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SUPPLEMENT ARTICLE

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A review of recent research on *Theileria parva*: Implications for the infection and treatment vaccination method for control of East Coast fever

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Abstract

The infection and treatment (ITM) live vaccination method for control of *Theileria parva* infection in cattle is increasingly being adopted, particularly in Maasai pastoralist systems. Several studies indicate positive impacts on human livelihoods. Importantly, the first detailed protocol for live vaccine production at scale has recently been published. However, quality control and delivery issues constrain vaccination sustainability and deployment. There is evidence that the distribution of *T. parva* is spreading from endemic areas in East Africa, North into Southern Sudan and West into Cameroon, probably as a result of anthropogenic movement of cattle. It has also recently been demonstrated that in Kenya, *T. parva* derived from cape buffalo can 'breakthrough' the immunity induced by ITM. However, in Tanzania, breakthrough has not been reported in areas where cattle co-graze with buffalo. It has been confirmed that buffalo in northern Uganda national parks are not infected with *T. parva* and *R. appendiculatus* appears to be absent, raising issues regarding vector distribution. Recently, there have been multiple field population genetic studies using variable number tandem repeat (VNTR) sequences and sequencing of antigen genes encoding targets of CD8+ T-cell responses. The VNTR markers generally reveal high levels of diversity. The antigen gene sequences present within the trivalent Muguga cocktail are relatively conserved among cattle transmissible *T. parva* populations. By contrast, greater genetic diversity is present in antigen genes from *T. parva* of buffalo origin. There is also evidence from several studies for transmission of components of stocks present within the Muguga cocktail, into field ticks and cattle following induction of a carrier state by immunization. In the short term, this may increase live vaccine effectiveness, through a more homogeneous challenge, but the long-term consequences are unknown.

KEYWORDS

cape buffalo, East Coast fever, infection and treatment (ITM), live vaccination, molecular epidemiology, population genetics, *Theileria parva*

1 | INTRODUCTION

Theileria parva is a tick-transmitted apicomplexan parasite that causes East Coast fever (ECF) resulting in frequently lethal infections in cattle, with serious economic consequences in eastern, central and southern Africa (Norval et al., 1992). *Theileria parva* infection was the most prevalent tick-borne disease in cattle in Uganda according to reverse line blot assay (RLB) data (Oura, Bishop, Lubega, & Tait, 2004). A recent longitudinal study in an endemic area in Kenya revealed that *T. parva* was the single most important pathogen inducing calf mortality in a cohort of African zebu calves kept by poor livestock keepers and monitored for health and infection status during their first year (Thumbi et al., 2013).

There have been a number of reviews of *Theileria parva* biology and genomics (Bishop et al., 2009; Dobbelaere & Mckeever, 2002; Weir et al., 2009) and numerous reviews relating to approaches to live vaccination and attempts to develop recombinant vaccines, most recently by Nene and Morrison (2016). There have also been several reviews of the infection and treatment (ITM) vaccination method, notably that by Di Giulio, Lynen, Morzaria, Oura, and Bishop (2009) which overviews the methodology and focuses on the first large-scale delivery of the live vaccine to pastoralists in Tanzania, and more recently by Perry (2016) which documents the historical and institutional context of the development of the live vaccine and the current status of commercial deployment and production. A recent important addition to the ITM literature was the first publication of a detailed protocol for large-scale production of a million doses of the live vaccine for field use (Patel et al., 2016). Infection and treatment, as applied to *T. parva*, is not new and was first conceptualized as long ago as the 1920s. However, two key innovations, approximately 40 years ago, were crucial to making this a practical technology: (a) the ability to cryopreserve sporozoites from whole ground up ticks (Cunningham, Brown, Burridge, & Purnell, 1973) and (b) the commercial availability of an effective long-acting formulation of oxytetracycline (Radley et al., 1975; reviewed by Radley, 1981). Subsequently, a variety of logistical issues and concerns among veterinary policy makers and regulatory bodies delayed widespread adoption of the ITM vaccination method until the mid-1990s.

This article is not intended to duplicate the above reviews, to which the reader is referred for further details and primary references, but is intended to provide an update on recent research on *T. parva* population genetics and genomics, parasite and vector distribution, and the challenge posed by the cape buffalo (*Syncerus caffer*) wildlife reservoir for live vaccine effectiveness in areas where buffalo interface closely with cattle. Improvements to genotyping for quality control of ITM stabilates and the feasibility of improving the composition of the trivalent vaccine are also covered. Potential priority areas for future research designed to support and improve understanding of *T. parva* population genomics and the biological impact of ITM against a background of increased deployment are highlighted.

2 | THE IMMUNOPATHOLOGY OF *THEILERIA PARVA* INFECTION AND LIVE VACCINATION

It used to be assumed that the mortality and pathology associated with ECF were due to the capacity of the *T. parva* schizont to induce immortalization of host lymphocytes, a phenomenon which has been intensively studied in vitro (Dobbelaere & Mckeever, 2002). The schizonts initially divide in synchrony with host lymphocytes, which are thought to ultimately invade multiple host tissues in an uncontrolled fashion in vivo, in a process with similarities to the metastasis of tumours. Although schizont-infected lymphocytes are found in many tissues during acute infection (Kessy & Matovelo, 2011), recent immunohistochemical studies suggest that mortality, which is the result of respiratory failure (Irvin & Mwamachi, 1983), in fact involves activation and dysregulation of macrophages and resultant severe vasculitis (Fry et al., 2016). In this, macrophages activated during the acute disease course infiltrate and destroy vessels within the lungs, lymph nodes, spleen and liver. The macrophages are strongly positive for the scavenger receptor, CD163, and for the pro-inflammatory cytokine IL-17. Macrophage-mediated vessel destruction in turn causes significant fluid leakage into surrounding tissues. Within the lung, this results in obstruction of airways by pulmonary oedema and restriction of lung expansion due to pleural effusion. These changes culminate in respiratory failure and death of the affected bovine, a hallmark of severe ECF (Fry et al., 2016). In addition to pulmonary changes, animals that succumb to ECF also exhibit marked lymphocyte death within all lymphoid tissues (Mbassa et al., 2006). These findings, coupled with the observation that acute ECF leads to severe peripheral blood lymphocyte decline, rather than expansion (Fry et al., 2016; Irvin & Mwamachi, 1983) as one would expect in a lymphocytic tumour-like disease, support the assertion that non-lymphoid components of the immune response play a significant role in ECF mortality.

