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Study of Virulence and Vector Transmission of *Babesia bovis* by Use of Cloned Parasite Lines

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Cloned lines of *Babesia bovis* were prepared from the avirulent vaccine strain, Ka, by an in vivo limiting dilution procedure. The virulence of these clones for adult *Bos taurus* cattle varied from completely avirulent to highly virulent. This suggests that the parent strain, Ka, is composed of a mixture of subpopulations of varied virulence. Passage of the avirulent clone K-19-47 in intact (nonsplenectomized) cattle resulted in its full reversion to virulence. This suggests that two mechanisms are operating to enable virulence to be a readily modified characteristic in this parasite: differential gene expression and phenotypic selection of subpopulations. A series of experiments demonstrated that all clones were non-tick transmissible. This lack of vector transmission was a stable characteristic and could not be altered by the passage of K-19-47 in intact cattle, despite the fact that passage in intact animals caused this clone to revert to the fully virulent phenotype. A mechanism is suggested for the virulence and vector transmission variations observed in *B. bovis*.

Babesia bovis is an intraerythrocytic protozoan parasite of cattle which causes babesiosis, a disease of economic importance in parts of the world where the tick vector, *Boophilus microplus*, occurs. Recent attempts to produce nonliving *B. bovis* vaccines (8, 9, 15, 17) have not succeeded because the immunity produced by these vaccines is unsatisfactory, at least by comparison with the immunity provided by attenuated live vaccines (15). While attenuated live vaccines provide solid immunity, they do have other potential problems including (i) the potential of vaccine strains to revert to virulence; (ii) a relatively short shelf life; (iii) the possibility of contamination with other blood parasites, bacteria, and viruses. The potential reversion to virulence is the most significant problem in many vaccination programs.

Callow et al. (1) described the attenuation of *B. bovis* by serial blood passage in splenectomized calves. The vaccine strain currently used in Australia, Ka, is produced by this mechanism and is found to be very effective. These same investigators also observed the ability of calf-attenuated vaccine strains of *B. bovis* to revert to full virulence after blood passage in intact cattle (1). They commented that "the fact that attenuation can be reversed by returning a strain to passage in intact cattle has not proved important in practical vaccination." Kahl et al. (6) confirmed the reversion to virulence of the Ka strain after passage in intact animals and ticks and used protein and antigen profiles to show the similarity of strains which had reverted to virulence and the original virulent K strain. However, these investigators were unable to determine whether these changes were due to differential gene expression within a population and/or selection of subpopulations from the mixed parent strain.

Recently, several groups have produced cloned lines of *B. bovis* parasites with the aim of gaining a better understanding of the virulence and immunogenicity mechanisms in this parasite. Rodriguez et al. (12) used an in vitro cloning procedure and compared their clones with regard to in vitro characteristics such as parasite growth rates. By comparison, Gill et al. (5) used an in vivo cloning procedure and were able to analyze their clones with a range of in vitro and in vivo tests. Both groups suggested that the isolation of avirulent clones and subsequent growth of homogeneous populations has application in developing safer live *B. bovis* vaccines.

This report describes the production of a series of clones from Ka strain *B. bovis* and the analysis of these clones with regard to virulence for adult cattle and their ability to be vector transmitted. The potential of an avirulent clone to revert to virulence on passage in intact animals is also examined. Finally, a mechanism for virulence and vector transmission variations in *B. bovis* is suggested.

MATERIALS AND METHODS

Animals. *Bos taurus* steers were obtained from a *B. microplus*-free area of Queensland and were 15 to 18 months of age at the start of the experiment. They were maintained under conditions which precluded infestation with ticks. Before use, the steers were allotted to groups of seven on the basis of body weight. Calves (4 to 8 weeks of age) were used in preparing infected blood. The calves originated from the same area as the steers and were housed in pens with concrete floors.

***B. bovis* strains and clones.** The attenuated Australian vaccine strain of *B. bovis*, Ka, was used in all aspects of the study. The K strain was originally isolated from a clinically normal cow in southeastern Queensland and was subsequently attenuated by 20 to 30 consecutive syringe passages in splenectomized calves (1).

