Theileria parva live vaccination: parasite transmission, persistence and heterologous challenge in the field

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SUMMARY

The 'Muguga cocktail' live vaccine, delivered by an infection and treatment protocol, has been widely deployed in Eastern, Central and Southern Africa to protect cattle against East Coast fever, caused by *Theileria parva*. The vaccine contains 3 component stocks (Muguga, Serengeti-transformed and Kiambu 5). In a previous study, parasites from vaccinated and unvaccinated animals were genotyped with a panel of micro- and minisatellite markers (Oura *et al*. 2004*a*) and it was shown that only the Kiambu 5 stock establishes a long-term carrier state but there was no evidence for the transmission of this stock. Also parasite genotypes different from the 3 component vaccine stocks were identified in vaccinated animals. We now report a follow-up study on the same farm, some 4 years after the initial vaccination, aimed at establishing the source of the novel parasite genotypes identified in vaccinated cattle, determining the longevity of the carrier state established by the Kiambu 5 vaccine stock and re-examining whether vaccine transmission can occur over a longer time-scale. To do this, samples were taken from vaccinated and unvaccinated cattle and the parasites were genotyped with a series of micro- and minisatellite markers. The data indicate that the vaccine stabilates contain at least 6 parasite genotypes, the Kiambu 5 stock can be detected in many but not all vaccinated cattle for up to 4 years and can be transmitted to unvaccinated cattle which share grazing and that some of the vaccinated animals become infected with local genotypes without causing overt disease.

Key words: *Theileria parva*, 'Muguga Cocktail' vaccine, carrier state, mini- and microsatellites.

INTRODUCTION

The most economically important bovine tick-borne disease in Eastern and Central Africa is East Coast fever (ECF) caused by the intracellular protozoan haemoparasite *Theileria parva*. The disease causes high mortality, especially in exotic and cross-bred cattle, as well as indigenous calves below 6 months of age. Apart from the regular use of acaricides to control ticks, the only effective method of protecting cattle is by infection and treatment immunization, which involves the simultaneous inoculation of a live, potentially lethal dose of *T. parva* sporozoites and a long-acting oxytetracycline (reviewed by Radley, 1981). Protection is partially stock specific and combinations of stocks have been used to provide broad protection. The most widely used is the 'Muguga cocktail' (Radley *et al*. 1975*a*, *b*) composed of *T. parva* Muguga, Kiambu 5 and Serengeti-transformed stocks. This cocktail combination has been used in Uganda, Malawi, Tanzania and, more recently, in Kenya (see Morzaria and Williamson (1999) for summaries).

Live vaccination against ECF, by infection and treatment, induces a carrier state, which may be important in maintaining immunity (Bishop *et al*. 2002; Kariuki *et al*. 1995). However, it has been shown that the component Muguga stock does not cause a long-term carrier state (Bishop *et al*. 1992; Skilton *et al*. 2002). In a recent field study, Oura *et al*. (2004*a*) investigated the carrier state induced by 'Muguga cocktail' immunization in Uganda and showed that the Muguga and Serengeti stocks were highly related but did not induce persistent infections, whereas the Kiambu 5 stock was very distinct and was detectable by PCR for up to 303 days post-vaccination. The vaccine stocks were identified using strain-specific PCR based on primers designed to the single copy PIM gene (Oura *et al*. 2004*a*), which is highly polymorphic (Geysen *et al*. 2004). No evidence was obtained for the transmission of the vaccine stocks to animals that shared grazing during the time-period of the study; however, in a number

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of the vaccinated animals there was evidence for novel parasite genotypes. It was postulated that these either represented infection with local genotypes or that these novel genotypes could have been derived from the vaccine stabilates, if the latter contained additional genotypes.

In this paper we present data from a follow-up study of the cattle on the farm sampled by Oura *et al.* (2004*a*), as well as an analysis of the genotypes present in the vaccine stabilates. This has allowed us to address the following questions. (1) How many distinct *T. parva* genotypes are present in the 'Muguga Cocktail' vaccine? (2) Are all the vaccine component stocks circulating in vaccinated calves? (3) How long do vaccinated cattle remain infected with the Kiambu 5 vaccine stock? (4) Is there evidence that vaccine stocks are transmitted from vaccinated to unvaccinated cattle? (5) Does vaccination prevent infection by local circulating parasites and, if infection is detected, does the vaccine prevent disease?

MATERIALS AND METHODS

Parasite material

Two batches of 'Muguga Cocktail' sporozoite stabilates were prepared from seed stabilates using the method described by Morzaria *et al.* (1999) at the International Livestock Research Institute (ILRI) and these batches were designated FAO 1 and FAO 2. These were prepared 2 weeks apart from similar batches of ticks. The 'Muguga cocktail' FAO 1 stabilate used to vaccinate the cattle in this study was obtained from the Centre for Ticks and Tick-borne Diseases in Malawi via the ECF Immunisation Project in Entebbe, Uganda. Cell lines had been established as separate macroschizont-infected cell lines of each component stock (Serengeti-transformed, Muguga and Kiambu) as described by Bishop *et al.* (2001) and DNA was isolated from these cell lines as described by Conrad *et al.* (1987).