This recent paradigm shift in ECF pathogenesis has given way to the discovery of potential correlates of ECF progression and severity in cattle. Such correlates will allow objective assessment and comparison of new ITM formulations and/or dosages, and of next-generation vaccines for *T. parva*. Although the development of a CD8+ cytotoxic T-lymphocyte response to *T. parva* schizont-infected cells is required for protection against *T. parva* (McKeever et al., 1994), this response develops late in the disease course and is thus not a useful measure of early ECF progression. Recently, a subset of the authors (Fry, Knowles, Bishop) found that cattle undergoing lethal *T. parva* infection, but not non-lethal infection, developed significant changes in peripheral blood monocyte phenotype and function, and that these changes could be used to predict development of severe ECF (manuscript under review). Moving forward, the use of these disease correlates, and the development of additional correlates representing the entire breadth of the bovine immune response to *T. parva*, is paramount to the development of improved premiumization strategies for ECF.

3 | DEPLOYMENT AND IMPACT OF LIVE VACCINATION

To date in excess of 1.6 million cattle have been immunized using the Muguga cocktail trivalent version of ITM (Perry, 2016). The vaccine was initially produced at ILRI, Nairobi, Kenya, in the mid-1990s using FAO funding and again in 2008 at the request of the African Union Inter African Bureau for Animal Resources (AU-IBAR). Subsequently, production has been transferred to the AU-IBAR International Centre for Ticks and Tick-borne Diseases (ICTTBD) in Malawi, which was formally opened in December 2014, with support from GALVmed, a public–private partnership facilitation organization based in Edinburgh, UK. Funding has been provided by the Department for International Development (DFID), UK, and the Bill and Melinda Gates Foundation (BMGF), USA.

Deployment of the vaccine to date has been primarily in pastoralist systems in Tanzania and southern Kenya, but it has also been successfully tested in a field trial in cross-bred cattle in central Tanzania (Lynen et al., 2012). On-farm trials of the antigenically diverse (Hemmink et al., 2016) Marikebuni stock have also been performed in western Kenya (Wanjohi, Ngeranwa, Rumberia, Muraguri, & Mbogo, 2001). Pilot trials of the cocktail have been made in other countries, particularly Uganda, which is currently hosting a larger scale trial, which will likely influence regulatory approval in that country. In addition, 'local parasite stocks' have been used for vaccination in Zambia and Zimbabwe, in the latter country, sometimes without the use of oxytetracycline, which is possible due to the low abundance of infection of the Boleni (Zimbabwe) stock in ticks (Di Giulio et al., 2009; Latif & Hove, 2011).

Vaccination campaigns in Zambia, using the locally isolated Katete stock, were the result of a Belgian initiative that provided a successful alternative to the use of the trivalent Muguga cocktail. By 1997, 130,000 cattle had been immunized in the eastern province of Zambia (Manangi, 1999). Economic analysis demonstrated that it was a cost-effective strategy for control of ECF in the traditionally managed Sanga cattle (Minjauw & Mcleod, 2003). Production of the Katete stock has now been transferred to the ICTTD vaccine factory in Malawi. One factor potentially contributing to the success of the local stock is that, as indicated by genotyping using Southern blotting with repetitive DNA probes, including Tpr and minisatellite 221, combined with PCR RFLP of antigen genes, there appears to be a relatively homogeneous parasite population and therefore presumably a homologous challenge, in much of eastern Zambia (Geysen, Bishop, Skilton, Dolan, & Morzaria, 1999). However, there may be additional *T. parva* epidemiological situations within East Africa where a local stock may be an appropriate alternative to the Muguga cocktail.

As mentioned above, an alternative to the infection and treatment approach is the use of the Boleni stock of *T. parva* which has been delivered in Zimbabwe, without concurrent administration of oxytetracycline, thereby greatly reducing the cost of vaccination. The pros and cons of this approach are discussed in detail by Latif and Hove (2011). Although the stock was virulent in cattle when first isolated, after experimental passage through ticks it produced stabilates with low numbers of sporozoites relative to other stocks, 8–9 times less than Muguga and Serengeti from East Africa, and 14 times less than Kasoba from Malawi (Latif & Hove, 2011). This allowed use of the stock without antibiotics with considerable reductions in the cost of vaccination. Boleni also protected well against infected tick challenge within Zimbabwe and thus represented a very attractive option for less wealthy farmers, and was used for ECF control in the country for at least 10 years. However, one problem was that the number of sporozoites within a stabilate batch was highly variable, making vaccine standardization difficult. In addition, in other laboratories the virulence was found to vary, for example a cloned Boleni stabilate generated at ILRI (Morzaria, Dolan, Norval, Bishop, & Spooner, 1995) was highly virulent. Thus, wider scale out of the Boleni vaccine would be unlikely to meet with regulatory approval. However, the goal of creating a whole-sporozoite-based vaccine that does not require simultaneous administration of oxytetracycline remains a desirable option.