A total of 14 cloned lines were produced from the Ka strain by using a limiting dilution procedure in splenectomized calves as follows. Jugular blood was collected from a splenectomized calf infection with Ka, and the parasite

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concentration was determined by the methylene blue staining method of Parker (11). Uninfected bovine blood was used to dilute the *B. bovis*-parasitized blood to one parasite per milliliter. A series of 10 4- to 8-week-old splenectomized calves were then inoculated intravenously with 1 ml of this diluted, Ka-infected blood and screened over the next 28 days for the presence of *B. bovis* parasites in Giemsa-stained films of tailtip blood. When parasites were detected, blood was collected into heparin and held at 4°C for up to 7 days, or mixed with dimethyl sulfoxide and frozen at −196°C for long-term preservation of the cloned lines.

These primary clones were designated K-*n*, where *n* is the number of the relevant splenectomized calf. Primary clones K-19 and K-13 were recloned by the same procedure to produce five secondary clones, designated K-*n*-*n*. Secondary clones K-13-60 and K-13-61 were again recloned to produce three tertiary clones, designated K-*n*-*n*-*n*. The molecular and biological characteristics of two of the clones used in this study (K-14 and K-13-68) have previously been described (5).

Virulence of clones compared with Ka strain. The virulence of the Ka strain, one primary clone (K-14), two secondary clones (K-13-68, K-19-47), and two tertiary clones (K-13-60-29, K-13-61-33) was assessed by inoculating groups of seven intact *B. taurus* steers subcutaneously with 10^7 parasites per animal. To prepare infective blood for the inoculations, we inoculated splenectomized calves intravenously with 10-ml lots of the respective liquid nitrogen stabilate. Infected blood was collected into heparin at the peak of the reaction by the animal, the parasite concentration was determined (11), and the blood was then diluted to give 10^7 parasites per 2-ml dose. This diluted blood was then used immediately to inoculate groups of steers.

To assess the severity of the response of the steers to inoculation, we measured parasitemia (percentage of erythrocytes infected), depression of packed cell volume (PCV), and rise in rectal temperature daily (2) (from day 2 postinoculation to clinical recovery).

Effect of blood passage in intact cattle on virulence of Ka strain and clone K-19-47. Callow et al. (1) showed that avirulent strains of *B. bovis* remain avirulent after blood passage in splenectomized calves but are restored to full virulence after only one to five rapid blood passages in intact cattle. The present experiment was designed to confirm this observation with Ka strain *B. bovis* and to determine whether avirulent cloned parasite lines would also revert to virulence after passage in intact cattle.

Two splenectomized calves were infected with stabilates of either *B. bovis* Ka or clone K-19-47. When the parasitemia exceeded 1%, blood was collected into heparin and the parasite concentration was determined (11). Diluted samples of these infected bloods were used to intravenously inoculate two intact *B. taurus* steers with 10^7 parasites per animal. Both animals experienced mild reactions, with peripheral blood parasitemias peaking at 0.06 to 0.3% erythrocytes infected. Blood from these animals was collected into heparin, the parasite concentration was estimated, and 2- to 10-ml samples (equivalent to 10^7 parasites) were used to intravenously inoculate two groups of seven intact *B. taurus* steers. A flow diagram of the passage details is given in Fig. 1.

The severity of the reactions was measured daily as described above. Cattle were treated with 1 ml of amicarbalide isethionate (Diampron) per 50 kg of body weight if any of the following criteria were met: (i) PCV less than 12%; (ii) total temperature rise of more than 7.5°C over three consecutive days; (iii) parasitemia of more than 2% erythrocytes infected (15).

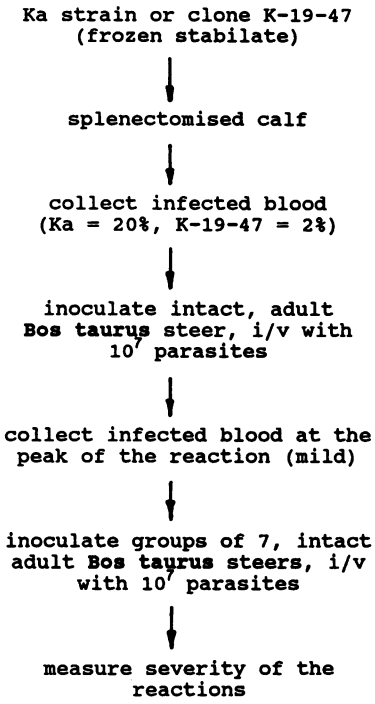

FIG. 1. Procedure used to determine the effect of blood passage in intact cattle on the virulence of *B. bovis* Ka and clone K-19-47. i/v, Intravenous.

