Abstracts presented at a workshop on genetic characterization of *Theileria parva* strains, January 19-20, 2017, Nairobi Kenya.

1. Extent and nature of antigenic and genetic diversity in the *Theileria parva* parasite populations compared with that of the Muguga cocktail live vaccine

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Abstract:

The tick-borne protozoan parasite *Theileria parva* causes an acute, often fatal disease in cattle known as East Coast fever (ECF), which is a major constraint to livestock production in an area extending over 16 countries in eastern, central and southern Africa. African buffalo is the main reservoir of the *T. parva*. Immunization of cattle with a mixture of 3 isolates of T. parva (the Muguga cocktail) by infection with T. parva sporozites and simultaneous treatment with long-acting tetracycline results in long term immunity against the homologous parasite strain but variable protection against challenge with heterologous parasite strains. Hence, efficient vaccination of cattle in the field by this method requires a match between vaccine and field parasite strains. In order to understand the basis of strain restricted immunity to *T. parva* and provide data that will enable further optimization of the parasite strain content of the live vaccine to achieve more robust immunity, recently identified *T. parva* antigens, as well as a panel of micro- and mini-satellite, were used to determine the extent and nature of antigenic and genetic diversity in the parasite populations and compare with those of the Muguga cocktail vaccine.

The results reveal extensive polymorphism in *T. parva* isolates but they are not associated with their geographical origin or mammalian host species. However, the extent of diversity is much greater in *T. parva* isolates originating from buffalo than in isolates known to be transmissible among cattle whereas *T. parva* parasites maintained in cattle represent a subset of the overall *T. parva* population, which has become adapted for tick transmission between cattle. However, the mechanism driving this diversity is still unknown. Compared with the field population, each of

the three component stocks of the cocktail contains limited parasite genotypic and antigenic diversity, with single alleles detected at many gene/satellite loci, and, moreover, that the Muguga and Serengeti components show a very high level of similarity. Thus, the vaccine incorporates very little of the genetic and antigenic diversity observed in field populations of *T. parva*. The presence of alleles at low frequency (<10%) within vaccine component populations also points to the possibility of variability in the content of vaccine doses and the potential for loss of allelic diversity during tick passage.

Overall, the results demonstrate that there is scope to modify the components of the vaccine in order to enhance its diversity and thus its potential for providing broad protection. The ability to accurately quantify genetic diversity in vaccine component stocks will facilitate improved quality control procedures designed to ensure the long-term efficacy of the vaccine.

Source:

1. Hemmink JD, Weir W, MacHugh ND, Graham SP, Patel E, Paxton E, Shiels B, Toye PG, Morrison WI, **Pelle R**. Limited genetic and antigenic diversity within parasite isolates used in a live vaccine against Theileria parva. Int J Parasitol. 2016: S0020-7519(16)30023-6.
2. Norling M, Bishop RP, **Pelle R**, Qi W, Henson S, Drábek EF, Tretina K, Odongo D, Mwaura S, Njoroge T, Bongcam-Rudloff E, Daubenberger CA, Silva JC. The genomes of three stocks comprising the most widely utilized live sporozoite Theileria parva vaccine exhibit very different degrees and patterns of sequence divergence. BMC Genomics. 2015 Sep 24;16(1):729.
3. **Pelle R**, Graham SP, Njahira MN, Osaso J, Saya RM, Odongo DO, Toye PG, Spooner PR, Musoke AJ, Mwangi DM, Taracha EL, Morrison WI, Weir W, Silva JC, Bishop RP. 2011. Two Theileria parva CD8 T cell antigen genes are more variable in buffalo than cattle parasites, but differ in pattern of sequence diversity. PLoS One 6(4):e19015.