Cattle

The cattle sampled in this study were from a farm in the Iganga district of Central Uganda. They were cross bred (African Shorthorn × Friesian), they shared grazing, were born on the farm, were visibly free from ticks and were in moderate health. The adult cattle were dipped weekly and the calves were sprayed weekly with acaricide (Supona Extra). Two groups of cattle were sampled in August 2004: Group (1) 13 unvaccinated adult cattle more than 5 years old and Group (2) 23 vaccinated cattle: 8 animals were vaccinated in 2000/01, 10 vaccinated in 2002 (Oura *et al.* 2004*a*) and 5 vaccinated in 2003.

DNA extraction and marker analysis

DNA was purified from 100 μl of the FAO 1 and FAO 2 Muguga Cocktail vaccine stabilates using the Wizard Genomic DNA Purification kit (Promega, UK) according to the manufacture's instructions. The DNA was resuspended in 100 μl of distilled H_2O. This was analysed by PCR amplification with 6 minisatellite markers (MS 3, MS 7, MS 19, MS 25, MS 27 and MS 46) using nested primers as described previously (Oura *et al.* 2003, 2004*a*, 2005). Amplified products were separated on 1·5% agarose gels in TAE buffer or Spreadex gels (Oura *et al.* 2003), stained with ethidium bromide and the DNA bands visualized on a UV light box and photographed.

DNA was purified from cattle blood samples spotted onto FTA filter paper (Whatman Bio-Science) as described previously (Oura *et al.* 2003). Specific nested primers, designed in the conserved 5′ and 3′ regions on the PIM gene, were used to discriminate the Muguga/Serengeti and Kiambu vaccine stocks, using the PCR protocol described by Oura *et al.* (2004*a*). Additionally the samples were genotyped using PCR amplification of 5 minisatellite markers (MS 3, MS 7, MS 19, MS 25, MS 27) as described above. The PCR amplified products generated from the Muguga/Serengeti and Kiambu derived nested primers were separated on 1·5% agarose gels and those from the minisatellite nested primer PCR were separated on high resolution Spreadex gels.

RESULTS

Characterization of T. parva *stocks comprising the 'Muguga cocktail' vaccine*

In order to characterize the genotypes present in the FAO 1 and FAO 2 'Muguga Cocktail' vaccine sporozoite stabilates, PCR was carried out using 6 minisatellite markers and the amplified PCR products were compared with products amplified from *in vitro* cell cultures of the original seed vaccine stabilates of Muguga, Kiambu 5 and Serengeti used to prepare the vaccine. The results for 2 of the minisatellites (MS 7 and MS 19) separated on high resolution Spreadex gels are shown in Fig. 1. Six distinct PCR products (alleles) were amplified with the minisatellite MS 7 and MS 19 primers from the FAO 1 and FAO 2 stabilates of which 3 were of identical size to those amplified, respectively, from the Muguga, Serengeti and Kiambu 5 tissue-culture generated component stocks. These data indicate that there are at least 6 genotypes of *T. parva* present in the 'Muguga Cocktail' vaccine.

In order to investigate whether the novel alleles previously identified in cattle vaccinated with the FAO 1 stabilate at 17 days post-vaccination (Oura *et al.* 2004*a*) were identical to those amplified from

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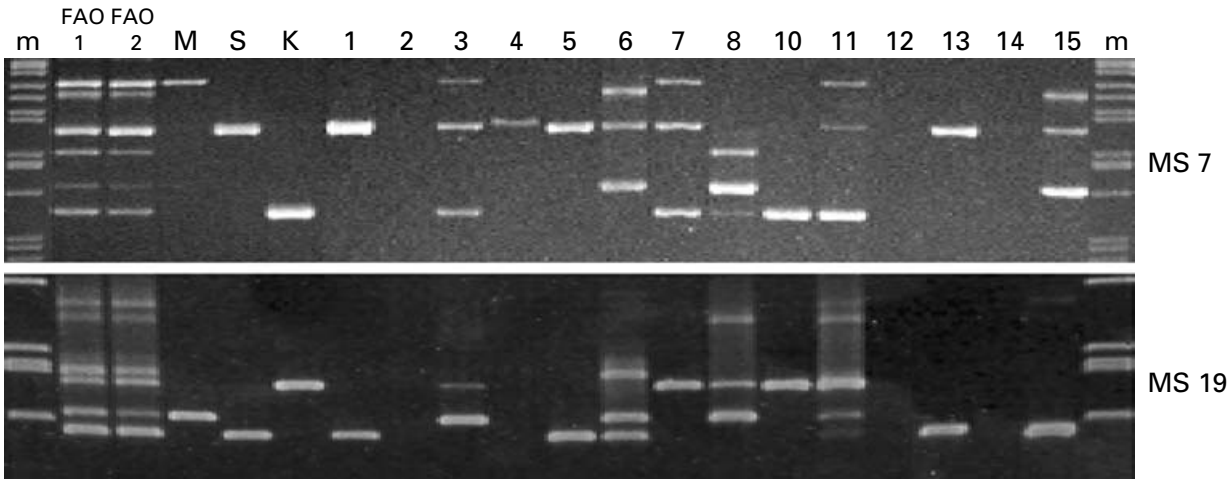

