

IMMUNOGENICITY AND PATHOGENICITY OF THREE SOUTH AFRICAN STRAINS OF *BABESIA BOVIS* IN *BOS INDICUS* CATTLE

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ABSTRACT

DE VOS, A. J., 1978. Immunogenicity and pathogenicity of three South African strains of *Babesia bovis* in *Bos indicus* cattle. *Onderstepoort Journal of Veterinary Research* 45 (2), 119-124 (1978).

A strain of *Babesia bovis*, which has been routinely used in the locally produced babesiosis vaccine for two decades and maintained by needle passage, was of low virulence and therefore safe, but it induced poor protection to challenge with two field isolates. Animals infected with this attenuated strain and subsequently challenged heterologously with a field strain were solidly immune when challenged later with a 2nd field strain. The two field strains, though more virulent, conferred a high degree of immunity to heterologous challenge.

Résumé

IMMUNOGÉNÉICITÉ ET PATHOGÉNÉICITÉ DE 3 SOUCHES SUD-AFRICAINES DE *BABESIA BOVIS* CHEZ LE BOEUF *BOS INDICUS*

Une souche de *Babesia bovis*, qu'on avait couramment employée depuis vingt ans pour la production locale de vaccin anti-babésiose en la maintenant par passage intraveineux, était de faible virulence et donc sans danger, mais n'a offert qu'une mauvaise protection quand on l'a éprouvée avec deux souches sauvages. Des animaux que l'on avait infectés avec cette souche atténuée et subséquentement éprouvés de façon hétérologue avec une souche sauvage ont témoigné une solide immunité lorsqu'on les a éprouvés plus tard avec une 2e souche sauvage. Quoique plus virulentes, ces deux souches sauvages ont conféré un haut degré d'immunité à l'application de l'épreuve hétérologue.

INTRODUCTION

A babesiosis vaccine consisting of pooled blood obtained from cattle harbouring both *Babesia bigemina* and *Babesia bovis* has been issued by this Institute for many years. Until fairly recently the vaccine was prepared from splenectomized carriers which were bled for only 4 weeks after the infection had become patent. The vaccine was produced once a week and had to be administered subcutaneously in 5 ml doses within 4 days of production. This vaccine was not entirely satisfactory, however, and complaints about poor protection after immunization or severe vaccine reactions were received from time to time.

The purpose of this investigation was to compare the protective and pathogenic effects of the original vaccine strain of *B. bovis* with those of two recently isolated field strains. In addition, an attempt was made to determine the minimum number of organisms required to ensure infection following subcutaneous inoculation.

MATERIALS AND METHODS

Animals

Sixty *Bos indicus* animals comprising 32 oxen and 28 heifers 12-15 months old were obtained from a Government experimental farm at Vaalharts, Western Transvaal. The farm is located in an area with an annual rainfall of less than 400 mm, a condition which is considered unsuitable for the establishment of *Boophilus* spp. (Theiler, 1949). The animals were kept under tick-free conditions at this laboratory.

Detection of natural infections

Since no serological tests were available at this laboratory, other methods were employed to detect possible natural infections.

After they had received prednisolone* as immunosuppressant at a dose rate of 0.05 mg/kg for 5 consecutive days, brain biopsies were performed on Day 10 on all the animals by the method described by Johnston & Callow (1963). The smears prepared from the biopsy material were fixed in methanol, stained

with 5% Giemsa stain for 30 min and examined for the presence of *Babesia* parasites in capillaries for a maximum of 30 min.

Thick blood smears (Mahoney & Saal, 1961) and standard thin blood smears were made from all the animals for 21 days from the start of prednisolone medication. Both smears were stained with 5% Giemsa stain for 20 min and the thick smears examined for a maximum of 10 min.

Fifteen animals that were not destined to be used in subsequent studies were splenectomized and thick blood smears prepared from them were examined for the presence of *Babesia* spp. for the next 28 days. In addition, 500 ml of blood was transfused from each animal into an equal number of fully susceptible splenectomized cattle which were likewise examined for the presence of parasites in blood smears for 28 days.