2. Genetic and antigenic diversity and population structure of *Theileria parva* in South Sudan

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Theileria parva is a parasitic protozoan that causes East Coast fever (ECF; theileriosis) an economically important disease of cattle in eastern, central and southern Africa. In South Sudan, ECF is considered a major constraint for development of livestock in regions where the disease is endemic. In an attempt to provide insight into the dynamics of the *T. parva* parasite in South Sudan, the sequence diversity among Tp1 and Tp2 antigens, as well as population genetic analysis was performed. A total of 178 *T. parva* positive blood samples collected from cattle from four regions in South Sudan (Bor = 62; Juba = 45; Kajo keji = 41 and Yei = 30) were genotyped using 14 microsatellite markers spanning four chromosomes. For sequencing analysis, 81 samples were used. Eight positions (1.97%) in Tp1 and 78 positions (15.48%) in Tp2 were shown to be polymorphic, giving rise to four and 14 antigen variants in Tp1 and Tp2, respectively. The overall nucleotide diversity in the Tp1 and Tp2 genes was π= 1.65% and π= 4.76%, respectively. Linkage disequilibrium was evident when populations from the four regions were treated as a single genetic stock. But, when analyzed separately, linkage disequilibrium was observed in Bor, Juba and Kajo keji. Juba region had a higher multiplicity of infection than the other three regions. The principal component analysis revealed a degree of sub-structure between isolates from each location. This indicates that populations are distinct with genetic exchange and gene flow between parasites in the four region studied. The sub-structuring observed, along with the evidence of panmixia in the populations, could have been due to high transmission rates. It is concluded that *T. parva* population genetic analyses from four populations in South Sudan showed a low level of genotype exchange between the populations, little evidence of genetic differentiation but a high level of genetic diversity within each population, implying genetic and geographic sub-structuring between the populations.

3. *Theileria parva stocks* characterization in Burundi

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Abstract

Livestock has been identified by Burundi Government as a key component of national development program (CAADP) as it contributes to 13% of GDP (Strategic Guidance Document, 2007). In the last decades, improved breeds have been disseminated and given to rural farmers to increase animal productivity and thus meet the challenges of food security. More than 10.000 of cattle were distributed to rural farmers (DGE, 2015). However, according to study conducted by Tama and Banuma (1987), 80% of improved animals succumb from theileriosis once in a rural environment. Moreover, theileriosis hinders the expansion of local cattle breeds. East Cost fever (ECF) is a devastating disease affecting livestock development in Burundi since 1907 corresponding with the first report of the presence of *Theileria parva* in the South Kagera River. To cope with this problem, vaccination against theileriosis could be the efficient way to protect animals and increase livelihoods of farmers.

In 1987th, a national research program was initiated in Burundi to develop a vaccine based on inoculation of *T. parva* live sporozoites and simultaneous treatment with a long acting oxy-tetracycline as tools for controlling ECF. A study based on the above strategy was evaluated in calves using three local strains (Gatumba, Gitega and Ngozi) and on challenge 96% of the immunized animals were found to be protected (Tama and Banuma, 1987). Unfortunately, these results have not been confirmed.

Recently, a study to identify the isolates responsible of Theileriosis in Burundi was carried out by the National Veterinary Laboratory under the initiative of the Global Alliance of Veterinary Medicine (GALVmed) to assess the feasibility of implementing the available Muguga Cocktail vaccine or to answer the question whether Burundi isolates could serve as immunizing strains instead of the Muguga Cocktail. The results of epidemiological study indicated a high prevalence of ECF in Burundi, both in ELISA (60.1%) and parasitology (29.9%).

As cross-protection between *T. parva* stocks is limited, precise evaluation of genetic diversity in field populations of the parasite will allow deployment of appropriate isolates of *T. parva* (Muguga Cocktail) to protect cattle against ECF in Burundi. For this purpose, 1000 samples from clinical cases will be collected in 10 different provinces representing agro-ecological zones of Burundi. Prevalence and distribution will be determined by ELISA and p104 nested PCR, *T. parva* diversity by Tp1 and Tp2 PCR and sequencing. Bioinformatics analysis (phylogenetic and multiple alignments) will be conducted to analyze similarity and diversity. Cross-matching the local strains of *T. parva* in Burundi with Muguga cocktail vaccine strains would

indicate whether the Muguga cocktail could be used to protect animals against ECF or further work would be required to characterize new isolates that can be incorporated into the Muguga cocktail. The result of this study will provide essential baseline for deployment of ECF vaccine in Burundi.