Effect of tick passage on virulence of Ka strain. Clone K-19-47 could not be tested as it proved to be non-tick transmissible (see below). Blood infected with the Ka strain was prepared in a splenectomized calf (as described above), and 10 ml of this *B. bovis*-infected blood was used to infect a second splenectomized calf on which uninfected *B. microplus* ticks were feeding. The second calf was inoculated by the intravenous route 16 days after the initial release of *B. microplus* larvae. When this calf reacted with a peripheral blood parasitemia of 0.1%, fully engorged female ticks were collected and cultured at 30°C and 90 to 100% relative humidity. A 2- to 4-g portion of the resultant viable larval progeny was released onto each of seven intact *B. taurus* steers. A flow diagram of the passage details is given in Fig. 2. The severity of the reactions was measured daily as described above.

Tick transmissibility of *B. bovis* clones. The ability of the tick vector *B. microplus* to transmit two primary, four secondary, and two tertiary clones was assessed by the method of Dalgliesh and Stewart (3).

Briefly, 2 to 4 g of uninfected *B. microplus* larval ticks was released onto clean splenectomized *B. taurus* calves housed in moated pens. Eighteen days later, individual calves were intravenously inoculated with blood parasitized with *B. bovis* Ka or clones K-14, K-19, K-13-60, K-13-68, K-19-47, K-19-49, K-13-60-29, and K-13-61-33, so that peak *B. bovis* blood parasitemias occurred at the time of tick engorgement. Adult female ticks that dropped from the calves during this period were collected and cultured at 30°C and 90 to 100% relative humidity. A 2- to 8-g portion of the resultant viable larval progeny was released onto susceptible splenectomized calves. Thick blood films were collected between days 7 and

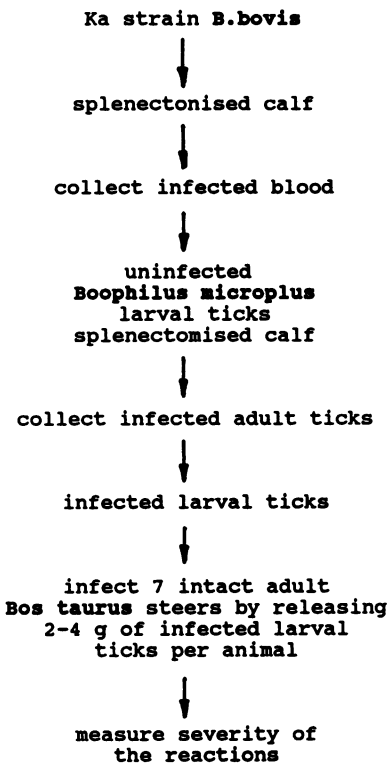

FIG. 2. Procedure used to assess the effect of tick passage on the virulence of *B. bovis* Ka.

28, Giemsa stained, and examined microscopically for *B. bovis*.

As a consequence of the negative results obtained above, two further attempts were made to tick transmit *B. bovis* clones. A four-clone mixture consisting of K-14, K-19-49, K-13-60-29, and K-13-61-33 was tested by the procedures outlined above. Parasitized blood from clone K-19-47 after blood passage in intact cattle (that is, after reversion of K-19-47 to virulence) was also tested by similar procedures.

Statistical analysis. Significant differences between groups for the parameters measured after virulence comparisons were determined by analysis of variance.

RESULTS

The preparation of the clones and their relationship to each other and to Ka are shown in Fig. 3.