Fig. 1. Characterization of component stocks of the 'Muguga Cocktail' vaccine. Spreadex gel separation of the PCR products generated using minisatellite MS 7 and MS 19 primers to amplify DNA from the 'Muguga Cocktail' vaccine stabilate (FAO 1 and FAO 2) and the 3 cell line component stocks in the vaccine, Muguga (M), Serengeti-transformed (S) and Kiambu (K) as well as samples from 14 calves (lanes 1–14) on day 17 post-vaccination with the FAO stabilate. Alleles were sized by direct comparison with the M3 marker (Elchrom Scientific) run in the left and right hand lanes of each gel (m).

the vaccine stabilates, DNA from the blood of the same 14 calves (calf 9 was not sampled at this time-point and so is not included) was genotyped with the minisatellite markers MS 7 and MS 19 and the products were separated on Spreadex gels (Fig. 1). Three of the samples showed no PCR product (2, 12 and 14), while 6 samples (1, 3, 5, 7, 10 and 13) amplified alleles of the same size as those identified in the 3 component stocks of the vaccine. The samples from the remaining 5 calves (4, 6, 8, 11 and 15) showed alleles that differed in size from the 3 component vaccine stocks, but in 4 of these samples (6, 8, 11 and 15) the additional alleles were identical in size to those amplified directly from the vaccine stabilates (FAO 1 and FAO 2). However, in 1 sample (number 4) a novel allele was identified that was not present in either the sporozoite vaccine stabilates or the component cell lines. Therefore all the genotypes present in the vaccinated calves (with the sole exception of sample 4) were present in the vaccine stabilate (Fig. 1), although not all were detected as circulating in all the calves at this single time-point. These data show that the novel alleles identified in these calves are likely to have been derived from immunization rather than from infection with local strains of *T. parva* as a result of tick challenge.

Carrier state of component stocks of the 'Muguga Cocktail' vaccine

Previous analysis of this group of vaccinated calves showed that the majority of calves carried the Kiambu 5 stock for 303 days, while the Muguga and Serengeti stocks were largely cleared by 48 days post-vaccination (Oura *et al*. 2004*a*). The farm was re-visited in 2004, 15 months after the previous vaccine trial was terminated. Blood samples were taken from 2 groups of cattle, the first comprised animals that had been vaccinated (8 cattle that were vaccinated in 2000 and 2001, 5 cattle that were vaccinated in February 2003 and 10 cattle from the original vaccine trial in 2002) and the second comprised unvaccinated cattle that had been co-grazed with the vaccinates over the whole period. The samples from the vaccinated animals were genotyped by PCR amplification using the primers for the PIM gene that are specific for Muguga/Serengeti and Kiambu 5 stocks and the MS 7 minisatellite, and the results are shown in Fig. 2A. The PIM gene primers specific for the Kiambu 5 stock amplify 3 bands of different size, as previously described (Oura *et al* 2004*a*). One of the primers was designed to a sequence within the hypervariable region of the gene to obtain stock specificity but also anneals to 2 other sequences within the same region. Out of the 10 cattle vaccinated in 2002, 5 gave an allele pattern identical to the Kiambu 5 stock (Fig. 2A, samples 1, 3, 5, 6 and 10), none of the cattle were positive for the Muguga/Serengeti stocks and 7 (1, 2, 3, 5, 6, 8 and 10) were positive for *T. parva* with minisatellite MS 7 (Fig. 2A). Two PCR products were amplified by the Kiambu-derived primers from sample 2; however, these products were of a different size to those amplified in the Kiambu 5 stock, showing that this isolate is distinct from Kiambu 5, although 1 of the 2 alleles amplified with the MS 7 primers was of identical size to that in Kiambu 5. Sample 8 shows no amplification with the Kiambu 5-specific primers but 2 alleles are detected for MS 7, one of which is of the same size as that detected in Kiambu 5. Thus, these samples have multilocus genotypes that are different from Kiambu 5. Out of the 8 cattle vaccinated in 2000 and 2001, 7 gave an