4 | THE SOCIO-ECONOMIC IMPACT OF LIVE VACCINATION

There have now been three different herd and homestead level impact studies of the trivalent Muguga cocktail version of ITM delivered on a cost recovery, or fully commercial basis to pastoralists in northern Tanzania and southern Kenya (Homewood, Trench, Randall, Lynen, & Bishop, 2006; Marsh, Yoder, Deoch, McElwain, & Palmer, 2016; Martins, Di Giulio, Lynen, Peters, & Rushton, 2010). All three concluded that the intervention was profitable and resulted in increased yields of meat and milk and disposable household income that translated into increased human capital, including female education (Marsh et al., 2016). The Homewood study concluded that wealthier pastoralists were more likely to adopt ITM initially, which is a likely scenario, given that the resource-poor pastoralists are typically least able to afford implementation of new technologies. However, significantly, although ITM is generally regarded as a private good, the Marsh study indicated that the subsequent use of acaricides for tick control and antibiotics for treatment of animals was reduced as result of vaccination, suggesting the accrual of broader societal benefits.

The cost of ITM is relatively high at an estimated US$ 7 per animal (Lynen et al., 2012). However, even in cross-bred dairy systems, where the economies of scale due to the smaller numbers of animals (3–7) kept are less than in pastoralist systems, the intervention is potentially profitable, since the cost of treatment is much higher at approximately US$ 40 per animal. Field trials in two ecologically distinct small dairy production systems in Hanang (highland) and Handeni (coastal) districts in Tanzania resulted in annual savings of US$ 4.77 per animal due to increased acaricide application intervals (Lynen et al., 2012). Despite the potential benefits highlighted by this

pilot study, adoption of ITM has to date been relatively limited in the small dairy sector.

Large-scale deployment of the ITM vaccination method and formal analysis of socio-economic impact have primarily been in pastoralist systems in Tanzania and southern Kenya. In Tanzania which has a cattle population of 18 million in total, with large numbers in the pastoralist sector, an analysis of 100 cattle (50 immunized and 50 unimmunized) from a Maasai homestead revealed a profit estimated to be US$ 5.58 per immunized calf (Martins et al., 2010). This was based on an increase in calf trade, an increased value of immunized calves in the market and higher milk production stimulated by increased letdown due to improved calf survival. It should be noted that in this study, control animals with symptoms of ECF were treated immediately, which is not a realistic scenario in normal production situations, given the requirement for early diagnosis and expense of treatment. A more recent study in southern Kenya, which encompassed a range of production systems from pastoralist to agropastoral, used a conceptual framework that went beyond the direct benefits of vaccination including increased meat and milk yields and reduced expenditure on acaricides, to examine indirect benefits on human education, health and nutrition (Marsh et al., 2016). The conclusions of the Marsh et al. study were that for the average household of 15 people in southern Kenya with a mean herd size of 66 cattle, the net benefit of ITM was US$ 35.80 at a vaccination cost of US$ 6.5 per head, with an average increase on educational expenditure of US$ 38.95 over a 4-month period.

Despite the benefits, the cost of vaccination of US$ 7 per animal may still be beyond the reach of poorest households as pointed out by Homewood et al. (2006). One solution to this constraint is that production and delivery (which is currently a commercial enterprise) should be subsidized by major development agencies and donors such as FAO, as was the case in the eradication of rinderpest through live vaccination (Njeumi et al., 2012).

5 | VACCINE STABILATE COMPOSITION

The Muguga cocktail trivalent ITM vaccine was designed following experiments performed over 40 years ago (Radley et al., 1975) that indicated that a panel of three stocks exhibited broader protection against heterologous *T. parva* challenge than any single constituent stock. A variety of recent studies using 'deep' NGS sequencing of variable number tandem repeats, antigen genes and complete genomes have indicated that: (a) this cocktail contains only a very small proportion of the diversity within the *T. parva* gene pool (Hemmink et al., 2016; Norling et al., 2015; Pelle et al., 2011), (b) the Serengeti-transformed and Muguga *T. parva* stocks are very similar to one another, whereas the third stock Kiambu V is very distinct with close to 40,000 single nucleotide polymorphisms (SNPs) when the whole genome is compared to that of the reference *T. parva* Muguga genome (Norling et al., 2015). Virtually, all known *T. parva* antigen-encoding genes are identical between Muguga and Serengeti including the polymorphic immunodominant molecule (PIM). Interestingly, the Serengeti stock does exhibit some non-synonymous substitutions relative to the Muguga reference genome, primarily in polymorphic multicopy gene families, but also in ATP-binding cassette transporter genes that are located as single copies immediately interior to the two major arrays of sub-telomerically encoded multicopy gene families.

The two main techniques for stabilate characterization are the use of a panel of micro- and minisatellites (variable tandem number variable repeats, acronym VNTRs) originally developed from the draft *T. parva* Muguga reference sequence (Oura et al., 2003). They are informative when applied to characterization of vaccine stabilates, especially when next-generation sequencing technologies are applied to specific VNTR loci to generate high coverage data that reveal the presence of 'minor' alleles (Hemmink et al., 2016).

CD8+ T-cell responses induced by ITM live vaccination have been shown to be important in mediating protection following adoptive transfer of a highly enriched population of CD8+ T cells between two identical twin calves generated by embryo splitting technology (McKeever et al., 1994). Genes encoding proteins recognized by CD8+ T cells induced by ITM, known as the 'Tp' (Tp1-Tp10) antigens, were cloned by screening libraries transfected into cell lines using an ELISPOT assay that measures production of gamma interferon by T-cell lines stimulated by autologous *T. parva*-infected T cells as the readout for positivity (Graham et al., 2006). To date, convincing protection induced by a defined Tp antigen has only been demonstrated for Tp1 (Svitek et al., 2018) and amounted to 36% protection in a group of cattle whose class I MHC loci were selected to bind the single currently known epitope encoded by the Tp1 gene. Following ITM vaccination, responses detected in an individual animal using assays designed to detect and selectively enrich for CD8+ T cells typically identify an 'immunodominant' antigen that is strongly associated with the MHC haplotype of the individual animal. However, it is possible that an aggregation of weaker T-cell responses to minor antigens contributes to the protection mediated by a CD8+ T-cell population such as those used in the adoptive transfer study (McKeever et al., 1994).

