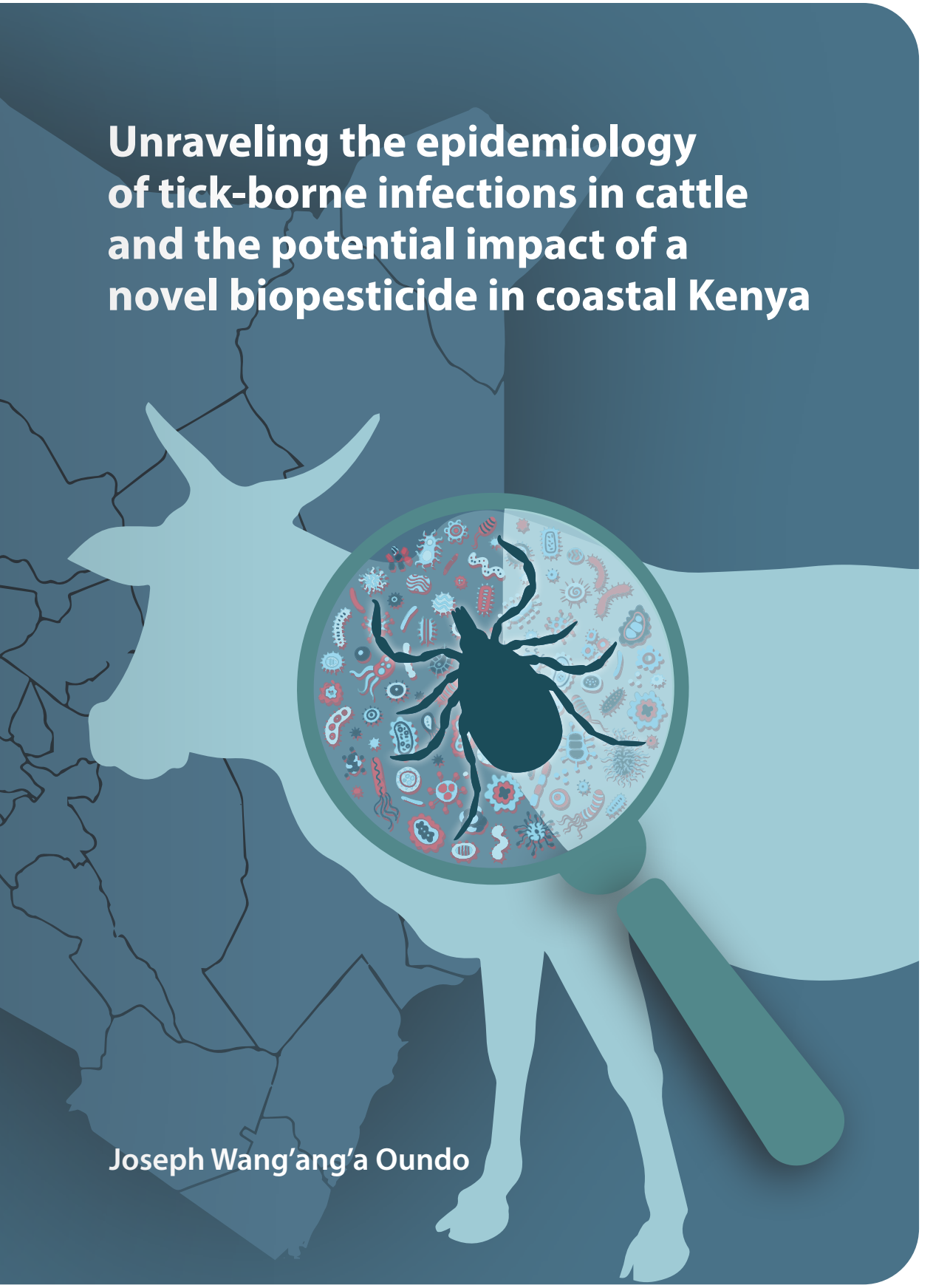
Unraveling the epidemiology of tick-borne infections in cattle and the potential impact of a novel biopesticide in coastal Kenya