B. bovis strains

Three strains were used in this study:

The "V" strain, which had been used as vaccine strain for 20 years and had been syringe-passaged in susceptible intact and splenectomized cattle in the process, the exact location of its original isolation in South Africa being unknown.

The "F" and "S" strains were recently isolated from experimentally-produced tick-transmitted cases of *B. bovis* infection at this laboratory. The *Boophilus microplus* ticks that transmitted the "F" strain originated from the Eshowe district, Natal, while those transmitting the "S" strain were obtained from the Pretoria district, Transvaal.

All three strains were preserved in the gas phase of a liquid nitrogen refrigerator with DMSO as cryoprotectant. The "V" strain was also maintained by subinoculation of blood and the "F" and "S" strains by passage through *B. microplus* ticks.

Method of infection

To exclude possible variations in virulence between parasites in the blood of acutely infected animals and those of carriers (Callow & Tammemagi, 1967), only the former was used for inoculation purposes. The

* Deltacortil (Pfizer)

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number of parasites/ml of infected blood was calculated from the percentage of infected erythrocytes, as determined from thin smears made from jugular blood, and the red cell count obtained with the aid of a Coulter Counter*.

The parasitaemia was adjusted to the required number of infected erythrocytes per 2.5 ml aliquot by the addition of known non-infected blood, a 10% sodium citrate solution being used as anticoagulant. All injections were made by the subcutaneous route.

Experimental design

Three successive experiments were conducted.

Experiment 1 was designed to determine the minimum infective dose for *B. bovis* when administered subcutaneously. It also provided an opportunity to study the pathogenicity of the "V" strain.

The 35 head of cattle used in this experiment were divided into 7 groups, each consisting of 5 animals. The animals in Groups 1-5 were inoculated with 2.5 ml volumes of ten-fold dilutions of infected blood, starting with 1×10^7 parasitized cells, those in Group 6 were inoculated with the same volume of uninfected, diluent blood only, and those in Group 7 served as uninoculated controls.

Brain biopsies were performed on all the animals 60 days post-inoculation and the brain smear findings were compared with those of blood smears.

Experiment 2 was designed to determine the degree of immunity of animals infected with the "V" strain to subsequent challenge with the "F" strain. Observations were also made on the pathogenicity of the "F" strain.

Three groups of cattle were selected, namely, Group 1, consisting of 10 animals which had been infected 60 days previously with the "V" strain in Experiment 1, Group 2 of 5 previously uninfected animals, and Group 3 of 5 uninoculated controls. Animals in the Groups 1 and 2 were each inoculated subcutaneously with approximately 1×10^7 parasitized cells of the "F" strain.

Experiment 3 was primarily designed to compare the degree of immunity that developed after primary infections with either the "V" or the "F" strains to challenge with the "S" strain. In addition, the degree of immunity to challenge with the "S" strain was studied in animals previously infected with both the "V" and "F" strains in sequence. Observations were also made on the pathogenicity of the "S" strain.

Five groups of 5 animals each were used in this experiment. Group 1 consisted of animals which had been infected 122 days previously with the "V" strain in Experiment 1, Group 2 of animals which had been infected 62 days previously with the "F" strain in Experiment 2, Group 3 of animals which had been infected 122 days previously with the "V" strain and challenged 60 days later with the "F" strain in Experiment 2, Group 4 of previously uninfected animals, and Group 5 of animals which served as uninoculated controls.

All the cattle in Groups 1-4 were inoculated subcutaneously with 2.5 ml blood containing approximately 1×10^7 parasitized cells of the "S" strain. Brain smears prepared from brain biopsies performed on all the animals prior to inoculation were examined for the presence of *B. bovis*.