4. Eco-epidemiology of East Coast fever in the Eastern Congo, Rwanda and Burundi: cross-sectional, longitudinal and genetic studies of *Rhipicephalus appendiculatus* and *Theileria parva*

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Abstract

Theileria parva is a protozoan parasite which causes East Coast fever (ECF), a lethal lympho-proliferative disease of cattle. ECF is an economically important disease in eastern, central and southern Africa due to a high rate of morbidity and mortality in cattle. The epidemics usually strike with high case-fatality; causing major cattle loss and significant social and economic distress to the individual farmer and can have negative effect on food production and food security in affected areas. Cattle can be immunized against the parasite by infection and treatment method (I&T), but immunity has been demonstrated to be strain specific. Knowledge of the genetic diversity and population structure of *T. parva* is critical for predicting pathogen evolutionary trends in view of developing effective control strategies. Cattle movements for sale and grazing within and between countries (DRC, Rwanda and Burundi), associated to the ecological and climatic variations, constitute a major risk of spreading infected ticks and *T. parva* strains. Recent outbreaks of the disease in the Great Lakes region have been associated with animal movements. In more recently infested areas in Democratic Republic of Congo, a large percentage of both adult and calf populations are still susceptible to ECF and major epidemics cause high losses of cattle each year in the Lowland. A different situation is observed in Highlands where only the calf population is the most affected and mortality is generally confined to calves. Successful control strategies of ECF in this region depends on a clear understanding of the genetic diversity of *T. parva* and *R. appendiculatus* and its importance in the epidemiology of ECF.
The overall objective of the project is to study the epidemiological status of ECF and the genetic diversity of *T. parva* and *R. appendiculatus* populations in The Great Lakes region of Central Africa.

This study investigated the genetic and antigenic diversity of *T. parva* in cattle and the genetic diversity of *R. appendiculatus* from different agro-ecological zones

(AEZ) of Eastern DRC, Burundi and Rwanda. Blood samples were collected from 800 cattle from three AEZ in Eastern DRC and two AEZ in Burundi. The overall percentage of *T. parva* positive samples determined by PCR using the p104 antigen gene primers was 45.6%, corresponding to 56.2% in Eastern DRC and 28.5% in Burundi. Sequence analysis of two antigen genes recognised by CD8+ T-cells (Tp1 and Tp2) were applied on p104 PCR positive samples. The *T. parva* antigenic diversity observed will provide some clues on the relevance to initiate I&T in the study area and which *T. parva* strains to target.
To assess the phylogenetic relationship between *R. appendiculatus* populations from Democratic Republic of Congo, Rwanda and Burundi, this study examined the DNA sequence variation of the mitochondrial genes Cytochrome C Oxidase subunit I (COI) and 12S Ribosomal DNA (12S rDNA) of 240 ticks collected from cattle and vegetation across different geographic locations in eastern DRC, Rwanda and Burundi. The description of the genetic diversity of the tick will contribute to a better understanding of the epidemiology of ECF in the region.

The genetic information from this study are useful to link the genetic diversity of the vector to the prevalence and transmission dynamic of *T. parva* and to evaluate the vectorial competence of *R. appendiculatus* originating from different agro-ecological conditions using mathematical modelling.

5. Study of bovine theileriosis (*Theileria parva* infection) in Bututsi region (Southern Burundi)

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The authors present an epidemiological study carried out in Bututsi region (Southern Burundi) about the distribution of tick population and *Theileria parva* infection prevalence in cattle between October and November 2013. A total of 239 bloods duplicated with sera samples and 143 ticks were randomly collected from asymptomatic cattle. In this study, analysis of ticks by cytochrome c oxydase sub unit 1 identified a tick population composed of *Rhipicephalus appendiculatus* (59%), *Rhipicephalus decoloratus* (*Boophilus*) (32%), *Amblyomma variegatum* (5%) and