Joseph Wang'ang'a Oundo

Propositions

1. The feasibility of using entomopathogenic fungi for the control of bovine tick-borne diseases is underestimated.
   (this thesis)
2. Understanding vector-host interactions in different environments is as essential as having an efficacious vector control tool.
   (this thesis)
3. The rise of peer-reviewed journals without an impact factor metric erodes the quality of science.
4. PhD programs that prioritize interdisciplinary collaboration are more effective in producing independent researchers than those with a specialized disciplinary focus.
5. The carbon credit system will delay the implementation of direct emission reduction measures and sustainable practices.
6. To enhance personal productivity, it is better to confront your self-doubt than focusing on personal strengths.
7. Access to internet and social media is the greatest instigator of the current migration crisis.

Propositions belonging to the thesis, entitled

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Thesis committee

Promotors
Prof. Dr M.C.M. de Jong
Professor of Quantitative Veterinary Epidemiology
Wageningen University & Research

Dr C.J.M. Koenraadt
Associate Professor, Laboratory of Entomology Group
Wageningen University & Research

Co-promotors
Dr Q. ten Bosch
Assistant Professor, Quantitative Veterinary Epidemiology Group
Wageningen University & Research

Dr D. Masiga
Principal Research Scientist, and Head of the Human and Animal Health Theme
International Centre of Insect Physiology and Ecology

Other members

Prof. Dr D.K. Aanen, Wageningen University & Research
Prof. Dr S. Vanwambeke, Université catholique de Louvain, Belgium
Prof. Dr G.P. Pijlman, Wageningen University & Research
Dr H.J. Esser, Wageningen University & Research

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Unraveling the epidemiology of tick-borne infections in cattle and the potential impact of a novel biopesticide in coastal Kenya

Joseph Wang'ang'a Oundo

Thesis

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Abstract

Although ticks and tick-borne pathogens (TBPs) have long been recognized for their negative effects on livestock production, there are relatively few robust epidemiologic studies documenting their occurrence, diversity, predisposing factors, and control strategies practiced in different endemic settings in Kenya. Chemical control is often relied upon to manage tick populations, but it faces challenges from the widespread emergence of tick resistance and the contamination of the environment, milk, and meat products. Biological control using entomopathogenic fungi (EPF) presents a promising alternative to synthetic chemicals, yet their effectiveness in extensive grazing systems has not been established. Additionally, we have little quantitative understanding of how EPFs can impact tick-borne disease transmission. In this thesis, I generate information on tick-borne diseases (TBDs) epidemiology and the effectiveness of EPF formulation as a control means in coastal Kenya. In Chapter 1, the existing knowledge on the epidemiology of ticks and TBPs, the control strategies that have been explored, as well as the gaps present in the existing knowledge are described in detail. Chapter 2 provides comprehensive information on tick species infesting cattle and their associated pathogens in coastal Kenya. Our results indicated that eight tick species are parasitizing cattle, and were infected with several pathogens of zoonotic and veterinary importance, including *Rickettsia africae*, *Ehrlichia ruminantium*, and *Theileria parva.* In Chapter 3, we characterize the epidemiology of TBD and management factors among extensively grazed zebu cattle for informed decision-making on the control and prevention strategies. In Chapter 4, we conducted a randomized controlled field trial to evaluate the effectiveness of Tickoff biopesticide (a formulation of the entomopathogenic fungus *M. anisopliae* ICPE 7) for control of tick infestations and transmission of tick-borne infections in extensively grazed zebu cattle. We show that Tickoff biopesticide can kill ticks on treated animals, but is insufficient to result in a significant reduction of tick infestation and incidence of tick-borne infections in cattle. We also show that the toxicity of Tickoff is delayed, which would hamper direct protection of treated animals, but could result in indirect effects by preventing onward transmission. We further highlight the challenges of randomized controlled field trials and the complexity of assessing the impact of vector control products on both direct and indirect impacts on pathogen transmission. In chapter 5, we use a modeling approach to explore the impact of EPFs on the transmission of tick-borne infections when deployed at a population level. We show that under the assumed product profile, EPF derives most of its impact on East Coast fever (ECF) through the delayed mortality effect. This delayed mortality will also cause a reduction in the tick-to-host ratio and thus cattle exposure to ticks. We further show that sufficiently high levels of population coverage and treatment frequency are needed to reduce the tick population size and reach meaningful epidemiological impact in cattle populations. A further substantial impact can be obtained by increasing the persistence time of EPF on the cattle skin. In the final Chapter 6, the main results in Chapters 2 to 5 are discussed and integrated with the current knowledge on epidemiology and control of ticks and TBPs. The future directions for research on the epidemiology and control of TBDs are also highlighted. Overall, this thesis has provided insight into the epidemiology and control of TBDs in cattle that will be useful in the design of evidence-based control strategies.