To date, a limited number of target populations have been immunized by infection and treatment. A recent study of an Ankole (an African taurine cattle breed) herd revealed that their class I MHC molecules were functionally distinct to those characterized previously, in terms of predicted peptide-binding specificities, and none of the *T. parva*-infected cattle were positive using ELISPOT when their PBMC was sensitized with overlapping peptides derived from Tp1, Tp2, Tp3, Tp4, Tp5, Tp6, Tp7 and Tp8 (Obara et al., 2016). There are likely thousands of as yet uncharacterized bovine class I MHC loci in Africa, for example a recent project using next-generation sequencing which genotyped predominantly Holstein-Friesian cattle, but also included some African cattle identified 140 novel alleles (Vasoya et al., 2016). Furthermore, there are 211 cattle breeds documented in the FAO database (http://dad.fao.org) for the region of Africa where ECF occurs. It therefore seems likely that there could be many more *T. parva* CTL target antigens, which potentially contribute to the protection mediated by ITM, awaiting discovery.

A recent experiment using MHC-matched animals demonstrated that *T. parva* Muguga alone provides an equivalent level of protection to challenge with the trivalent Muguga cocktail, as animals immunized with complete cocktail (Steinaa et al., 2018). These authors also note that the original experiment of Radley used Kiambu 1 (presumably representing a distinct stock that has not yet been genotyped or characterized phenotypically) as the heterologous challenge parasite (Radley et al., 1975). Collectively, these data suggest that it would be worthwhile to perform additional experiments to revisit whether a single stock, either *T. parva* Muguga, Serengeti or Kiambu V (which induces a long-term carrier state (Oura et al., 2007)), unlike Muguga (Skilton, Bishop, Katende, Mwaura, & Morzaria, 2002) could replace the trivalent cocktail. Kiambu V is the most clonal of the three vaccine components according to deep sequencing (Hemmink et al., 2016) and might therefore be the best candidate. This would simplify the production process and in addition reduce the risk of recombination between vaccine components.

6 | STABILATE PRODUCTION AND DELIVERY: POTENTIAL IMPROVEMENTS

Major avenues for improving production and delivery of ITM were discussed at a GALVmed convened meeting of all major stakeholders held at the International Livestock Research Institute (ILRI), and the recommendations are summarized by Toye and Ballantyne (2015). These included relatively easily implementable measures such as simplification of the composition and stabilization at room temperature of the diluent. An additional goal was increasing post-reconstitution stability of the cryopreserved sporozoites from four to 12 hr. More challenging would be an attempt to thermostabilize the sporozoites so that they remain viable at room temperature, using lyophilization, a strategy which is effective for live attenuated viral vaccines, but has proved challenging for eukaryotic pathogens. Another recommended modification was packaging containing smaller doses than the current 40 dose straw, for dairy systems with smaller numbers of more valuable taurine or cross-bred animals. In this context, an innovation has very recently been described that would allow delivery of 4–8 doses per straw (Patel et al., 2019).

A longer term option would be to try to attenuate the parasite with the goal of removing the requirement for the use of oxytetracycline in live vaccination. Irradiation of sporozoites has proved successful *for Plasmodium falciparum*, and these provide protection in humans (Hoffman, Subramanian, Collins, & Venter, 2002). Irradiation has been attempted for purified *T. parva* sporozoites using a ^{60}Co source at doses between 4.0 and 10 kilorads, without any apparent attenuation in vivo in cattle (Cunningham et al., 1973). Chemical attenuation involving treating *Plasmodium* sporozoites with the DNA alkylating agent centanamycin has also worked well in rodent malaria models (Purcell, Yanow, Lee, Spithill, & Rodriguez, 2008), but has not yet been attempted for *Theileria*. Parasite transfection and genetic modification is a further option and has worked for *Plasmodium*. However, this has proved technically difficult in *Theileria*. Transient transfection systems have been reported for *T. parva* (De Goeyse et al., 2015), with reporter genes being expressed in sporozoites, driven from parasite promoters following nucleofection to introduce the constructs into the parasite. There is also an earlier protocol published for the related *Theileria annulata* involving transient expression of green fluorescent protein from several parasite promoters in sporozoites treated with a lipofectin reagent (Adamson et al., 2001). There have not been any further publications in this area to date. However, with the development of more sensitive reporter molecules such as nanoluciferase, and CRISPR-CAS9 technology for precise genome editing (reviewed by Kim, 2016), which has been applied successfully to protozoan parasites including *Toxoplasma* and *Plasmodium* (reviewed by Suarez, Bishop, Alzan, Poole, & Cooke, 2017), this approach may now be worth revisiting.

One recent initiative which may increase the sustainability of delivery, by ultimately reducing the dependence on liquid nitrogen, involves simple modifications to the structure and handling of liquid nitrogen dewars that are designed to minimize temperature fluctuation in straws that are briefly exposed to ambient temperatures, when the flask is opened. This has worked well for improving artificial insemination by ensuring that the straws, although their contents are not thawed, do to reach the critical transition temperature of water of −137°C, which results in reorganization of water molecules and damage to membranes when the contents are returned to the liquid nitrogen (Lieberman, McClure, Harston, & Madan, 2016). As an extension of this work, the Seattle-based company Intellectual Ventures is exploring the potential of keeping sporozoite stabilates on dry ice at −80, for various periods of time with support from the Intellectual Ventures Global Good Fund (funded by the Bill and Melinda Gates foundation). The rationale for this is that many countries in the endemic ECF region, including Tanzania and Uganda, do not currently have sustainable liquid nitrogen production industries but do produce dry ice, which has a wide range of industrial applications.

