of 75 % to 80% and with natural day and night conditions.

The nymphs which develop are then placed to feed on susceptible sheep, usually 2, at the same time. When engorged, nymphs are collected, sorted (all the dead and obviously diseased ticks are removed) and the remainder counted. Batches of up to 10 000 nymphs have been processed in this way.

The nymphs are then washed and sterilized externally using 10 % alkyl-dimethyl-benzyl-ammonium chloride for 30 min, rinsed in 70 % ethanol and copious amounts of sterile water. The nymphs and washing solutions are continuously stirred.

Finally, BLP-containing penicillin G at 240 mg/ℓ, dihydro-streptomycin at 200 mg/ℓ, polymixin E at 10 million units/ℓ and amphotericin B at 10 μg/ℓ as well as 0,5 % Silicone Antifoam Fluid[1] at 50 mℓ/ℓ is added before the nymphs are homogenized at a concentration of 5 nymphs/mℓ. This concentrated nymph suspension is then filtered under vacuum through a series of 3 gauze filters ranging from 35 to 625 pores/cm$^2$. Homogenization is done in a blender which is kept packed in ice. The duration of homogenization, which fluctuates according to the number of nymphs in a batch, varies between 1 and 3 min. Thereafter the filtered homogenate is bottled in 50 mℓ plastic bottles and snap frozen in a dry ice/ethanol mixture. It is then transferred and stored in liquid nitrogen-containing freezers.

The quality of the concentrate is evaluated during preliminary trials which include all the tests made on the final vaccine (see below) and infectivity tests done in sheep. Susceptible sheep are inoculated with diluted concentrate representing 1/80th, 1/160th and 1/320th of a nymph. The results determine the concentration at which the final vaccine will be made. Once a suitable dilution has been established the concentrate is diluted to 20 times the highest dilution at which it was found to be infective, e.g. if the sheep which was inoculated with the 1/320th of a nymph dilution reacted then the concentration of the final vaccine will be 20 × 1/320 = 1/16th of a nymph/dose.

(c) *Bottling of the final vaccine*

Immediately prior to bottling, the required amount of frozen nymph suspension is thawed and further diluted as previously determined in the original diluent. Either this material or freshly collected, diluted, infected blood is then dispensed into suitably labelled containers and snap frozen in a dry ice/ethanol mixture. Each container holds at least 2 doses, i.e. 10 mℓ blood or 4 mℓ nymph suspension. Thereafter the bottled vaccine is stored at at least −76 °C, but usually in liquid nitrogen (−196 °C).

**Quality control**

Each batch of vaccine produced has to pass a battery of quality control checks before it is sold. These are:

(a) *Freedom from bacterial and fungal contamination*

At least 5 final vaccine containers are randomly selected and the contents mixed; 0,25 mℓ of the pooled contents is inoculated into each of 2 tubes of thioglycollate and 2 tubes of soyabean medium (0,25 mℓ vaccine in 40 mℓ medium per tube). The thioglycollate cultures are incubated at 37 °C for 7 days and the soyabean cultures at 20 °C for 14 days.

Control cultures inoculated with known bacteria and fungi should show active growth. Cultures inoculated with the vaccine should remain sterile. If there is evidence of contamination the test is repeated with another 5 final containers. If the repeat tests prove completely negative the vaccine is passed but should they too show evidence of contamination the batch is discarded.

(b) *Inocuity and absence of adventitious agents*

The contents of 5 final vaccine containers are pooled and injected as follows:

Two mature guinea pigs are injected with 1 mℓ each intraperitoneally. The animals are observed for a period of 14 days. They should remain healthy.

The pooled material is also inoculated intraperitoneally (0,25 mℓ) and intracerebrally (0,03 mℓ) into groups of at least 6 adult mice and intraperitoneally (0,1 mℓ) into 2 groups of 7 infant mice. If the vaccine is to pass the test the mice should remain healthy during an observation period of 14 days. If there is any evidence of specific mortality the vaccine batch is disqualified and destroyed.

The pooled blood is also inoculated into the yolk-sac of 10 eight-day-old embryonated eggs. The eggs are incubated at 37 °C for 8 days and candled daily. Embryos which die within 24 h are not taken into account, but the test should be repeated if less than 70 % of the embryos survive for 24 h. The cause of embryo mortality during the subsequent observation period should be determined

[1] Union Carbide Corporation, Silicone Div. New York.

by setting up the appropriate sterility and haemagglutination tests and examination of yolk-sac smears. On the 4th day of observation 4 eggs are opened. The embryos and membranes are examined for lesions and the allantoic fluid is subjected to the haemagglutination test with guinea pig and chicken red blood cells at 4 °C and 37 °C. The remaining eggs are opened on the 8th day of incubation and examined for lesions on the membranes and abnormalities of the embryos. Specific embryo mortality, haemagglutinating activity of the allantoic fluid, any lesions on the membranes or abnormalities of the embryos will disqualify the batch of vaccine.