Quantification of reactions

Parasitaemia

Thick smears were examined as described by Mahoney & Saal (1961) but, to simplify examination, this technique was only used to calculate parasitaemias below 0.05%. In heavier infections the number of parasitized cells per 500 erythrocytes was established in thin smears and expressed as a percentage. Parasitaemias were classified according to the following scale:

1. <0.0005%
2. 0.0005-0.005%
3. 0.005-0.05%
4. 0.05-0.2%
5. 0.2-1%
6. >1%

Febrile reactions

The rectal temperatures of all the animals were recorded daily between 08h00 and 10h00 throughout the period of observation. The average daily temperature of the uninoculated control group served as the average normal temperature for that day. The cumulative temperature rise in the other groups was determined as described by Callow & Pepper (1974), that is, by adding all values of 0.3 °C and greater above the normal.

Packed cell volume (PCV)

PCV determinations were done in duplicate three times a week, using Clay Adams microhaematocrit tubes and a Christ Microfuge. After the animals had been drafted into the experiments, their PCV's were allowed to stabilize (Gartner, Callow, Grazien & Pepper, 1969) for 2 weeks prior to inoculation. The average of the readings during the pre-patent period was taken as the norm for a particular animal. The maximum PCV depression was expressed as a percentage of this norm and the mean for each group determined from these figures.

The Students "t" test was used to determine the significance of differences between means of different sets of figures obtained during this study.

RESULTS

Susceptibility of experimental animals

No *B. bovis* parasites were observed in the thick blood smears of any of the animals, even of the 15 that were splenectomized. Brain biopsies are considered to be more reliable than blood smears for the detection of latent *B. argentina* infections (Callow & Johnston, 1963), and unpublished observations indicate that this also applies to *B. bovis* infections, but no parasites were seen in the brain smears of these animals. Blood transfusions from the splenectomized animals likewise failed to reveal the presence of *B. bovis* and it was therefore assumed that these animals were fully susceptible to this organism.

Effect of tenfold dilutions of *B. bovis* infected blood on infectivity and pathogenicity

The infectivity of various concentrations of *B. bovis* infected blood administered by the subcutaneous route is summarized in Table 1. Although as few as 1×10^3 infected cells were capable of establishing infections in some of the animals, it is evident that 1×10^6 parasitized cells are necessary to ensure that infection occurs. There was a tendency for lower parasite numbers to result in longer pre-patent periods, but this feature was not statistically significant ($P < 0.05$).

* Coulter Electronics Inc.

TABLE 1 Infectivity of tenfold dilutions of *B. bovis* infected erythrocytes inoculated subcutaneously ("V" strain)

Inoculum size	No. of cattle/ group	No. of reactions	Pre-patent period (days)		Brain biopsies	
			Range	Mean	Positive before infection	Positive after infection
1×10^7	5	5	7-13	10	0	5
1×10^6	5	5	10-18	14	0	5
1×10^5	5	3	11-20	16	0	3
1×10^4	5	3	12-18	15	0	3
1×10^3	5	2	8-28	18	0	2
0	5	0	—	—	0	0

TABLE 2 Effect of differing inoculum sizes on the pathogenicity of *B. bovis* ("V" strain)

Inoculum size	No. of cattle/ group	Reaction			
		Mean patent period (days)	Mean maximum parasitaemia	Mean maximum PCV depression	Mean total temperature rise ($^{\circ}$ C)
1×10^7	5	8	2,6 ⁽¹⁾	10 ⁽²⁾	2,78
1×10^6	5	8	2,4	10	2,12
1×10^5	3	7	2	8	2,5
1×10^4	3	9	3	11	2,7
1×10^3	2	6	3	6	2,2

⁽¹⁾ Based on score ranging from 1-6⁽²⁾ Depression given as percentage of normalTABLE 3 Primary reactions of three strains of *B. bovis* in *Bos indicus* cattle