Hyalomma marginatum (4%). An ELISA test and a nested PCR were carried out to identify *T. parva* infection. There was a relationship between the distribution of *R. appendiculatus* ticks and *T. parva* infection in cattle by serological and molecular techniques (PCR with p104, Tp1 and Tp2 antigen genes) that showed a prevalence of 60.7; 52.7; 43.5 and 39.7%, respectively. The infection prevalence by *T. parva* was higher in the extensive farming system (68.9%) compared with the intensive ($p<0.001$). A sequence analysis was performed. Among the 45 sequences of *R. appendiculatus* used to assess genetic diversity, 24 haplotypes defined by 83 polymorphic sites were obtained. There was a large haplotype and the diversity of the haplotype was 0.82 between the 24 haplotypes defined. The nucleotide diversity was 0.01 with an average number of nucleotide differences of 4.95. For Tp1, 61 sequences were used. The total number of sites obtained was 298 and 4 sites were variable. There were 7 haplotypes with diversity of 0.71. For Tp2, a total of 46 sequences defined by 430 sites was used and among them 182 sites were variable. There were 12 haplotypes with diversity of 0.83. Samples were randomly taken at each class level and compared to the different strains in the Muguga cocktail vaccine.

The following study will examine the genotype of *T. parva* in cattle in five regions of Burundi, identify the strains that exist in the country, introduce vaccines according to the strains identified and evaluate their effectiveness.

6. Prevalence and risk factors associated with *Theileria parva* infection in cattle in three regions of Tanzania

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Ticks and tick borne diseases (TBDs) are serious constraints to cattle production in Tanzania and other tropical and subtropical countries. Among the TBDs, East Coast fever (ECF) is the most important TBD known to cause significant economic losses in cattle in Tanzania. However, control of ECF in Tanzania is becoming difficult due to inadequate epidemiological information. The main objective of this study was to determine the epidemiological situation of *T.parva* infection in cattle kept under traditional farming systems in Mara, Singida and Mbeya regions of Tanzania. Blood samples were collected from 648 cattle in the three regions. In addition, information was collected on the possible risk factors of the disease. Genomic DNA from each cattle was extracted from 100 µl of whole blood sample using Quick –gDNA^{TM} Blood Mini Prep kit catalog Nos D3073 based on the manufacturer's instructions and then eluted in 100 µl elution buffer. All samples were screened using a nested polymerase chain reaction (nPCR) using *T. parva* specific primers targeting the 104-kD antigen (P104) gene. The prevalence of *T. parva* across the three regions was 14.2% (92/648). There was variation in prevalence among the three regions with Mara having a significantly higher (21.8%, 47/216, p = 0.001) prevalence followed by Singida (13.4%, 29/216) and Mbeya the lowest prevalence (7.4%, 16/216). Factors found to be significantly associated with the likelihood of an animal being PCR positive for *T.parva* were region (p = 0.001) and tick burden (p = 0.003). The present study showed high variation in tick burden and *T.parva* prevalence between regions. The upcoming study will involve antigenic and genetic analysis of *T.parva* using Tp1 and Tp2 antigen genes and min and microsatellite markers respectively in order to identify the existing strains in three regions of Tanzania. Samples from all the three regions will be compared with different vaccine strains. Vaccines will be introduced in the country based on the identified strains and evaluate their efficacy under field condition.

7. Characterization of *Theileria parva* in Zambia

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Theileriosis caused by a protozoan parasite known as *Theileria parva* has been the cause of over 10, 000 losses of cattle per year in Zambia. It is an important economical disease. In Zambia, theileriosis greatly hinders the development of the livestock industry especially in the well known endemic areas of the southern and eastern provinces. In recent years, theileriosis has been steadily spreading from the traditional endemic areas of eastern and southern provinces to previously naive areas. This spread has been attributed to the migration of cattle keepers from endemic areas into Lusaka, central, copperbelt and western provinces in search for better pastures and absence of major cattle diseases that have plagued the southern province. In these new areas, ITM, the main control strategy for theileriosis in Zambia, cannot be implemented because of the lack of information on the strain and diversity of the causative agent *T. parva*. In this regard, molecular DNA based tools such as sequence and microsatellite analysis have been implemented.

