Blocking *Babesia bovis* vaccine reactions of dairy cattle in milk

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The use of 1.16 mg/kg (one third) of the recommended dose of diminazene aceturate, administered indiscriminately to cattle on day seven of the unfrozen *Babesia bovis* and *Babesia bigemina* bivalent live blood vaccine reaction, was an infection and block treatment method of immunisation used successfully with no known adverse effect on the parasites or the development of protective immunity. Continuing with this practice after replacement of the unfrozen vaccine with deep-frozen monovalent *B. bovis* and *B. bigemina* live blood vaccines resulted in reports of vaccine failure. Laboratory investigation indicated the harmful effect of block treatment in preventing the development of durable immunity against *B. bigemina* as opposed to the much lesser effect it had on *B. bovis*. Consequently the practice was no longer recommended. A *B. bovis* vaccination attempt aimed at controlling the disease of dairy cows in milk (n = 30) resulted in 20% fatalities during the expected vaccine reaction period. The practice of block treating *B. bovis* was therefore reinvestigated, this time in a field trial using dairy cattle in milk (n = 11). Using 0.88 mg/kg (one quarter) of the recommended dose of diminazene administered on day 12 of the *B. bovis* vaccine reaction resulted in only two animals (n = 5) testing ≥ 1/80 positive with the indirect fluorescent antibody test (IFAT) although parasites could be demonstrated in three. In the untreated control group, by contrast, five of the vaccinated animals (n = 6) tested ≥ 1/80 positive with IFAT and parasites could be demonstrated in all. The unsatisfactory outcome obtained in this study, combined with that of the earlier investigation, indicated that there are more factors that influence successful vaccination than previously considered. It is therefore concluded that block treatment of the live frozen South African cattle babesiosis vaccines reactions is not recommended.

Introduction

Babesia bovis is an arthropod-transmitted pathogen in cattle, rated as being the tick-borne disease with the biggest economic impact on cattle farming in the tropical and subtropical areas of Africa, Asia, Australia and South America (Bock *et al.* 2004). The prevalence of the disease is closely related to the tick vectors' distribution, which is confined by humidity and temperature (Gothe 1967; Yeoman & Walker 1967). In South Africa, the disease is transmitted by the one-host tick, *Rhipicephalus* (*Boophilus*) *microplus*, naturally inhabiting the savanna climatic regions of wooded grasslands that are also used as cattle pasture (Walker *et al.* 2003).

Disease control by tick eradication is not a solution in those areas where the vector is already well established (De Vos 1979). An alternative, more acceptable practice is to limit the degree of tick control, thereby allowing natural endemic stability to develop. However, relying on this as a realistic approach is made impossible by the multiple tick-borne disease ecosystems of southern Africa (Bezuidenhout 1985; Perry *et al.* 1985). The control of multi-host ticks invariably also affects the one-host ticks and ultimately has a negative effect on the epidemiology of bovine babesiosis through reduction of the tick population to the extent that endemic stability cannot be maintained (De Waal 1996). The advisable approach to managing the disease would therefore be to integrate the strategic use of acaricides and the application of the appropriate vaccine, which should also prove the most cost-efficient method of control (De Waal & Combrink 2006).

For many years, the only available babesiosis vaccine was a chilled bivalent live blood vaccine containing parasites of both *B. bovis* and *Babesia bigemina*. As calves up to nine months of age usually show highest resistance to the disease and seldom develop serious symptoms, it is generally recommended that vaccination be restricted to this age group (Trueman & Blight 1978). Despite this recommendation, a large proportion of vaccines are administered to older, more susceptible animals. Although the vaccine strains used were attenuated, the shortcoming of the chilled vaccine was the fatal reactions it caused in older cattle if left untreated. For this reason a method of infection and block treatment was investigated, whereby one third (1.16 mg/kg) of the recommended dose of diminazene aceturate is administered indiscriminately to cattle on day seven after inoculation of the vaccine, which proved to be quite successful with no known adverse effects to the parasites or to the development of protective immunity (De Waal 1996).