7 | THE SPREAD OF *THEILERIA PARVA* WITHIN SUB-SAHARAN AFRICA

The distribution of *T. parva* is not as wide as that predicted for the vector according to climate-based models (Norval et al., 1992), so there is theoretically potential for spread of *T. parva*, especially given the largely un-regulated transboundary cattle movements between certain countries within the East and central African region. *Theileria parva* is moving north in the Sudan (Malak et al., 2012; Marcellino et al., 2017) and has recently been discovered for the first time in Cameroon (Silatsa et al., article in this special issue). *T. parva* can therefore be considered an emerging pathogen.

It is likely that anthropogenic cattle movement has played a major role in the spread of the parasite in both instances, but some aspects of the epidemiology are distinct between Cameroon and South Sudan. In South Sudan, the tick vector *Rhipicephalus appendiculatus* is present in all of four cattle populations surveyed and

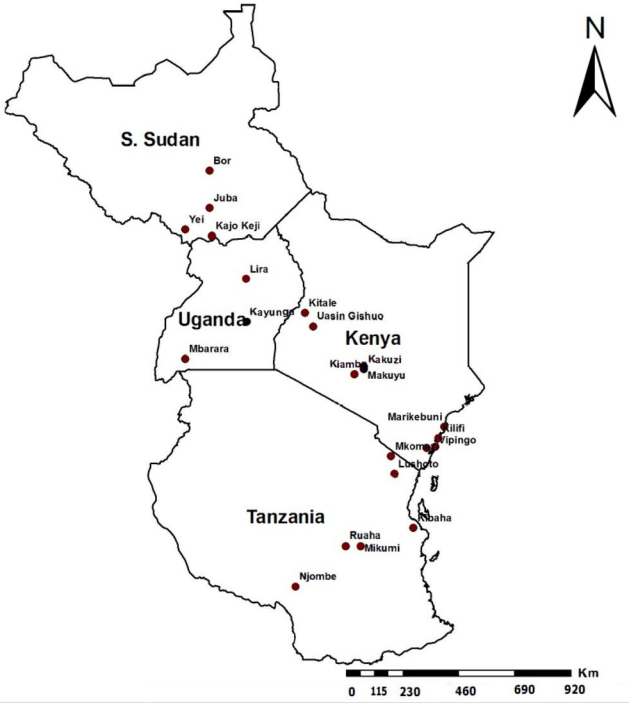

FIGURE 1 Map showing location of VNTR genotyping studies in Kenya (Odongo et al., 2006), Uganda (Oura et al., 2005), Tanzania (Mwega et al., 2015) and South Sudan (Salih et al., 2018)

25% were infected with *T. parva* according to a sensitive PCR assay based on the p104 antigen (Marcellino et al., 2017; Odongo, Sunter, Kiara, Skilton, & Bishop, 2010). The PCR assay revealed a 13% infection rate in cattle blood and the PIM ELISA (Katende et al., 1998) demonstrated 23% seroprevalence. Genotyping using 14 micro- and minisatellites (Salih et al., 2018) revealed very high levels of diversity with an average of 9.57 alleles per locus and a range of between 6 and 15 alleles per locus. Principal components analysis indicated a degree of substructure suggesting that the populations were partially distinct with limited gene flow between them. This might suggest that there have been multiple migrations of *T. parva*-infected cattle, together with the tick vector, into different regions of South Sudan. The fact that the vector is present suggests that ECF is likely to be a serious problem for cattle keepers in South Sudan. In a participatory survey, farmers from one of two districts regarded ECF as their most serious animal health problem (Malak et al., 2012). Live vaccination should therefore seriously be considered as a control option in this country, especially given that sequencing of the Tp1 and Tp2 genes from Sudanese isolates revealed close genetic similarity of these antigens to stocks within the Muguga cocktail (Salih et al., 2017).

By contrast, in Cameroon, all animals surveyed were asymptomatic and a parallel comprehensive countrywide tick survey did not reveal the presence of the major vector *R. appendiculatus*, although the other expected ixodid tick species associated with livestock were present (Silatsa et al., submitted). Among animals surveyed using ELISA, 23.76% were serologically positive and of 1,324 DNA samples from blood assessed by PCR using the p104 nested assay, 1.86% representing 25 animals were positive. Sequencing of the p104 gene has generally received limited use as a polymorphic marker to date, perhaps because it is the standard diagnostic PCR gene (Odongo et al., 2010; Skilton et al., 2002). The p104 sequences of *T. parva* from Cameroon revealed seven genotypes, some of the rarer ones not having been described previously. There were only two p104 alleles present in the complete genome sequences of the Muguga cocktail component stocks, while the most frequent genotype was identical to that of the Kenyan coastal stock *T. parva* Marikebuni (Morzaria et al., 1995) that was field tested as an ITM vaccine on farms near Kitale in western Kenya (Wanjohi et al., 2001). The Tp1 alleles present were identical to those in the three stocks comprising the ITM stabilate. This scenario may indicate spread of *T. parva* by vaccination as suggested by McKeever (2007). However, the distances involved for cattle migration would be very substantial and additional analyses of VNTRS and Tp antigens using high-resolution next-generation sequencing (NGS) will be required to determine the origin of the *T. parva* parasites in Cameroon. More detailed monitoring of transboundary cattle movements will also contribute to our understanding of how *T. parva* has reached Cameroon.