Strain	Inoculum size	No. of cattle/ group	Reaction			
			Mean patent period (days \pm S.D.)	Mean maximum parasitaemia	Mean maximum PCV depression	Mean total temperature rise ($^{\circ}$ C)
V	1×10^6 - 1×10^7	10	7 \pm 2,2	2,5 ⁽¹⁾ \pm 1,41	10 ⁽²⁾ \pm 8,4	2,45 \pm 2,72
F	1×10^7	5	11 \pm 3,2	5,8 \pm 1,64	34 \pm 12,6	8,76 \pm 1,65
S	1×10^7	5	18 \pm 4,1	5,8 \pm 1,14	27 \pm 6,6	7,80 \pm 1,09

⁽¹⁾ Based on score ranging from 1-6⁽²⁾ Depression given as percentage of normalTABLE 4 Primary and challenge reactions of *B. bovis* in susceptible and immunized *Bos indicus* cattle

Primary infection	Interval after initial infection (days)	1st heterolo- gous challenge	Interval after 1st challenge (days)	2nd heterolo- gous challenge	No. in group	Reaction			
						Mean patent period (days \pm S.D.)	Mean maximum parasitaemia	Mean maximum PCV depression	Mean total temperature rise ($^{\circ}$ C)
F	—	—	—	—	10	11 \pm 3,24	5,8 ⁽¹⁾ \pm 1,64	34 ⁽²⁾ \pm 12,62	8,76 \pm 1,65
V	60	F	—	—	5	9 \pm 1,84	4,5 \pm 2,17	26 \pm 11,2	6,66 \pm 2,86
S	—	—	—	—	5	18 \pm 4,09	5,8 \pm 1,14	27 \pm 6,58	7,80 \pm 1,09
V	122	S	—	—	5	17 \pm 4,76	6,6 \pm 0,55	26 \pm 5,3	7,43 \pm 2,32
F	62	S	—	—	5	16 \pm 3,97	2,2 \pm 1,30	12 \pm 3,5	2,64 \pm 1,00
V	60	F	62	S	5	8 \pm 4,02	1,0 \pm 0	12 \pm 2,6	1,80 \pm 1,46

⁽¹⁾ Based on score ranging from 1-6⁽²⁾ Depression given as percentage of normal

Owing to the mild nature of the primary reactions caused by the "V" strain (Table 2), it was not possible accurately to determine the relationship between the number of parasites inoculated and the severity of the ensuing reaction. The small numbers of animals reacting to the higher dilutions of infected cells also precluded proper comparison. It is therefore not surprising that no significant ($P < 0.05$) differences could be detected between the values obtained in any of the criteria of pathogenicity studied, including patent period, maximum parasitaemia, percentage PCV drop and cumulative temperature rise (Table 2).

Pathogenicity of 3 different strains of B. bovis

The observations made on the criteria used to evaluate pathogenicity, namely, patent period, maximum parasitaemia, PCV depression and cumulative temperature rise, are summarized in Table 3. In addition, the mean parasitaemia of each of the 3 groups is plotted in Fig. 1. It is clear that the highly passed "V" strain consistently produced very mild reactions, but, though the "F" and "S" field isolates were markedly more pathogenic, none of the animals died.