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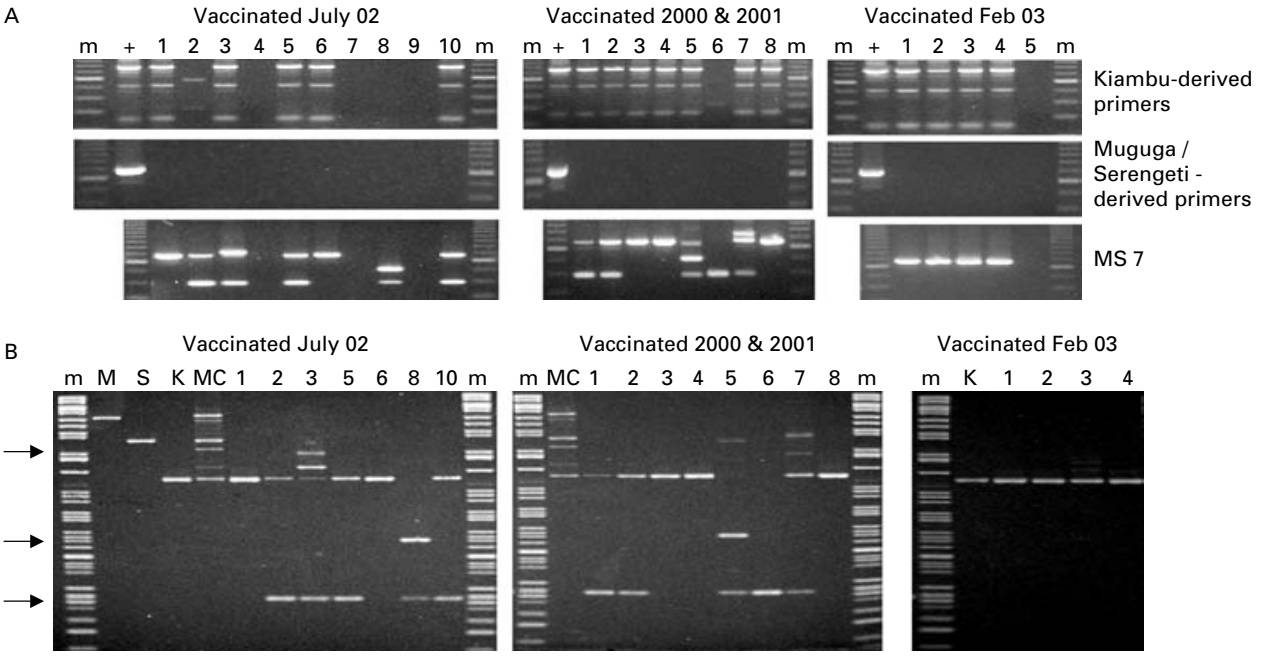

Fig. 2. Parasite genotypes present in carrier animals vaccinated with the 'Muguga Cocktail' vaccine. (A) Agarose gel separation of the PCR products amplified from blood samples of 10 calves vaccinated in 2002, 8 calves vaccinated in 2000 and 2001 and 5 cattle vaccinated in 2003. (A) PCR products amplified with Kiambu 5-derived primers (top panel) Muguga/Serengeti-derived primers (middle panel) and minisatellite MS 7 primers (bottom panel) are shown. Positive controls (+) using either Kiambu 5 or Muguga DNA are run to the left of the gels. (B) Spreadex gel separations of the PCR products generated using minisatellite MS 7 primers to amplify DNA from the 'Muguga Cocktail' vaccine stabilate (MC), the 3 component tissue-culture derived stocks in the vaccine, Muguga (M), Serengeti-transformed (S) and Kiambu (K) as well as 7 cattle vaccinated in 2002, 8 cattle vaccinated in 2000 and 2001 and 4 cattle vaccinated in 2003. Alleles were sized by direct comparison with markers (m) run in the left and right hand lanes of each gel.

allele pattern identical to Kiambu 5, none were Muguga/Serengeti positive and all 8 calves were *T. parva* positive with minisatellite MS 7, although non-Kiambu alleles were present (Fig. 2A). Sample 6 shows 2 feint bands with the Kiambu 5 primers but these are of different size to those of the Kiambu 5 stock, suggesting that this animal is infected with a different isolate. Of the 5 calves vaccinated in February 2003, 4 gave an allele pattern identical to Kiambu 5, all were negative for Muguga/Serengeti and 4 were *T. parva* positive and identical with the MS 7 marker (Fig. 2A). These data indicate that the Kiambu 5 carrier state can be detected in the majority (70%) of calves vaccinated with the 'Muguga Cocktail' vaccine for up to 4 years, but there is no evidence for the presence of the Muguga/Serengeti component of the vaccine.