What is clear from the data is that *T. parva* is present in asymptomatic cattle in Cameroon and that there is currently no proven tick vector in the country, although tick species that have been shown to be susceptible to *T. parva* infection in the laboratory (Norval et al., 1992) do occur. This situation could change either by migration of ticks into the country on cattle, or evolution of a novel vector. The data also raise the question of how long the parasite has been present in Cameroon, and whether there are other countries in the region where cattle may harbour cryptic *T. parva* infections.

8 | *THEILERIA PARVA* DIVERSITY IN THE FIELD

Theileria parva diversity in field populations has been assessed in multiple recent studies using Tp CD8+ target antigen gene polymorphism and analysis of VNTRs. Whole genome sequencing is emerging as a tool for the future (Hayashida et al., 2013; Henson et al., 2012). The initial and most comprehensive study on variation within the Tp antigens, in which 82 cattle and buffalo field isolates were examined, focused on Tp1 and Tp2 which contain one and six mapped epitopes, respectively (Pelle et al., 2011). Both antigens were highly variable, but much of this diversity was either in buffalo isolates or in cattle isolates that were obtained from animals that had been challenged by ticks that fed on co-grazing buffalo and succumbed to ECF (Bishop et al., 2015). Diversity among cattle isolates was limited. Interestingly, although analysis of the ratio of synonymous to non-synonymous substitution was consistent with selection in some domains in these two antigens, there was no evidence for positive selection within the mapped epitopes (Pelle et al., 2011).

Over the last 5 years, there have been a number of studies examining *T. parva* diversity in field populations (Figure 1). These have

used between 12 and 30 VNTRS, often in combination with the Tp1 and Tp2 antigens. The VNTR data typically reveal very high levels of diversity when applied to field populations from endemic areas in Kenya, Tanzania, Uganda and South Sudan (Oura, Asiimwe, Weir, Lubega, & Tait, 2005; Odongo et al., 2006; Nanteza et al., article in this issue; Mwega et al., 2015; Salih et al., 2018). For example, 5–11 alleles per locus and a total of 183 alleles at 30 loci were identified among schizont-infected lymphocyte isolates from three regions in Kenya (Odongo et al., 2006) and 82 multilocus genotypes were identified from three distinct regions in Uganda (Oura et al., 2005). In studies involving multiple geographically separated regions, analysis of the total data sets often reveals linkage equilibrium and linkage disequilibrium, which is presumably at least in some instances a consequence of epidemic structures, apparent when samples from smaller regions are examined individually. Analysis of molecular variance has revealed that typically the majority of the variation appears to be within rather than between populations. Additionally, the genotypes of isolates separated by large-scale (between countries or regions) and medium-scale distances (within countries) show little correlation with the geographical origin of the parasite.

The high levels of diversity in VNTRs and some antigen genes including p104 (Skilton et al., 2002) and PIM (Toye, Gobright, Nyanjui, Nene, & Bishop, 1995) can be attributed to the obligate sexual cycle of *T. parva* in the tick vector. Two experimental crosses have been performed involving co-infections of cattle with parasite clones and tick feeding on cattle and subsequent isolation of clones (Henson et al., 2012; Katzer, Lizundia, Ngugi, Blake, & McKeever, 2011). The first of these studies revealed a high frequency of recombination in the 35 progeny clones, with specific 'hotspots' of recombination identified (Katzer et al., 2011). The second study used 454 Roche pyrosequencing to analyse the progeny clones and identified a total of 64,000 SNPs when progeny and parents were compared to the *T. parva* Muguga reference strain. 50% of the crossovers were accompanied by gene conversion events. Such a high frequency of gene conversions means that meiotic recombination plays a significant role in the evolutionary process by not only re-distributing genetic material but also altering allelic frequencies (Henson et al., 2012).

By contrast, the Tp1 and Tp2 antigen genes exhibit rather limited variation with the alleles observed frequently being the same as those present in the Muguga cocktail, with occasional variants within Tp2 (Nanteza et al., article in this issue; Mwega et al., 2015; Salih et al., 2017). Since the class I MHC haplotype of the animals involved in these field studies is largely unknown, it is unclear whether Tp1 and Tp2 are actually CD8+ T-cell targets in the *T. parva* populations sampled in these studies.

9 | *THEILERIA PARVA* DIVERSITY IN THE WILDLIFE RESERVOIR HOST CAPE BUFFALO (*SYNCERUS CAFFER*)

The cape buffalo wildlife reservoir of *T. parva*, as already mentioned, harbours a much greater diversity of *T. parva* genotypes according to analysis of Tp antigen gene sequences (Pelle et al., 2011) and also p67 gene sequences (Obara et al., 2015). The greater genetic diversity in buffalo has been confirmed by whole genome sequencing of DNA isolated from partially purified schizonts from infected T-cell lines (Hayashida et al., 2013). Additionally, buffalo are almost invariably infected with multiple parasite genotypes (Oura, Tait, Asiimwe, Lubega, & Weir, 2011a).

Early studies involving ITM immunized cattle kept in paddocks together with buffalo indicated that *T. parva* parasites from ticks fed on buffalo could 'breakthrough' the immunity induced by live vaccination (Radley et al., 1979). Recently, two studies in ranches in the Central and Rift Valley Provinces of Kenya where buffalo grazed adjacent to cattle immunized with both the trivalent cocktail and the Marikebuni ITM vaccine showed that most vaccinated cattle were not immune to challenge from ticks that fed on buffalo and contracted severe disease (Bishop et al., 2015; Sitt et al., 2015).

By contrast in Tanzania, there are no documented cases of live vaccine 'breakthrough' in the Maasai areas where buffalo graze adjacent to ITM-vaccinated cattle (Homewood et al., 2006). The only study so far conducted of the genetic diversity of *T. parva* in buffalo in Tanzania was that of Rukambile et al. (2016) using VNTR sequences applied to a limited number of buffalo (30) from several geographically separated national parks. The mean number of alleles at 3.37 was relatively lower than would be expected for *T. parva* populations in buffalo in Kenya or southern Uganda. However, the animals were from national parks and not in pastoralist areas where buffalo co-graze adjacent to vaccinated cattle. The comparative analysis of parasites in buffalo and cattle in vaccination areas awaits a definitive study.