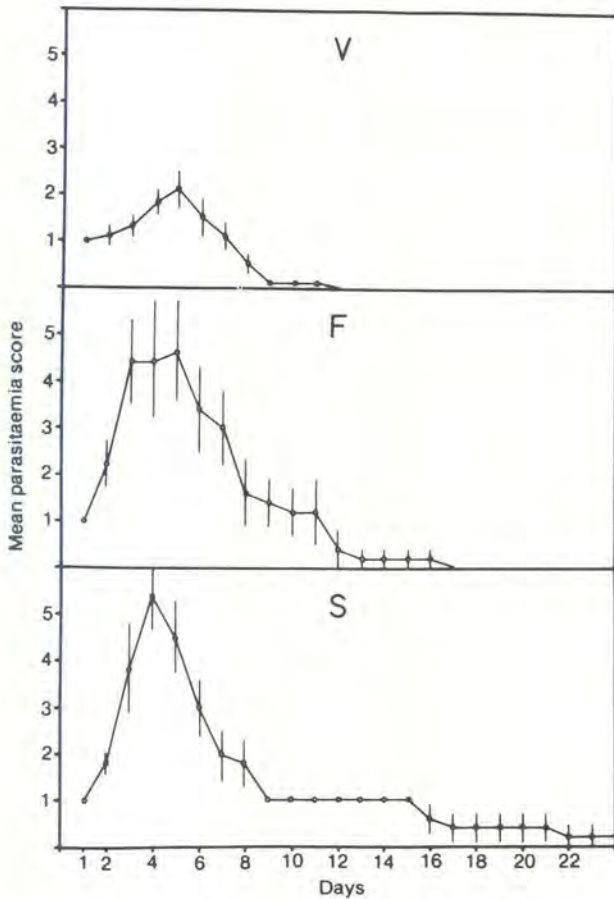


FIG. 1 Primary reactions of three strains of *B. bovis* in cattle starting on the day when parasites are first detectable in the blood smears. Vertical bars represent the standard deviation

If the reactions caused by the attenuated "V" strain are compared statistically with those of either of the field isolates, highly significant ($P < 0.01$) differences emerge with regard to all the criteria studied. The only significant ($P < 0.05$) difference between the "F" and "S" strains, however, lay in the patent periods.

Cross-immunity between three different strains of B. bovis (Experiment 3)

The results of this experiment are summarized in Table 4. The relevant data concerning the primary reactions of the 2 strains ("F" and "S") used for the challenges are included in the Table for comparative purposes. In addition, plots of the mean parasitaemias recorded during these challenges are given in Fig. 2.