In order to address the question of whether vaccination prevents infection with local parasite strains, the minisatellite MS 7 amplified PCR products from the vaccinated cattle samples were separated on high resolution Spreadex gels and the results are shown in Fig. 2B. Allele size comparison between the 'Muguga Cocktail' vaccine tissue-culture derived component genotypes and those from the FAO 1 vaccine stabilate (Fig. 2B, M, S, K and MC) with the alleles amplified in the vaccinated cattle revealed that at least 3 alleles (indicated by arrows in Fig. 2B) were present in the animals vaccinated between 2000 and 2002, but were not present in the 'Muguga Cocktail' vaccine or the animals vaccinated in 2003. These non-vaccine derived alleles were found in 10 of the 15 animals vaccinated between 2000 and 2002 and, based on this analysis, must have been derived from local stocks infecting the animals. Given that there is no evidence for such infections in the animals vaccinated in 2003, it must take more than a year for such infections to occur. These data show that local stocks are able to infect immunized cattle, although it is important to note that the immunized calves infected with local stocks did not show any clinical signs of overt disease, indicating that the Muguga Cocktail vaccine, coupled with acaracide treatment, protected these calves from disease but not infection.

Evidence for transmission of 'Muguga Cocktail' vaccine stocks to unvaccinated cattle

In 2002 ten unvaccinated cattle were sampled on the farm and no evidence was obtained for the transmission of vaccine stocks to these cattle over the period of 11 months since vaccination (Oura *et al.* 2004*a*). In order to examine whether transmission

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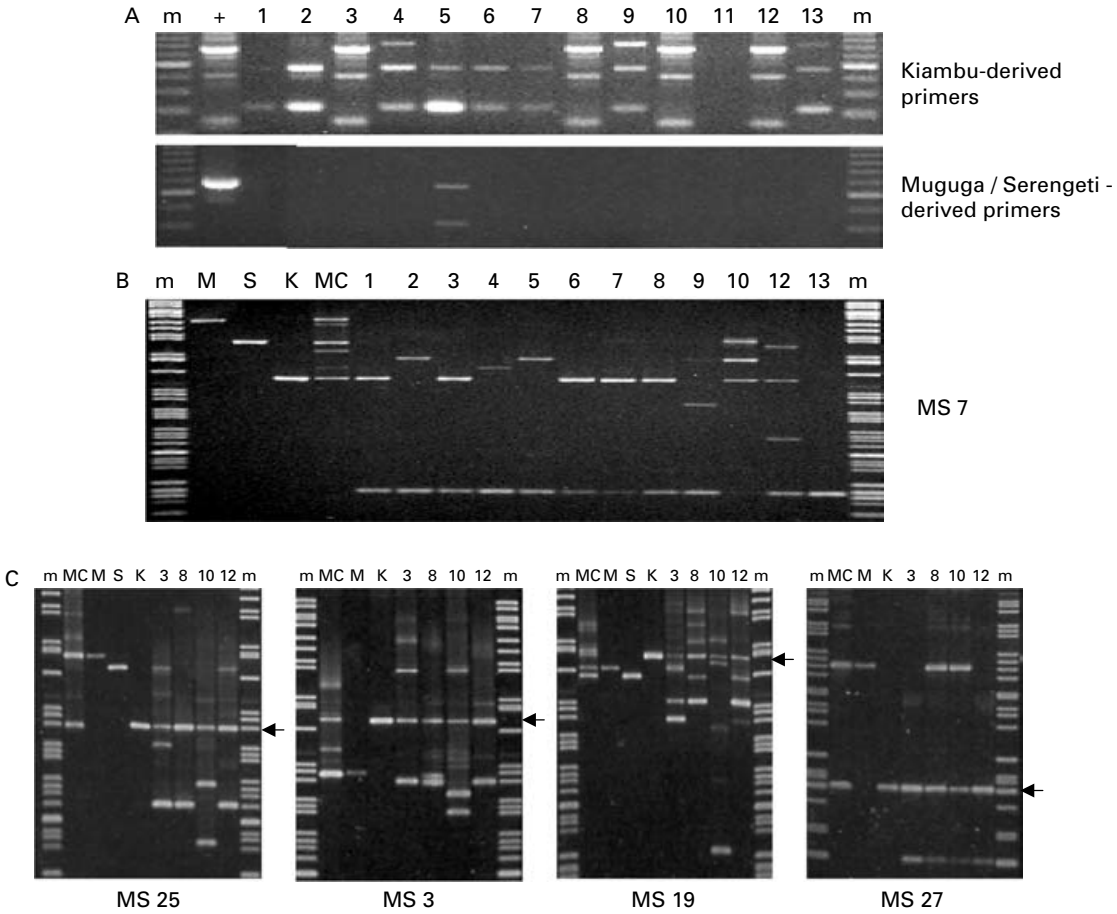