(c) *Minimum infectivity or potency*

This is tested by inoculation of 4 sheep, 2 with a 1:10 and 2 with a 1:20 dilution of a dose. The animals are observed for 3 weeks and rectal temperatures are recorded daily. Both sheep (1:20) should show typical heartwater reactions.

## Discussion

The use of these vaccines, in particular the infected blood, has played a major role in the advances made in livestock farming and improvement in the heartwater endemic areas of South Africa. They are the only vaccines against heartwater produced on any appreciable scale but despite many improvements, particularly regarding production techniques, there remain many problems hampering larger scale production and wider use.

*The cost of production*

To produce the approximately 175 000 doses of heartwater vaccine (infected blood) which are used annually in Southern Africa about 700 sheep are required. Each of the 50 batches of this vaccine produced annually requires 10 sheep for production and 4 for quality control.

The nymph suspension is considerably cheaper to produce, requiring only 8 sheep per batch and 2 batches per year, i.e. 16 sheep, if there are no production problems.

Both techniques require the use of highly trained technical staff and strict veterinary supervision.

*The lability of the organism*

Due to the extreme lability of *Cowdria* and the need to keep the vaccine at at least −76 °C, storage and transport are problematic and expensive.

*The route of administration*

The fact that the vaccines can only be effectively administered intravenously is perhaps the greatest limiting factor to their use. Only farmers skilled in this technique and veterinarians use the vaccines. Also, farmers who have to engage professional help have considerably increased expenditure.

This route of vaccination also increases the likelihood of adverse reactions (Bezuidenhout, 1981). Occasionally shock is reported with inoculation with the blood vaccine (Fick & Schuss, 1952; Barnard, 1953; Van der Merwe, 1979). L. van der Merwe (unpublished data, 1986) reports severe histamine-like reactions in 53 % of kid goats and 16 % of lambs at the first inoculation of the nymph suspension. These reactions occur sporadically, being far more severe on certain farms. J. D. Bezuidenhout & P. T. Oberem (unpublished data, 1985) inoculated 105 lambs younger than 1 week old and 100 Angora kids less than 3 months old using the nymph suspension without any adverse reactions. Calves, adult sheep and cattle very rarely develop these reactions.

*The virulence of the vaccine*

The virulence of the vaccine increases the expenses incurred due to losses of vaccinated animals where supervision is lax, increased supervision time and quality required, and the consequent need to treat older animals. The use of the vaccine in pregnant animals is, for the same reason, not advised. The fact that this is a live virulent vaccine also precludes its use in heartwater-free areas where potential vectors exist, e.g. the United States (Uilenberg, Barré, Camus, Burridge & Garris, 1984).

*The possible transmission of other diseases*

Where the quality control procedures are not adapted to local conditions and requirements, there remains a possibility that other diseases may be transmitted in the sheep blood or nymph suspension. In areas where *Ehrlichia ovis, Anaplasma ovis* and *Theileria ovis* are problems the above quality control techniques would be insufficient.

*Possible solutions*

Despite the problems and drawbacks it must be borne in mind that these are the only vaccines against heartwater which are effective and available. Nevertheless, there are both short- and long-term solutions which must be considered.

(a) *Short-term solutions*

The nymph suspension has gone a long way in reducing production costs but this will only become feasible when the problems of adverse reactions in kids and lambs are solved, either by the addition of antihistamines or by some other means of removing the toxic fraction such as differential centrifugation (J. D. Bezuidenhout & J. A. Olivier, unpublished data, 1986). The success achieved by Bezuidenhout, Paterson & Barnard (1985) in culturing the organism should also prove advantageous in this regard. If the Welgevonden isolate (Du Plessis, 1985) is used the costs could further be reduced by obviating the need to use sheep in the quality control tests. This, however, should only be considered after more information on cross-protection, and the possible consequences, have been collected and evaluated.

Birnie, Endris & Logan (1986) have already shown the way to reduce the lability of the organism by comparing various diluents for maintaining the viability of *Cowdria ruminantium*. The protection afforded by the diluents during freezing must also be considered. J. L. du Plessis (unpublished data, 1986) achieved limited success with freeze-drying of the organism. A combination of these 2 approaches should enhance the chances of success.

The problem of virulence could perhaps be overcome in the short term by the repeated administration of a more purified, inactiviated antigen at higher concentrations than was previously possible when only crude blood or tick extracts were available.

The possibility of transmitting the blood parasites *Ehrlichia ovis, Anaplasma ovis* and *Theileria ovis* in the vaccine can be reduced by examination of blood smears of the animals just prior to bleeding for the vaccine and even more so by the use of the tissue-culture antigen.

(b) *Long-term solutions*

The long-term solutions may involve the use of biotechnology, more specifically the isolation of *Cowdria* DNA, and the identification of a sequence or sequences which code for immunogenic products of the organism, thus leading to the production of a recombinant vaccine.

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