There have been several studies on the population genetics of the parasite in buffalo. It was shown using VNTRs that in the area adjacent to, and within, Lake Mburo National Park in south-west Uganda, *T. parva* populations in cattle and buffalo were distinct and presumably reproductively isolated (Oura et al., 2011a). This is consistent with infrequent transmission of parasites from buffalo to cattle that subsequently become tick-transmissible between cattle. A longitudinal study is required in order to ascertain how frequent transmission from buffalo to cattle is in this area, and whether the typical buffalo-derived *T. parva* parasite 'corridor disease' syndrome of rapid death, with low schizont parasitosis and piroplasm parasitaemia in the blood, is the typical outcome in infected cattle.

A recent comparison of Tp antigen gene diversity, using six Tp antigens, including Tp1 and Tp2 between parasite populations present in buffalo from central Kenya and South Africa revealed that there was between 10% and 69% nucleotide variation between different genes. However, although there was extensive allelic variation within individual cattle, analysis of molecular variance suggested that much of the underlying genetic variation was ancient and preceded divergence of the two populations (Hemmink et al., 2018). The selective pressures driving divergence of the Tp antigens remain unknown, but are not necessarily due to the immune response of the mammalian host. A study of Tp antigen gene sequence variation in a buffalo population in central Kenya (Sitt, Pelle, Chepkwony, Morrison,

& Toye, 2018) was notable in that certain Tp antigens revealed very little polymorphism indicating that this is not an invariable property of CD8+ T-cell target antigens.

It is interesting to note that cape buffalo are capable of mounting CD8+ T-cell responses to *T. parva*-infected lymphocytes (Baldwin, Malu, & Grootenhuis, 1988). However, it has not yet been demonstrated whether these responses contribute to the *T. parva* tolerance phenotype exhibited by buffalo. Class I MHC haplotypes have yet to be analysed in buffalo and the extent to which peptide-binding specificity overlaps that in cattle is unknown.

10 | THE CARRIER STATE AND DISSEMINATION OF PARASITES USED FOR INFECTION VACCINATION INTO FIELD TICKS AND CATTLE

Because the ITM vaccination or natural infection of cattle typically induces a tick-transmissible carrier state (Bishop et al., 1992; Kariuki et al., 1995; Oura et al., 2004; Skilton et al., 2002), it is likely that immunizing parasite genotypes may transfer into field ticks and cattle. The presence of a vaccine component in unvaccinated cattle was first definitively described using molecular markers specific for the Kiambu V stock using primers derived from the PIM gene (Oura et al., 2007). This work was performed at a Muguga cocktail ITM vaccination trial site near Iganga in eastern Uganda. The re-sampling was done 3 years after of the initial sampling that demonstrated the Kiambu V carrier status in vaccinated animals (Oura et al., 2004). Based on this result, it was suggested that live vaccination could spread, as well as control, the disease (McKeever, 2007). This is a possibility, particularly in the case of vaccination in areas on the margins of the current *T. parva* distribution. However, the data from South Sudan and Cameroon suggest that the parasite may already be moving as a result of anthropogenic movement of immune carrier cattle from endemic areas. Further investigation will be required to test whether ITM may be involved in disseminating the parasite in some situations.

An additional study for which the primary data were collected approximately 20 years ago involved a 100 animal field trial of the Marikebuni live vaccine by the Kenya Agricultural Research Institute (Wanjohi et al., 2001). In the collaborative study involving ILRI and funded by DFID, 20 cattle were selected from the experimental group (10 vaccinated and 10 unvaccinated) and monitored using PCR-based assays. One set of PCR primers was derived from the hypervariable N-terminal end of the TpR locus (Bishop, Musoke, Morzaria, Sohanpal, & Gobright, 1997) and designed to be specific for the cloned Marikebuni stock (Morzaria et al., 1995). In addition, a p104-based nested PCR assay that detects all *T. parva* but not other *Theileria* species (Odongo et al., 2010; Skilton et al., 2002) was also used to monitor the cattle. The animals were monitored for 2 years and 8 months post-vaccination. With one exception, all of the vaccinated animals were carriers of the clonal Marikebuni component, and 4 of the unvaccinated animals were positive between 14 and 24 months with the Marikebuni-specific markers (Bishop et al., article in this issue). A panel of 24 of the 60 VNTR markers described by Oura et al. (2003) was applied to 19 clonal *T. parva*-infected T-cell cultures, prepared either directly from the cattle at the vaccination site or from ticks collected and subsequently applied to cattle for schizont-infected cell line isolation. The study revealed two Marikebuni clonal component profiles and two Kiambu V profiles in *T. parva*-infected cell lines isolated directly from the cattle. The seven cattle cell lines isolated following tick on cattle feeding all exhibited a profile that according to the MS7 VNTR locus could be either Serengeti or Muguga.