Virulence of clones compared with Ka strain. The Ka strain is well recorded as being avirulent for adult cattle, and this was confirmed in this work by the fact that all cattle inoculated with Ka had very mild reactions (Table 1). The clones could be divided into three virulence categories based on the severity of their reactions. Clones K-14 and K-19-47 were avirulent and had responses which were very similar to those from Ka. Clones K-13-60-29 and K-13-61-33 could be described as moderately avirulent. While the maximum parasitemia score of these clones (0.9) was not significantly different from that of Ka (1.1), both those tertiary clones did give a significantly higher maximum temperature rise (1.8 and 1.7°C, respectively) than with Ka (1.1°C). Clone K-13-60-29 also induced a significantly larger PCV depression (20.9%) than did Ka (17.6%). Clone K-13-68 was the only fully virulent clone with all parameters being significantly higher than for Ka.

TABLE 1. Virulence of *B. bovis* Ka and its derived clones measured in groups of intact *B. taurus* cattle ($n = 7$)

Strain or clone	Parameter[a]		
	Maximum parasitemia (score)[b]	Maximum temp rise (°C)	Maximum PCV depression (%)[c]
Ka	1.1[d]	1.1[d]	17.6[d]
K-14	1.0[d]	1.4[d]	16.0[d]
K-13-68	4.9[e]	2.2[e]	30.6[e]
K-19-47	0.9[d]	1.2[d]	14.6[d]
K-13-60-29	0.9[d]	1.8[e]	20.9[d]
K-13-61-33	0.9[d]	1.7[e]	14.5[d]

[a] All values given in the table are mean maximum values for groups of seven animals per strain or clone category.
[b] Parasitemia score according to Callow and Pepper (2).
[c] Maximum PCV depression is the percent depression below the normal PCV value of the animal.
[d,e] Values with different superscripts within vertical columns are significantly different ($P < 0.01$).

Effect of blood passage in intact cattle on virulence of Ka strain and clone K-19-47. A single blood passage of either Ka strain or clone K-19-47 in intact cattle caused full reversion to virulence (Table 2). All parameters measured were significantly higher ($P < 0.01$) after passage in intact animals. The reversion to virulence was just as pronounced in clone K-19-47 as it was in the Ka strain. The virulence of both modified lines is evident from the fact that 11 of 14 animals inoculated with the modified lines required treatment, whereas none of 14 animals inoculated with the lines before the single passage in intact cattle required treatment.

Effect of tick passage on virulence of Ka strain. The passage of strain Ka through *B. microplus* ticks, using splenectomized calves, resulted in a full reversion to virulence similar to that obtained by blood passage in intact cattle (Table 2).

TABLE 2. Virulence of *B. bovis* Ka and clone K-19-47 before and after one blood passage in intact *B. taurus* cattle or Ka after one tick passage on splenectomized calves[a]

Strain or clone	Maximum parasitemia (score)[b]	Maximum temp rise (°C)	Maximum PCV depression (%)[c]	No. of animals treated
Ka				
Before passage	1.1[d]	1.1[d]	17.6[d]	0/7
After one intact blood passage	10.0[e]	2.5[e]	43.7[e]	5/7
After one tick passage on splenectomized calf	10.0[e]	3.1[e]	54.6[e]	7/7
K-19-47				
Before passage	0.9[d]	1.2[d]	14.6[d]	0/7
After one intact blood passage	13.6[e]	3.1[e]	49.0[e]	6/7

[a] All values are mean maximum values for groups of seven animals per strain or clone category.
[b] Parasitemia score according to Callow and Pepper (2).
[c] Maximum PCV depression is the percent depression below the normal PCV value of the animal.
[d,e] Values with different superscripts in vertical columns are significantly different ($P < 0.01$).