Fig. 3. Parasite genotypes present in unvaccinated carrier cattle. (A) Agarose gel (1%) separation of the PCR products from DNA extracted from the blood of 13 unvaccinated adult cattle on the farm. PCR products were amplified with Kiambu 5-derived primers (top panel) and Muguga/Serengeti-derived primers (bottom panel). Positive controls (+) using either Kiambu 5 or Muguga DNA are run to the left of the gels. (B) Spreadex gel separations of the PCR products generated using minisatellite MS 7 primers to amplify DNA from the 'Muguga Cocktail' vaccine stabilate (MC), the 3 tissue-culture derived component stocks in the 'Muguga cocktail' vaccine [Muguga (M), Serengeti-transformed (S) and Kiambu 5 (K)] as well as 13 unvaccinated adult cattle (Lanes 1–13). (C) Spreadex gel separation of PCR products amplified using 4 minisatellite primers (MS 25, 3, 19 and 27) to amplify DNA extracted from the 'Muguga Cocktail' vaccine stabilate (MC), the 3 tissue-culture derived component stocks in the 'Muguga Cocktail' vaccine Muguga (M), Serengeti-transformed (S) and Kiambu 5 (K) and the blood of 4 unvaccinated cattle (3, 8, 10 and 12) that were Kiambu 5 positive on PCR amplification with the Kiambu 5 derived primers (A, top panel). A band of identical size to the Kiambu 5 stock (K) is amplified from samples 3, 8, 10 and 12 (arrow). The Serengeti-transformed stock is not run with MS 3 and MS 27 but amplifies an identically sized PCR product to the Muguga stock. Alleles were sized by direct comparison with markers (m) run in the left and right hand lanes of each gel.

of the vaccine stocks could occur over a longer time-period, blood samples were taken from 13 unvaccinated adult cattle on the farm (over 2 years after the previous sampling) including 3 of the animals sampled in 2002. These cattle had shared grazing for over 4 years with 43 cattle, vaccinated between 2000 and 2003. Blood samples were genotyped, using the Muguga/Serengeti and Kiambu 5-derived primers that amplify regions of the PIM gene. Four of the unvaccinated cattle (numbers 3, 8, 10 and 12) amplified PCR products of the same size and pattern as the Kiambu 5-specific PCR products from the vaccine stabilates (Fig. 3A). No evidence was found for transmission of the Muguga or Serengeti stocks to the unvaccinated cattle (Fig. 3B), although 2 PCR products were amplified by the Muguga/Serengeti-derived primers in sample 5. However, these were of a different size to the Muguga-specific PCR product from the vaccine stabilates. These data provide evidence that the Kiambu 5 component of the vaccine is being transmitted to unvaccinated cattle. In 8 (samples 1, 2, 4, 5, 6, 7, 9 and 13, Fig. 3A) of the 13 samples, PCR products were amplified that were of different size/pattern to the Kiambu-specific PCR product from the vaccine stock (Fig. 3A). This suggests that local genotypes of *T. parva* present in these cattle can also be amplified with the Kiambu-derived primers, however, the pattern of bands

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amplified were always found to be distinct from the unique pattern of bands amplified by the Kiambu 5 stock in the vaccine stabilate. Similarly the Muguga-derived primers were not specific for the Muguga stock (sample 5, Fig. 3A) and amplified PCR products of a different size.

To test the possibility that certain local *T. parva* strains amplify alleles of identical size and pattern to the Kiambu 5 stock with the Kiambu-derived primers, the parasites from the 12 unvaccinated cattle together with the 'Muguga Cocktail' vaccine stabilate (MC) and the 3 component vaccine stocks were genotyped with a panel of 5 minisatellite markers (MS 7, 25, 3, 19 and 27). The genotypes obtained with the minisatellite MS 7 are illustrated in Fig. 3B. PCR products of identical size to the Kiambu 5 stock were amplified in samples 1, 3, 6, 7, 8, 10 and 12, but samples 2, 4, 5, 9 and 13 showed non-Kiambu size alleles, which presumably represent local non-vaccine derived strains. In addition to the Kiambu size alleles in samples 1, 3, 6, 7, 8, 10 and 12, novel sized alleles were observed (Fig. 3B). As samples 1, 6 and 7 have an MS 7 allele of the same size as observed with Kiambu 5 but alleles of a different size from the Kiambu stock using the Kiambu-derived primers (Fig. 3A), these are presumed to be infections with local stocks. Thus samples 3, 8, 10 and 12 have an identical genotype, using 2 markers, to that of the Kiambu 5 component of the vaccine stabilates and this finding provides evidence for the transmission of this vaccine stock to unvaccinated cattle. However, each of these animals are also infected with local stocks based on the data with minisatellite MS 7. To provide further evidence for this conclusion, samples 3, 8, 10 and 12 were genotyped with a further 4 minisatellite markers (MS 25, 3, 19 and 27) and the results are shown in Fig. 3C. While all the samples showed multiple alleles of different size to the vaccine stabilates (MC, Fig. 3C), they also all showed alleles of the same size as the Kiambu 5 vaccine stock (K, shown with an arrow in Fig. 3C). The data with the 5 minisatellite markers, along with the positive PCR products amplified using the Kiambu 5-derived primers (summarized in Table 1), provide very strong evidence that the Kiambu 5 component of the 'Muguga Cocktail' vaccine is present in 4 of the unvaccinated cattle and is thus being transmitted from vaccinated cattle. In addition, the parasites from the unvaccinated cattle sampled in 2002 (Oura *et al.* 2004*a*) did not share any alleles with the Kiambu 5 vaccine stock, although they amplified with the PIM gene primers but gave a distinct pattern, thus indicating they were derived from local stocks and were distinct from the vaccine. The 4 cattle (3, 8, 10 and 12) carrying the Kiambu 5 stock were confirmed by the farmer as being both unvaccinated and born on the farm so the only way these cattle could have been infected with the Kiambu 5 stock would have been by tick transmission.