It is known that Muguga does not generate a long-term PCR-detectable carrier state, only being detectable experimentally by PCR of blood for 90–130 days, and being apparently unable to transmit to ticks (Skilton et al., 2002). The ability of Serengeti to induce a carrier state has never been investigated. Although, as already mentioned, very similar to *T. parva* Muguga, Serengeti does contain 53 loci with non-synonymous SNPS relative to the *T. parva* Muguga reference genome at certain highly polymorphic loci (Norling et al., 2015). There had been no application of Muguga cocktail ITM vaccination anywhere in Kenya at the time this study was performed. It therefore seems possible that Muguga cocktail parasites originated from small-scale pilot trials across the border in Uganda, Tororo, being the closest live vaccine trial site. Alternatively, these genotypes could have been circulating naturally in western Kenya. A recent longitudinal study (Gwakisa et al., article in this issue) of cattle in northern Tanzania also demonstrates the transfer of Serengeti/Muguga ITM components into unvaccinated cattle, providing a third example of Muguga cocktail genotypes being transmitted to unvaccinated animals. This longitudinal study also demonstrates that although the number of PCR-detectable animals declines with age, one animal was still positive by p104 PCR 14 years post-vaccination. It has never been investigated how important the carrier state is in enhancing protection by modulating the kinetics of recall of protective T cells in cattle upon infected tick challenge. If the carrier state is important, a carrier state of this longevity would suggest that induction of lifetime immunity is a real possibility at least for some ITM-vaccinated cattle.

11 | POPULATION GENETICS AND DISTRIBUTION OF THE TICK VECTOR *RHIPICEPHALUS APPENDICULATUS*

A multilocus VNTR genotyping study of *Rhipicephalus appendiculatus* from 10 populations within Kenya revealed a lack of genetic structure in the field populations (Kanduma, Mwacharo, Githaka, et al., 2016; Kanduma, Mwacharo, Meaura, et al., 2016). This is consistent with *T. parva* population analyses that revealed a similar situation for *T. parva* in Kenya (Odongo et al., 2006). The data suggest that anthropogenic cattle movement rapidly homogenizes tick populations. Another illustration of this is the rapid spread north of *T. parva*-infected *R. appendiculatus* in South Sudan.

It was recently reported that there are two major haplotypes of *R. appendiculatus* that are sympatric within Kenya (Kanduma,

Mwacharo, Githaka, et al., 2016; Kanduma, Mwacharo, Meaura, et al., 2016). A similar phenomenon was subsequently described in the Great Lakes region (Amzati et al., 2018). We do not yet know whether there is any functional significance in these two distinct haplotypes. However, what is known is that there are major differences between *R. appendiculatus* lines in their ability to acquire and transmit *T. parva* (Ochanda, Young, Medley, & Perry, 1998; Odongo et al., 2009).

It is generally considered that distribution of *T. parva* is primarily limited by the tick vector which is in turn determined by climate (Norval et al., 1992). Oura, Tait, Asiimwe, Lubega, and Weir (2011b) performed a reverse line blot (RLB) survey of buffalo in the two northern Uganda national parks, Murchison Falls and Kidepo Valley, and surprisingly, no *T. parva* was detected using this method although the parasite was readily detectable in the south-western Uganda national parks. Recent work (Obara et al., article in this issue) confirms this result using the PIM ELISA (Katende et al., 1998) and the more sensitive nested p104 PCR assay. A team of Ugandan and ILRI scientists (including the ILRI tick unit manager) twice visited Kidepo and Murchison parks to sample ticks in buffalo adjacent areas and found that, although other expected livestock ticks were present, *R. appendiculatus* was absent. This is surprising because both these parks superficially appear climatically and ecologically suitable for the tick. It does, however, parallel the result of the countrywide Cameroon tick survey (Silatsa et al., submitted), where *R. appendiculatus* was also absent. These data suggest that there may be some biotic factor constraining the distribution of the tick. On a practical note, these results also indicate that buffalo-derived *T. parva* challenge of ITM-vaccinated cattle in northern Uganda should not pose serious problems for ITM effectiveness.

On a different theme, but likely relevant to the success of ITM in northern Tanzania, a study of vaccinated cattle and unvaccinated control cattle adjacent to Tarangire National Park (Kazungu et al., 2015) revealed an incremental increase in seropositivity, assessed by PIM ELISA, the closer cattle were to the park. *Theileria parva* prevalence using p104 nested PCR was also higher closer to the park. This suggests that challenge by ticks that have fed on wildlife (including buffalo) can have a positive effect in boosting immunity.

12 | FUTURE FOCUS AREAS FOR RESEARCH RELEVANT TO ITM DEPLOYMENT AND EFFICACY

Suggestions for priority future research include: (a) a comparative survey of *T. parva* prevalence and diversity in buffalo, ticks and cattle in Tanzania, (b) a meta-analysis combining data from the major *T. parva* VNTR population genetic studies performed to date in order to gain a broader overall overview of parasite population genetics, (c) establishment of whole genome sequencing as a tool for genotyping *T. parva* field isolates, (d) application of population genomics using both deep sequencing of individual loci and whole genome sequencing to provide insight into parasite diversity, (e) application of population genomics would be particularly informative at sites adjacent to previous vaccine trials where baseline monitoring has already been performed, and (f) surveys for *T. parva* in additional countries that are potentially exposed to transboundary cattle movement from endemic (or transiently affected) countries. These might include: Central African Republic, South West Ethiopia and Nigeria, (g) more in-depth analysis of Cameroonian *T. parva* parasites and cattle migration patterns to provide insight into the origin of *T. parva* in central Africa, (h) analysis of the limits of distribution of *T. parva* infection in Cape buffalo in countries including South Sudan, Rwanda and Democratic Republic of Congo, (i) sampling of carrier cattle to include lymph node biopsies to determine whether apparent loss of the carrier state based on PCR detection in blood is reflected in lymphoid tissues, (j) assessment of whether induction of a long-term infection (carrier state) is required to sustain immunity against field challenge, and (k) re-assessment of the possibility of replacing the trivalent cocktail with one of the component stocks. One issue this could affect positively would be ease of vaccine registration. Given the nature of the production of ITM stabilates, good manufacturing practice (GMP) as strictly defined is almost impossible to achieve. However, a vaccine based on a single stock would potentially be easier to standardize between different production runs.

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ETHICAL STATEMENT

Ethical statement is not applicable as there was no sample collection and questionnaire data from animals/humans were not gathered.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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