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Chapter 1

General introduction

Ticks represent a major threat to livestock health and productivity in Africa, Asia, and Latin America (Jongejan & Uilenberg, 2004). Their obligate blood-feeding habit can affect livestock, directly, by causing irritation, weight loss, anemia, and damage to the udder and skin; or, indirectly, by transmitting various pathogens including bacteria, viruses and protozoa causing tick-borne diseases (TBDs) (Jongejan & Uilenberg, 2004; Jonsson, 2006; Walker et al., 2003). Major TBDs of livestock in Africa include anaplasmosis caused by rickettsia *Anaplasma marginale* and transmitted by one-host ticks *Rhipicephalus decoloratus and Rhipicephalus microplus*; babesiosis caused by the protozoans *Babesia bigemina and B. bovis* and transmitted by *R. decoloratus* and *R. microplus*; East Coast fever (ECF) caused by the protozoan *Theileria parva* and transmitted by a three-host tick *Rhipicephalus appendiculatus*; and heartwater caused by rickettsia *Ehrlichia ruminantium* and transmitted by a three-host tick *Amblyomma variegatum* (Walker et al., 2003). Additionally, *A. marginale* can be transmitted mechanically by biting flies of the genera *Tabanus* and *Stomoxys*, and blood-contaminated fomites such as needles, dehorning saws, nose tongs, and ear-tagging devices (Kocan et al., 2010). All mentioned diseases can cause morbidity or mortality in cattle, along with reduced milk and meat production, thus resulting in huge economic losses, especially to resource-poor livestock keepers (Gachohi et al., 2012; Kasaija et al., 2021; Kivaria, 2006).

To date, multiple tick control strategies have been explored across the globe. Of these, the use of synthetic chemical acaricides (George et al., 2004), anti-tick vaccines (Merino et al., 2013), immunization of cattle (Aubry & Geale, 2011; Nene et al., 2016), and breeding of tick-resistant livestock (Shyma et al., 2013), are amongst the primary strategies. Additionally, biological control using natural enemies such as entomopathogenic fungi, entomopathogenic nematodes, parasitoids and predators, and plants in the form of botanical extracts, have shown great potential for tick control (Kaaya, 2003; Samish et al., 2004). Whilst no control method is 100% effective, existing evidence demonstrates that considerable success in tick control can be realized when one major tick species or disease is intensively targeted with a particular control method, within a given space, in a particular host species or population. For instance, major advances in management of the one-host cattle tick *R. microplus*, have been made in Australia, Cuba, Mexico and Brazil, by consistent use of commercial vaccines against the species (de la Fuente et al., 2007). In North America, control of Lyme disease in humans has been achieved by reducing Ixodes scapularis by modifying its habitats and strategic acaricide application to its wildlife reservoirs (Poland, 2001).