Table 1. Multilocus genotype (MLG) identical to Kiambu 5 vaccine stock present in unvaccinated cattle

(MS, Minisatellite; –, non-Kiambu 5 multilocus genotype.)

Sample number	Identical sized alleles to Kiambu amplified with Kiambu-derived primer PCR (number of alleles)	Identical sized allele to Kiambu with MS 7 (number of alleles)	Identical sized allele to Kiambu with MS 25, 3, 19 and 27
1	– (2)	K (2)	nd
2	– (3)	– (2)	nd
3	K (4)	K (2)	K
4	– (3)	– (2)	nd
5	– (2)	– (2)	nd
6	– (2)	K (2)	nd
7	– (2)	K (2)	nd
8	K (4)	K (2)	K
9	– (3)	– (2)	nd
10	K (4)	K (3)	K
11	–	nd	nd
12	K (3)	K (4)	K
13	– (3)	– (1)	nd

DISCUSSION

The results presented in this paper indicate that the two 'Muguga Cocktail' vaccine stabilates (FAO 1 and FAO 2) contain at least 6 distinct genotypes of *T. parva*, 3 of which are identical to the single distinct genotypes from each of the component stocks from which the vaccine stabilates were prepared. These results indicate that at least 3 additional genotypes are present in the vaccine and it is difficult to consider any other explanation, although formal proof of this would require analysing a set of cloned populations *in vitro*. All 6 genotypes were detected in vaccinated calves at 17 days post-vaccination and this provides an explanation for the previously observed novel alleles that were thought to be due to infection by local strains (Oura *et al.* 2004*a*). However, 1 of these calves did contain parasites with a novel allele, which was not detected in the vaccine stabilates and so could represent infection with a local genotype. The presence of multiple genotypes in the vaccine stabilates does not detract from the efficacy of the vaccine, as it provides good protection from disease. Before a vaccination policy was introduced on the sampled farm there were frequent cases of ECF in calves, whereas after the introduction of the vaccine the cases of ECF reduced dramatically (C. A. L. Oura, personal communication from farmer). Indeed the presence of additional parasite components that may be antigenically heterogeneous could, in principal, broaden the protection induced by vaccination. There has been concern about the use of the

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'Muguga Cocktail' due to the risk of introducing 'exotic' stocks of *T. parva*, which might be outside the immunological range of indigenous parasites and so the presence of these additional genotypes in the live vaccine could make national disease control authorities even more cautious. The results presented here highlight the need to fully characterize the vaccine stabilates and show that microsatellite markers provide a simple and effective means of doing this.

One of the component vaccine stocks (Kiambu 5) results in a carrier state that could be detected in the majority of vaccinated cattle for up to 4 years post-vaccination. The 'Muguga Cocktail' vaccine provides a significant level of protection against local genotypes of *T. parva* as none of the vaccinated cattle succumbed to disease. There are 2 pieces of evidence which show that the calves were being challenged with local *T. parva* stocks, firstly there was a serious ECF problem on the farm prior to vaccination (pre-2000) and secondly the unvaccinated cattle, which shared grazing with the vaccinated calves, were infected with many different *T. parva* genotypes. Given the use of acaricides on the farm, this finding is somewhat surprising, as one would predict that the level of transmission and challenge would be low, but this is consistent with the finding that the 5 calves vaccinated 18 months before sampling showed no evidence of infection with local genotypes of *T. parva*. However, there was evidence for infection by local genotypes of *T. parva* in cattle 2–4 years after vaccination. In this context, the original aim for this vaccine was to deploy it in the field under reduced tick control so that there would be a limited exposure to challenge and the immunity would be boosted and broadened by low parasite challenge (Radley, 1981; Radley *et al* 1975*a*, *b*). In this study the vaccine is performing exactly as would be predicted, in that it is allowing infection by local strains of *T. parva* in the field but these are not resulting in overt disease. This is in agreement with the broad cross-protection, with no breakthrough of disease observed, induced by the 'Muguga cocktail' vaccine against local Ugandan parasite strains reported by Ochiba *et al*. (1999).

Both this study and other studies (Oura *et al*. 2004*a*, *b*) show that non-vaccinated cattle in endemically stable areas are often infected with many *T. parva* genotypes, although disease symptoms are not detected. This is consistent with the concepts and analysis put forward by Young (1981) and Moll *et al*. (1984, 1986), where endemic stability was defined as an epidemiological state of a population in which clinical disease is scarce despite high levels of infection. It is possible that the level of parasite challenge from infected ticks in such endemic areas may be low and thus produce subclinical infections or alternatively, and more likely, that the immunity produced by multiple infections with heterologous infecting local genotypes of *T. parva* is sufficient to protect cattle from disease.