In April 1998, the bivalent chilled vaccine was replaced by deep-frozen monovalent live blood vaccines of *B. bovis* and *B. bigemina*. The block treating method was continued until reports of vaccine failures associated with the use of this practice prompted a reinvestigation of this approach. It was found that treatment of the frozen *B. bigemina* vaccine reactions with diminazene on day seven at dose levels as low as 0.35 mg/kg (one tenth the recommended dose) will still kill all parasites (Combrink & Troskie 2004) and thus prevent the development of a durable immunity. Although treatment at 1.16 mg/kg (one third the recommended dose) was not as harmful to the *B. bovis* parasites, it was found that the prepatent period of the vaccine reaction was extended for a longer period. Due to the detrimental effect of diminazene on the vaccine parasites as well as various other factors, such as the degree of natural resistance of different cattle breeds and individual animals to *Babesia* parasites, the infectivity of frozen vaccine being less predictable than that of unfrozen vaccine (due to demise of parasites during freezing and thawing), the non-conformance of diminazene preparations to manufacturer's label claims (Tettey *et al.* 2002) and the accuracy of the drug dose administered, all influencing successful immunisation, the block treating of *B. bovis* and *B. bigemina* vaccine reactions was no longer recommended.

In renewed efforts aimed at specifically controlling *B. bovis*, veterinarians have again started to vaccinate older cattle and have found that, although not completely safe, the risk involved in losing a few animals due to vaccination outweighs the risk of losing large numbers of animals due to natural disease outbreaks. Vaccination of 2134 nutritionally challenged adult beef cattle and 30 normally rationed adult dairy cows in milk with the *B. bovis* vaccine resulted respectively in 16 (0.75%) and six (20%) fatalities during the two to four weeks following inoculation (Nick Fischer pers. comm., February 2011). It has been suggested (Kuttler, Zaugg & Johnson 1984) that environmental and other stressors could compromise natural resistance to disease, but no published information could be found of this being of significance under field conditions. However, it is known that the effect of stressors is even more pronounced in the case of intensive lactating dairy cattle (Johnson & Vanjonack 1976), which may explain the higher fatality rate experienced in this group. Having thus observed a specific need for use of the *B. bovis* vaccine in milk-producing animals, and considering that laboratory block treating of this vaccine's reaction was found not as completely harmful to the parasites, we decided to reinvestigate the old practice.

Materials and methods

Animals

Eleven non-pregnant Friesian dairy cows, four to nine years old, between 54 and 83 days in milk, with body condition scores of 1.5 to 2.5 out of five, and which had no antibody titres to *B. bovis* antigen in the IFA test (Gray & De Vos 1981; Joyner *et al*. 1972), were used in the field trial to determine the effect of block treating *B. bovis* vaccine. Animals were randomly divided into two groups of which one consisted of six and the other of five cows each (Table 1). They were dipped for ticks before and for the duration of the experiment. Frost and cold weather experienced during this period restricted tick activity to a minimum.

Vaccination

The commercial frozen live-blood vaccine (De Waal 1996; De Waal & Combrink 2006) used in this study was the *B. bovis* 'S' strain (De Vos 1978; Callow, Mellors & McGregor 1979). The vaccine was dispatched from Onderstepoort on dry ice and directly before use thawed in water at 37 °C. All 11 cows were each vaccinated with 1 mL of the vaccine intramuscularly and were then returned to the farm's normal dairy herd management system.

Block treating vaccine reactions

Previously, vaccine reactions were block treated on day seven of the vaccine reaction, using one third (1.16 mg/kg) of the recommended dose of diminazene. This was done to correspond with the five to seven day pre-patent reaction period of the *B. bigemina* parasite used in the old bivalent, unfrozen vaccine. The practice was retained even after replacement of the bivalent, unfrozen vaccine with deep-frozen, monovalent babesiosis vaccines, in the event that both are used concomitantly. Considering that the pre-patent period of the *B. bovis* vaccine applied in the field ordinarily varies between 9 to 19 days (Combrink personal observation) the parameter for block treatment in this study was moved from day seven to 12 after inoculation. The rationale for treating later was to increase the potential of parasite survival, but still allow for the treatment to be effective in case of an early well-advanced reaction.

Very few farmers have access to animal weighing facilities and it has been observed by author Graham Carr that the weight estimation of clients in his practice is on average, an overestimate rather than an underestimate. Therefore, in the absence of determining a specific normal error in weight estimation for this study, and which may not be objectively

TABLE 1: *Babesia bovis* vaccine reactions of dairy cattle in milk.