DNA extracted from cattle blood is subjected to PCR amplification using *Theileria parva* specific p104 gene primers. Positive p104 PCR samples are used to sequence the TP 1 and TP 2 genes coupled with microsatellite analysis. The results produced will help in determining whether, either the Chitongo or Katete strain can be used to vaccinate cattle or a new vaccine is required in the new areas. This information will guide the implementation of better and more effective control strategies ultimately improving the livestock development and the livelihood of cattle keepers in Zambia.

8. Progress on the characterization of Rwanda *T. parva* isolates

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Theileriosis (East Coast Fever - ECF) is caused by a hemoprotozoan parasite known as *theileria parva*. It is transmitted by *Rhipicephalus appendiculatus* and it is widely distributed in the in eastern, central and southern Africa. Control of ECF is based on vector control, cattle movements, chemotherapy and immunization (Infection and Treatment Method - ITM). ITM induces immunity based on Cytotoxic T-Lymphocytes, which destroy cells infected with schizonts. ITM has been feared of causing recombination and as such characterization of field isolates in new areas is important prior to its introduction. Several

T. parva antigens have been identified and of particular importance with respect to characterization is TP1 and TP 2 which are major targets for CD8 T-cell immune response in infected cattle. The similarity of the TP 1 and TP 2 epitope of the vaccine candidates to the field strains warrants vaccine compatibility.

In Rwanda, ITM is not yet registered due to the unavailability of information on the type of strains of *T. parva* prevailing in the field. With this regard, samples (n = 11) were collected from Bugesera district, eastern province in 2016 from animals manifesting clinical disease. These animals were part of a vaccine challenge trial carried out at Karama station (Bugesera district). DNA was extracted followed by PCR using the *T. parva* specific p104 gene primers. Partial gene fragments of TP 1 (n = 7) and TP 2 (n = 9) were then sequenced from p104 gene positive samples.

Phylogenetic analysis of TP 1 showed two clusters, one cluster comprised of Muguga vaccine strains and the majority of Rwanda samples (n = 6) while in cluster two, a single sample clustered with isolates from South Sudan and Uganda. Amino acid sequence alignment showed that six (6) samples from Rwanda had similar epitope with Muguga cocktail strains and one (1) variant implying that a similar immune response can be elicited for the majority of samples based on TP 1 antigen.

TP 2 phylogenetic analysis showed two clusters. In the first cluster, five (5) Rwanda samples clustered with Muguga vaccine strains while in the second cluster, four (4) samples formed a cluster with isolates from South Sudan and Uganda. Amino acid sequence alignment showed that three (3) samples had similar epitope as Muguga vaccine strains, two (2) were variants and the remaining four (4) had a similar epitope as the Uganda strain implying that TP 2 epitope was polymorphic and variable immune response can be elicited on TP 2 for the majority of Rwanda samples.

The results from this preliminary study shows evidence of the presence of two clusters of Rwanda samples implying the possible existence of two strains, one strain similar to Muguga cocktail vaccine and the second being similar to the Uganda strain. In order to validate this, further research is required.

9. Challenges in the immunization against East Coast fever.

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The infection and Treatment method of ECF immunization is the only practical means of vaccinating cattle against East Coast fever. The technology has been available for over forty years but only in the last 10 years has it been commercially

available. Despite a great deal of effort by many players, adoption of the technology has been quite low. To date, only about one and half million cattle have been vaccinated against a target of almost 50 million cattle at risk in East and Central Africa. Many reasons have been advanced for this low adoption.

Challenges to the delivery of the technology fall under three broad categories; constraints associated with the product itself, those associated with the unfavorable business environment and lingering concerns on the vaccine's safety.

The ITM technology is considered complex by service providers. Unlike other veterinary vaccines, it requires additional inputs to deliver. Animals require to be weighed to accurately determine their weight, it requires simultaneous injection with an antibiotic in addition to the vaccine, and animals need to be ear tagged for identification. The additional activities require more personnel. Animal health service providers find this too complicated and opt for easier- to-deliver products. The ITM technology is also relatively expensive compared to other vaccines. On average the vaccine costs about US$10 dollars. This is quite expensive for small holder farmers whose incomes are often less than two US$ dollars per day. Furthermore, the vaccine has to compete with other effective, less complex and cheaper products in the market including chemical acaricides, antitheilerial drugs and husbandry practices that reduce the risk of the disease.