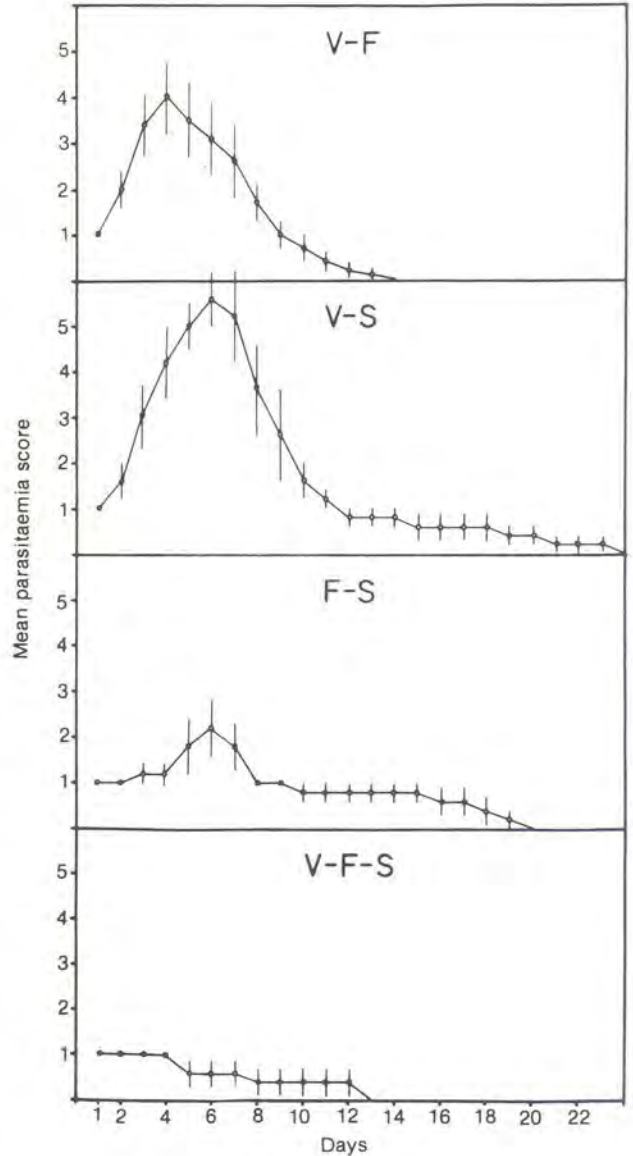


FIG. 2 A comparison of four challenge reactions

The reactions of animals challenged with the pathogenic "F" strain 60 days after inoculation with the less virulent "V" strain were as severe as the reactions observed in cattle that received the "F" strain only. The same applied when the "S" strain was used to challenge cattle inoculated with the "V" strain 122 days previously. It is evident therefore that the highly passed "V" strain afforded little or no protection against infection with the 2 field strains, in terms of the parameters used to measure pathogenicity.

Cattle primarily infected with the "F" strain, however, showed a noticeable resistance when challenged 62 days later with the equally virulent "S" strain. If the reactions to challenge are compared with

the primary reaction to the "S" strain, in terms of the parameters measured, the differences are highly ($P < 0.01$) significant, the exception being the patent period.

The animals that were infected primarily with the "V" strain and challenged 60 days later with the "F" strain, were solidly immune to challenge 62 days later with the "S" strain (Table 4). A comparison of the latter reactions with those obtained in cattle challenged with the "S" strain after immunization with the "F" strain revealed that the differences in the patent period ($P < 0.05$) and maximum parasitaemia ($P < 0.1$) were significant, but not those in PCV depression and cumulative temperature rise. Even though infection with the "V" strain conferred little protection to challenge with the "S" strain (see above), these results indicate that infection with the "V" and "F" strains resulted in the development of a higher level of immunity than that induced by the "F" strain alone.

DISCUSSION

Since there is little information in the literature on immunological and other aspects of *B. bovis* infections, reference to the reports on related species is necessary for the purpose of this discussion. Goldman & Rosenberg (1974) demonstrated a close serological relationship between *B. bovis* and *B. argentina* and also between these 2 species and *B. berbera*. More recently, basing their findings on fluorescent antibody studies, Callow, Quiroga & McCosker (1976), concluded that *B. argentina* is synonymous with *B. bovis*. The relevant information on *B. argentina* and *B. berbera* will therefore be compared with the results obtained in this study.

The finding that 1×10^6 infected erythrocytes are necessary to ensure infection after subcutaneous inoculation corresponds with observations made by Callow & Mellors (1966) on *B. argentina*. Mahoney (1973) has found that, when *B. argentina* is inoculated intravenously, very few parasites (< 10) are necessary to infect an animal. From the point of view of vaccine administration, the intravenous route is, however, unpractical. A babesiosis vaccine must therefore attain a level of at least 1×10^6 parasites per dose at the time of inoculation. To reach this level in a dose of vaccine consisting of 4 ml blood and 1 ml of an anticoagulant solution, a parasitaemia of approximately 0.005% in the donor cattle is required. This level is considerably higher than that encountered in the carrier animals with subpatent infections that were used in the past at this Institute for vaccine production purposes. Moreover, if allowance is made for the degeneration of parasites between the time of collection of the blood and inoculation, it is evident that poor infectivity was probably the main reason for so-called breakdowns in immunity after vaccination. This finding agrees with observations made by Callow & Tammemagi (1967) on *B. argentina*.

No attempts were made in this study to demonstrate the degree of immunity to challenge with homologous strains. Unpublished observations (De Vos, 1973) on immunity to the "V" strain, however, indicate that animals challenged with the same strain were solidly immune, irrespective of whether a latent infection was present or not, or whether the animals were intact or had been splenectomized. This observation corresponds with earlier reports on various other *Babesia* spp., as reviewed by Mahoney (1972). Brain biopsies

performed immediately prior to the heterologous challenges revealed the presence of a latent infection in all the animals. The possible influence of quantitative or qualitative differences between sterile and co-infectious immunity to heterologous challenge is therefore excluded.