***B. bovis* vaccination/block treatment**	**Group**	**Number of animals**	**Blood smear positive for *B. bovis***	**Serology positive ≥ 1/80 for *B. bovis***	**Febrile reaction ≥ 40 °C**	**PCV depression ≥ 20%**	**Treatment required**
Vaccine only	1	6	6	5	2	1	1
Vaccine and diminazene (0.88 mg/kg)	2	5	3	2	0	1[a]	0

B. bovis, Babesia bovis; PCV, packed cell volumes.
[a], Blood smear positive for *Babesia bovis* and coincidental *Anaplasma marginale* relapse infection parasites.

applicable to all farmers, it was decided to use one quarter (0.88 mg/kg) of the recommended dose of diminazene, found effective in controlling the vaccine parasite reaction (Combrink & Troskie 2004). The rationale for using a one quarter dose was that this would help restrict the total dose administered to less than one third, should the weight of the animal be greatly overestimated by 32%. For the sake of uniformity, a commercially obtainable weight measuring tape (Rondo™) was used.

Monitoring reactions to *Babesia* infection

Rectal temperatures, blood smears and packed cell volumes (PCV) of experimentally infected cattle were monitored daily. Antibody titres for *Babesia* were determined in sera collected before, and at 17, 24 and 30 days after vaccination, using the indirect fluorescent antibody test (IFAT) (Gray & De Vos 1981). The IFAT was until recently listed in the World Organization for Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals as being suitable for the diagnosis of disease within a local setting, but has now been largely replaced by the enzyme-linked immunosorbent assay (ELISA) as the test of choice for *Babesia* spp., mainly because of objectivity in interpretation of results. However, the ELISA has not yet been validated for use at the Onderstepoort Veterinary Institute and the IFAT is still used for research and serological surveys in South Africa, where only titres of dilutions 1/80 or higher are considered as positive (Bessenger & Schoeman 1983). The infectivity of the vaccine strain in cattle was determined by demonstrating the *Babesia* parasites in stained blood smears or positive seroconversion following vaccination.

Molecular genotyping of the *Babesia bovis* vaccine strain

Total genomic DNA was extracted from 200 µL of whole blood collected in ethylene-diaminetetraacetic acid (EDTA) tubes using automated MagNAPure protocols according to established diagnostic procedures and eluted in 100 µL of elution buffer (Mans *et al*. 2011). Genotypic analysis of *B. bovis* samples were conducted using the 1Bf (TGTGTTAATGTAACTCAGCCCG) and 2Br (AAAGCCTGTTAGTTGATGGACC) primers for the Bv80 gene as previously described (Lew *et al*. 1997a, 1997b). General polymerase chain reaction (PCR) reaction used 2.5 µL of genomic DNA, 25 µL GreenTaq (Fermentas), 20.5 µL PCR-grade water and 1 µL of each primer at 10 pmoles final concentration. The PCR protocol consisted of denaturation at 95 °C for 2 min, 45 cycles of denaturation at 95 °C (30 s), annealing at 58 °C (30 s) and extension at 72 °C (1.5 min), followed by a final extension at 72 °C (7 min). The amplified products were analysed by electrophoresis on a 1.2% agarose gel using standard conditions. Blood from the *B. bovis* vaccine production stock material (S22) was used as positive control and blood from a bovine kept under quarantined tick-free conditions as well as blood from the *B. bigemina* vaccine production stock material were used as negative controls. The positive control gave a single amplified PCR product of ~700 bp, whilst both negative controls yielded no PCR products. Blood collected from the animals before vaccination tested negative.

Ethical considerations

Ethical approval for the study was obtained from the Animal Ethics Committee of the ARC-OVI (ref. OV14/02/P001). Animal experimental and welfare work was performed by a veterinarian (Dr Graham Carr) registered with the SAVC.

Results

All six animals in group one that only received the vaccine (Table 1) were found *B. bovis* positive on blood smear examination. Five tested ≥ 1/80 positive on IFAT. Two experienced febrile reactions in excess of 40 °C, occurring on days 16 and 17 post vaccination for the one animal and between days 15 to 18 for the other, who also required treatment on day 18 due to an increasing parasitaemia and severe anaemia (PCV 14). Molecular genotyping of both animals experiencing febrile reactions confirmed the presence of the *B. bovis* vaccine strain, whilst no other genotypes were detected.