The other set of constraints are associated with the general unfavorable business environment that affects other animal health products but impacts more on ITM. These include lack of cold chain facilities such as liquid nitrogen and refrigeration, lack of trained personnel and until recently there were concerns on the sustainable supply of the vaccine because batches were produced on an ad-hock basis.

The final set of constraints that might have constrained adoption of ITM, which is relevant to this meeting, has been the lingering concerns among scientists on the long term impact of live *Theileria* parasites on the epidemiology of ECF. The main concerns have been the direct introduction of different parasite stocks into a cattle

population through immunization and the possibility of new, more virulent stocks resulting from sexual recombination. The other concern has been since the majority of immunizing stocks create a carrier state in immunized cattle, this might pose a risk of spreading the disease into new areas, or by increasing the risk of exposure of susceptible animals to the disease. The final concern has been whether these risks have been made worse by the use of a single vaccine (e.g., Muguga cocktail) across the region rather than using local isolates. This debate has been largely speculative because our knowledge of genetic diversity of *Theileria* parasites and its biological significance is lacking. More importantly, is an understanding of the implications of the diversity in terms of the efficacy of the vaccine and the epidemiological impacts of vaccination.

Some of the constraints could be addressed through additional research while others have to await the next generation of ECF vaccines.

10. Sequence diversity in the *T.parva* CTL antigens in buffalo-derived parasites

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This project aimed to determine the extent of sequence diversity among the genes encoding 10 antigens known to be recognized by CTL from cattle immune to *T. parva*. The sequences were derived from parasites infecting 28 buffalo-derived cell lines. The results showed that there was a substantial difference in the amount of sequence diversity between individual antigen families. The greatest nucleotide and amino acid diversity was observed in the antigens Tp1, Tp2 and Tp9. In contrast, the genes encoding Tp 4, 5, 6 and 7 showed least diversity. Interestingly, only Tp1, Tp2 and Tp9 showed variation in the sequences of the respective defined CTL epitopes.

Six of the cell lines were shown to be infected with *T.* sp. buffalo. In general, the antigen sequences from these cell lines did not display unique characteristics when comapred to the *T. parva* sequences.

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11. Genetic and antigenic diversity in Theileria parva in vaccine stabilate and African Buffalo

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An infection and treatment protocol is used to vaccinate cattle against Theileria parva infection. Due to incomplete cross-protection between different parasite isolates, a mixture of three isolates, termed the Muguga cocktail, is used for vaccination. While vaccination of cattle in some regions provides high levels of protection, some animals are not protected against challenge with buffalo-derived Theileria parva. Knowledge of the genetic composition of the Muguga cocktail vaccine is required to understand how vaccination is able to protect against field challenge and to identify the potential limitations of the vaccine. The aim of the current study was to determine the extent of

genetic and antigenic diversity within the parasite isolates that constitute the Muguga cocktail. This was investigated using high throughput multi-locus sequencing of antigen-encoding loci using 454 technolog. In parallel, satellite typing using a panel of 15 micro- and mini-satellite loci was performed. The former focused on genes encoding $CD8^+$ T cell antigens, believed to be relevant to protective immunity. The results demonstrate that each of the three component stocks of the cocktail contains limited parasite genotypic diversity, with single alleles detected at many gene/satellite loci and, moreover, that two of the components show a very high level of similarity.

In contrast, a high level of antigenic diversity was detected in samples of African buffalo from the Kruger National park (South Africa) and the Ol Pejeta conservancy (Kenya). High multiplicity of infection was found in individual buffaloes. AMOVA demonstrated that most of the overall diversity is found within populations (90-100%) with only a small proportion of the diversity found between populations. Thus, the vaccine incorporates very little genetic and antigenic diversity, especially when compared to *Theileria parva* populations in African buffalo.