We also provide evidence that the vaccine stock (Kiambu 5) is transmitted from vaccinated to unvaccinated cattle, which share grazing. This is in contrast to our data from a previous study carried out earlier on the same farm (Oura *et al*. 2004*a*) in which there was no evidence of transmission of vaccine stocks from vaccinated to unvaccinated cattle. The reasons for this may be 2-fold. Firstly, many more calves had been vaccinated thus increasing the probability of infecting the local tick population with resultant transmission to unvaccinated cattle and secondly, given the use of acaracide, the level of tick challenge will be low leading to an increased interval before the vaccine genotype would be established in the tick population. These considerations would explain why only local parasite genotypes were initially observed in the unvaccinated cattle (Oura *et al*. 2004*a*) with the vaccine genotypes observed some 2 years later. Once the Kiambu 5 genotype is established in the tick population, it is possible that it could be transmitted back into the vaccinated cattle as well. We consider this is unlikely, as Kiambu 5 carrier animals would be immune to further challenge by the identical strain, although this possibility cannot be excluded.

The evidence presented shows that 4 out of the 13 unvaccinated animals contain parasites with a multilocus genotype that is identical to that of Kiambu 5 and, as previous analysis of the genotypes of parasites from unvaccinated cattle on the same farm taken at an earlier time-point did not identify this genotype, we conclude that it has been introduced from the vaccinated cattle. However, several of these isolates from unvaccinated cattle contain additional alleles other than Kiambu 5, suggesting that there is a mixture of local and Kiambu 5 strains present in these cattle. This conclusion is based on the assumption that both the alleles of the PIM gene and those of the satellite markers are sufficiently stable that the alleles of different size to those of the Kiambu 5 vaccine stock do not arise by mutation from this stock. Although there is no direct experimental evidence for the stability of these markers, the fact that an identical MLG to Kiambu 5 is seen in vaccinated animals more than 300 days after vaccination (Oura *et al*. 2004*a*) suggests that the markers are stable and therefore backs up our conclusion that the novel alleles arise from local stocks. This opens up the possibility of recombination occurring between vaccine and local strains. To address this question, it would be necessary to isolate and clone lines from these animals and then undertake micro- and minisatellite genotyping. The presence of the Kiambu 5 vaccine stock in unvaccinated cattle on the farm means that, with extensive use, the vaccine component stocks, which are 'foreign' to a particular area, will be introduced into the local cattle and tick

populations. The situation in East Africa is very different to countries in Southern Africa, such as Zambia, where many isolates from the Northern and Eastern provinces appear virtually clonal (Geysen *et al*. 1999). In Zambia the local strain approach to vaccination (using *T. parva* Katete) has been used extensively in Eastern Province whereas immunization with the 'Muguga Cocktail' vaccine was used to a limited extent in the Southern province. Molecular characterization of *T. parva* field samples from the Southern Province of Zambia, several years subsequent to the Muguga cocktail vaccination campaign, were consistent with the hypothesis that disease was being caused by a clonally expanded strain of *T. parva* that was genetically very similar to the components within the 'Muguga Cocktail' vaccine (Geysen *et al*. 1999). This has resulted in debate about the risks of using the 'Muguga Cocktail' vaccine in Southern Africa. However, it should be noted that the immunization programme with this vaccine was stopped prematurely, resulting in a cattle population that was susceptible to infection and which might have been protected with continued immunization. In contrast to the situation in Southern Africa, in East Africa there is an extensive range of *T. parva* parasite stocks circulating in the field (Oura *et al*. 2005). This and the fact that the constituent stocks present in the 'Muguga Cocktail' vaccine originate from this region suggest that the introduction of vaccine stocks into the field will not result in exacerbation of the ECF problem.

Following improvements in the quality of stabilates used in the infection and treatment method of immunization against ECF and modifications to dosage of oxytetracycline, the 'Muguga Cocktail' is now being used successfully in East Africa. Results from more than 6 years of immunizations with this vaccine in Tanzania, predominantly in pastoral communities, have been highly successful resulting in a considerable reduction in mortality due to ECF (Lynen *et al*. unpublished communication). The findings in this and other studies (Bishop *et al*. 2001; Oura *et al*. 2004*a*) that the Muguga and Serengeti components of the vaccine are genetically very similar raises the possibility that the combination does not provide any additional cross-protection and so the vaccine could be simplified by removing the Serengeti component. This would have two advantages, firstly it would simplify vaccine production and secondly it would get around reservations about the inclusion of an exotic strain, when the vaccine is used in Kenya. Clearly further research would be required to test a Muguga/Kiambu 5 vaccine combination.

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