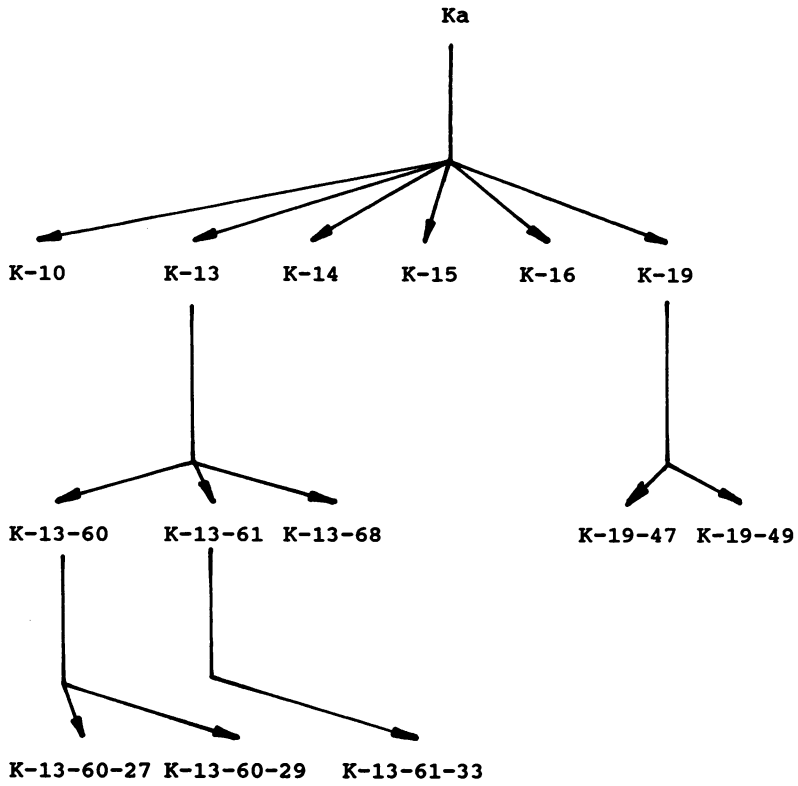

FIG. 3. Production of primary (K-*n*), secondary (K-*n*-*n*), and tertiary (K-*n*-*n*-*n*) clones from *B. bovis* Ka.

All seven recipients of the tick-passaged Ka strain required treatment to prevent death, and the other parameters measured were also significantly higher ($P < 0.01$) than for Ka strain *B. bovis* before tick passage.

Tick transmissibility of *B. bovis* clones. Eight different clones (K-14, K-19, K-13-60, K-13-68, K-19-47, K-19-49, K-13-60-29, K-13-61-33) tested a total of 15 times in five separate experiments failed to be transmitted by *B. microplus* ticks. By comparison, the parent Ka strain is reliably tick transmissible and was positively transmitted on both occasions that it was tested in this work. The four-clone mixture, as well as clone K-19-47 after passage in intact animals, also failed to be transmitted by *B. microplus* ticks.

DISCUSSION

The in vivo limiting dilution cloning technique used in this work resulted in a series of clones which differed widely in their virulence characteristics. Of the five clones tested, two were avirulent, two were moderately avirulent, and one was fully virulent. This confirms the previous results of Gill et al. (5), who showed that the Australian vaccine strain of *B. bovis* contained virulent as well as avirulent subpopulations. The present study used two tertiary clones, both of which were moderately avirulent. Interestingly, K-13-60-29, moderately avirulent in this study, was derived from the avirulent clone K-13-60 used by Gill et al. (5). It is not possible from the present results to say whether continued cloning selects for more virulent lines or whether K-13-60 was actually a mixture of both avirulent and moderately avirulent lines which were separated at tertiary cloning.

Whatever the mechanism of selection, the present results confirm that *B. bovis* strains contain mixtures of genetically diverse organisms. *B. bovis* clones have been shown by others to vary in such characteristics as pathogenicity, DNA hybridization pattern, protein and antigen composition, vector transmission (5), and growth rate in vitro (12). Similar results have also been found for *Plasmodium* species, from which clones with various pathogenicities (7), antigenicities (4), drug resistances (16), isoenzyme patterns (13), and morphologies (16) have been isolated. The genetic heterogeneity that exists in both these hemoprotozoa illustrates a need for these parasites to respond rapidly to the changing environment to ensure their continued survival.

Even though the eight *B. bovis* clones tested in this study exhibited variable pathogenicity, none were tick transmissible. This is in direct contrast to the parent strain, Ka, which is readily tick transmissible. The fact that a four-clone mixture was also not transmissible suggests that the clones were not of separate sexual types, with both types being required for sexual recombination for successful vector transmission. It seems more likely that the clones tested represent the nontransmissible subpopulations of the parent strain. The gene product required for vector transmission (possibly an enzyme necessary to penetrate the gut epithelial cells of the tick) has been permanently lost in these clones. The permanency of this loss of vector transmissibility is suggested by the fact that blood passage of K-19-47 in intact cattle caused a reversion to virulence but did not restore tick transmissibility. This is further supported by the work of Dalgliesh and Steward (3), who showed that *B. bovis* strains made nontransmissible by long-term blood passage in splenectomized calves failed to regain infectivity for ticks during long-standing infections in cattle. These researchers sug-

gested that repeated blood passaging had eliminated the tick-infective component of the parasite population.