This study confirms the presence of immunologically distinct strains of *B. bovis*. The poor protection conferred by the highly passaged, relatively avirulent "V" strain to challenge with heterologous field strains is of particular interest. It is known that artificial passage of *B. argentina* will result in a reduction in virulence (Callow & Mellors, 1966), but little is apparently known of the effect of passage on immunogenicity. Rogers (1971) observed reactions of almost the same severity as in the controls in cattle vaccinated with a passaged strain of *B. argentina* and subsequently challenged with a heterologous, low passage strain. Using the same vaccinating strain and challenging the animals heterologously with a strain maintained in ticks, Johnston & Tammemagi (1969), however, observed only a parasitaemia without accompanying clinical signs. In contrast to this, Callow (1968) recorded clinical reactions in some animals that were vaccinated against *B. argentina* and subsequently challenged artificially or naturally with a heterologous strain.

Todorovic, Lopez, Lopez & Gonzalez (1975) infected animals on several ranches in Colombia with a low passaged strain of *B. argentina* and found that they had a high level of immunity when exposed to natural challenges. Similar findings were made in this study using 2 field isolates from geographically different localities.

The evidence obtained in this study that vaccination with 2 heterologous strains resulted in a more solid immunity than that imparted by only 1 strain, corresponds with Australian observations on *B. argentina* (Emmerson, Knott & Callow, 1976).

Despite the fact that they had never been exposed to *B. bovis*, the cattle used in these experiments showed a moderate degree of resistance to infection, a feature which was well-illustrated by the primary reactions to the 2 field isolates. This may possibly be due to the fact that all the animals were *B. indicus*. Johnston (1967) observed that Zebu crosses in Australia were relatively more resistant to *B. argentina* parasitaemia than Hereford cattle. Both *B. bovis* field strains used in the present study are, however, known to be capable of producing severe or even fatal infections in *Bos indicus* cattle (De Vos, unpublished observations, 1973).

The reduced virulence recorded for the "V" strain that had been needle-passaged at infrequent intervals for many years, in comparison to the more virulent field strains, agrees with the observation of a decrease in the virulence of strains of *B. argentina* passaged rapidly through splenectomized calves (Callow & Mellors, 1966).

The number of reacting animals observed in the titration experiment was too small accurately to assess the effect of dosage on the degree of reaction. In addition, the strain used was of low virulence. No evidence of a direct relationship between the number of parasites inoculated and the degree of response was seen, however, as has been reported for *B. argentina* (Rogers, 1971) and *B. bigemina* (Kemron, Hadani, Egyed, Pipano & Neuman, 1964).

Though Rogers (1971), Gonzalez, Todorovic & Thompson (1976) and Pipano (1969) reported that the pre-patent and incubation periods are inversely related to the number of *B. argentina* and *B. berbera* parasites inoculated, no such relationship was observed in this study. This may be due, however, to the relatively small number of parasites used, as a relationship was seen when larger numbers (1×10^9 – 1×10^{12}) of the same ("V") strain of *B. bovis* were injected (De Vos, unpublished observations, 1973). The use of the pre-patent period as a measure of the number of viable organisms in a specific volume of blood at various intervals after collection has considerable application in the production of a vaccine against babesiosis. More work on this aspect is therefore indicated, especially when small numbers of viable organisms are involved.

As a result of this study a number of changes have been made to the Onderstepoort redwater (babesiosis) vaccine. Blood of acutely infected animals only is now being used for the production of the vaccine, with the number of infected erythrocytes standardized at approximately 1×10^7 per dose, that is, 10 times the minimum dose required to ensure infection when inoculated immediately. The volume of a dose of vaccine has also been reduced from 5 ml to 2 ml to reduce the risk of iso-immune haemolytic disease (Dimmock, 1973; Dimmock & Bell, 1970). Finally, the less passaged "F" strain of *B. bovis* is being used, which, although more pathogenic than the previous one, induces a higher level of immunity to heterologous challenge. Despite evidence of the development of a more satisfactory immunity after immunization with 2 strains administered consecutively, a single vaccination is advocated at present.

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