Three of the five animals in group two that received the vaccine and 0.88 mg/kg diminazene administered on day 12 post inoculation (Table 1) were found *B. bovis* positive on blood smear examination. Two tested ≥ 1/80 positive on IFAT. One experienced a very mild febrile reaction peaking at 39.5 °C on day 18, and *B. bovis* molecular genotyping of this animal indicated only the vaccine parasite being present. One of the blood smear positive animals also had a coincidental *Anaplasma marginale* relapse infection, which left untreated was instrumental in the anaemia (PCV 18) experienced.

The parasite prepatent period average of 15.5 ± 5.9 days obtained for group one (n = 6) was not dissimilar to the 17.3 ± 8.0 days of group two (n = 3).

Trustworthiness

Laboratory testing of samples was performed in compliance with SANAS ISO/IEC 17025:2005 accreditation requirements for a molecular and serology Diagnostic Laboratory (ref. V0017) and DAFF 001/DAFF 002 molecular (PCR) and serology section requirements for an Approved Veterinary Laboratory (ref. DAFF-30).

Discussion

Field vaccination of 30 cows in milk with *B. bovis* resulted in six (20%) fatalities occurring during the expected vaccine reaction period. Blood smear examination at the time revealed the cause as *B. bovis* but it was not confirmed whether it was from the vaccine strain or a field isolate (Nick Fischer pers. comm., February 2011). In another *B. bovis* vaccination attempt by Fischer, blood was then collected from a sick cow, which when tested with PCR showed the only parasite genotype present to be that of the *B. bovis* vaccine. In this study, although only six animals were used, vaccination of the dairy cows resulted in one (17%) of the six animals developing clinical disease that required treatment, which also showed that unmonitored vaccination of dairy cattle in milk is not advisable.

In the present study, six of the 11 cows used tested ≥ 1/40 for *B. bigemina* on IFAT prior to vaccination with *B. bovis* and during the course of the study circulating parasites for *B. bigemina* could be demonstrated microscopically on blood smears in three of these animals as well as in two of the five that tested completely negative. In 2010 Combrink *et al*. found that only 53% field cattle (n = 260) and 58% laboratory cattle (n = 12), which had previously been infected with a *B. bigemina* field isolate, seroconverted (≥ 1/80) after *B. bovis* vaccination, yet *B. bovis* parasites could be demonstrated microscopically in all of the laboratory cattle and when challenged with a *B. bovis* field isolate this group did not show clinical reactions compared with an unvaccinated control group (n = 6). Therefore, considering this effect that *B. bigemina* can have on *B. bovis* vaccine serology and in the present study having found only five of the six animals in group one positive on IFAT, although all were blood smear positive, it becomes clear that the microscopic demonstration of the parasite was the only reliable way of validating exposure of animals to the *B. bovis* vaccine organism.

Conclusion

In this study, then, failing to demonstrate *B. bovis* parasites on blood smears in two of the five cows of group two is indicative of the harmful effect that block treatment can have on the *B. bovis* vaccine organism. Comparing the unsatisfactory results from this study with those of Combrink and Troskie (2004), it is clear that there are more factors that influence successful vaccination than what was then considered by the authors. Probably the most obvious factors to be added is the effect that existing *B. bigemina* infection in an animal has on subsequent infection with *B. bovis* (Legg 1935; Wright *et al*. 1987) and the differences between animal husbandry practised during laboratory and field experimentation. Consequently, it can only be endorsed that block treatment of the live frozen South African cattle babesiosis vaccines reactions is not recommended.

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Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this paper.

Authors' contributions

M.P.C. (ARC-Onderstepoort Veterinary Institute) was the project leader. M.P.C. (ARC-Onderstepoort Veterinary Institute) and G.C. (Howick Veterinary Clinic) were equally responsible for experimental and project design. M.P.C. (ARC-Onderstepoort Veterinary Institute) performed laboratory microscopy and experimental data analysis. G.C. (Howick Veterinary Clinic) performed all animal experimental and welfare work (collection and preparation of samples, monitoring and treatment of animals). B.J.M. (ARC-Onderstepoort Veterinary Institute) performed PCR work. F.M. (ARC-Onderstepoort Veterinary Institute) performed serology diagnostic work. M.P.C. (ARC-Onderstepoort Veterinary Institute) wrote the manuscript and B.J.M. (ARC-Onderstepoort Veterinary Institute) assisted.

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