If it is assumed that *B. bovis* field strains contain both tick-transmissible and nontransmissible subpopulations, then the transmissible parasites must be capable of supporting the transmission of the nontransmissible parasites by providing some factor that is required for transmission. Stewart (14) showed that *B. bovis* strains made nontransmissible by long-term blood passage were unable to penetrate the tick gut epithelial cells, thereby preventing transmission. It seems feasible that the transmissible parasites might secrete an enzyme in the tick gut which enables both themselves and accompanying nontransmissible parasites to penetrate the gut epithelium and continue the cycle. Why only nontransmissible parasites were cloned in this work is uncertain. Perhaps they exist as a major subpopulation in the Ka strain, since this strain has already been blood passaged 20 to 30 times in splenectomized calves, thereby beginning the nontransmissible selection process described by O'Sullivan and Callow (10). It is also possible that for some unknown reason the non-tick transmissible subpopulation is more fit to survive and initiate an infection from a single parasite. The fact that all *Plasmodium* clones tested have been vector transmissible (7, 16) suggests a major difference in mechanisms between these otherwise closely related hemoprotozoa.

The results of this work suggest that the virulence phenotype in *B. bovis* is readily modifiable. In addition to subpopulation selection mechanisms, it is probable that differential gene expression is also occurring in this parasite. It has been shown previously that the blood passage-attenuated strain of *B. bovis* reverts to full virulence upon blood passage in intact animals or after tick passage on splenectomized calves (1, 6). These changes can be explained by the preferential selection of virulent subpopulations and are supported by the results of the Ka cloning in this work. The fact that the avirulent clone K-19-47 also reverted to virulence after one intact calf passage, however, suggests that differential gene expression is also operating in *B. bovis*. The virulence gene(s) must still be present, but not expressed, in both the Ka avirulent strain and the K-19-47 avirulent clone, since the virulence phenotype is rapidly restored for both lines after a single passage in intact animals.

While the molecular nature of virulence in *B. bovis* is not yet known, a mechanism can be suggested to explain the results observed. Field strains of *B. bovis* must consist of a range of subpopulations, each expressing their virulence genes to various degrees. The overall balance of subpopulations determines the observed phenotype. Parasites which express the virulence genes at very low levels, or not at all, may have a slightly faster growth rate and express other immunogenic proteins on the surface of the erythrocytes that they infect. Serial blood passage in splenectomized calves gradually selects a population enriched in the faster-growing avirulent phenotype. This corresponds to the Ka strain, and limit dilution of the Ka strain results in further selection of subpopulations (= clones) with a range of phenotypes.

Passage of the Ka strain through intact cattle results in rapid removal of the avirulent parasites because the immunogenic proteins on the surface of these parasitized cells are recognized and removed by the spleen. When avirulent clone parasites are passaged through intact animals, they must rapidly alter their genotypic expression to avoid being removed by the spleen. This results in their assuming a virulent phenotype.

The results presented here have implications for the development of improved live *B. bovis* vaccines. Contrary to possible predictions, clone K-19-47 was not totally stable with regards to virulence, reverting upon blood passage in intact animals. However, all clones, including K-19-47, were non-tick transmissible, and this characteristic does appear to be stable. If K-19-47 was used as a live vaccine strain, it could have certain advantages over the Ka strain. Even though K-19-47 becomes virulent in the original animal vaccinated with it, this animal controls its infection. If it was blood passaged at this stage, it would cause severe reactions in the recipients, but because it is non-tick transmissible, this cannot occur naturally in the field. By contrast, the Ka strain can potentially be tick transmitted as the virulent K strain to unvaccinated neighboring cattle. As noted by Callow et al. (1), this potential reversion has apparently not proved important in practical vaccination programs in Australia. This could be due largely to the enzootic nature of babesiosis in the vaccinating areas. Nevertheless, a non-tick transmissible *B. bovis* clone would be a much safer prospect, particularly in less enzootically stable areas.

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