However, in many developing countries, success stories against tick control are limited, possibly due to the complexity of agricultural production systems, uncontrolled transboundary livestock movement for transhumance, and poor tick control practices amongst other issues (Byaruhanga et al., 2015; Mutavi et al., 2021; Ouedraogo et al., 2021b; Vudriko et al., 2018). This highlights the need for an integrated tick management approach that is robust and that takes into account that no single solution may exist for tick control.

Epidemiology of bovine ticks and tick-borne diseases in Kenya

Several risk factors have been predicted to be associated with anaplasmosis and ECF infections in cattle in Kenya including the agro-ecological zone in which livestock resides, the livestock production system, as well as animal traits such as breed and age (Chiuya et al., 2021; Gachohi et al., 2012; Maloo et al., 2001a; Wesonga et al., 2014). For example, it has been reported that ECF is more severe in exotic (*Bos taurus*) and crossbred cattle (*B. taurus* × *Bos indicus*) than in the indigenous zebu cattle (*B. indicus*) in endemically stable areas (Nene et al., 2016). Cattle often become long-term asymptomatic carriers of *T. parva* following treatment or spontaneous recovery (Kariuki et al., 1995; Olds et al., 2018). On the other hand, all ages of cattle are susceptible to infection with *A. marginale*, but the severity of the disease increases with age (Aubry & Geale, 2011). Similarly to ECF, cattle that recover from anaplasmosis often remain persistent carriers of the infection and act as a source of infection for naïve cattle (Aubry & Geale, 2011). Although positive carrier status provides immunity to clinical disease, events associated with immunosuppression (e.g. advanced pregnancy and/or lactation) can cause a relapse of acute infection (Kocan et al., 2010).

Cattle production systems in Kenya have also been associated with variable levels of exposure to ticks and TBDs and are categorized into i) traditional extensive systems divided into traditional crop-livestock and livestock-dependent systems (pastoralism) and ii) intensive systems grouped into the commercial and intensive or semi-intensive smallholder dairy systems (Gachohi et al., 2012). In general, the traditional extensive system is the most common production system and is characterized by little or no tick control. As a consequence, cattle are continuously exposed to tick infestation with a consequent higher incidence of infection with pathogens. However, continuous exposure of indigenous breeds of cattle to infected ticks in endemically stable areas may facilitate the development of immunity to disease compared to non-endemic areas (Gachohi et al., 2012).

On the contrary, the intensive systems (practiced mainly in commercial farms) are characterized by intensive usage of acaricides in the farms which help to disrupt pathogen transmission in the cattle population. In the semi-intensive systems, cattle are exposed to any combination of both intensive and extensive management practices, either simultaneously, or varied according to changes in climatic conditions or physiological state of the cattle. Consequently, cattle in this system exhibit varying prevalence, incidence, and mortality rates (Gachohi et al., 2012).

Tick-borne zoonoses in Kenya

To date, some cases of tick-borne zoonoses have been reported in humans in Kenya, including Crimean-Congo hemorrhagic fever (CCHF) (Dunster et al., 2002), Spotted Fevers Group (SFG) rickettsiosis (Rutherford et al., 2004; Yoshikawa et al., 2005), and Q fever (Njeru et al., 2016). The burden and impact of these zoonoses remain unknown as the majority of zoonoses are underreported and/or misdiagnosed in clinical settings. This is mainly due to a lack of awareness, dedicated surveillance systems, specific diagnostic tests, and capacity in most rural and peri-urban areas (Brah et al., 2015; Ndeereh et al., 2016). This is exacerbated by the dearth of information on the level of risk posed to humans by infected ticks, and to what extent the bites from ticks are reported from humans. Nonetheless, there has been increasing serological evidence of exposure to these tick-borne zoonoses in patients with acute febrile illness in Kenya (Lwande et al., 2012; Maina et al., 2016; Njeru et al., 2016; Thiga et al., 2015). In addition, zoonotic pathogens have been detected in questing ticks collected in wildlife habitats in Kenya, including *Rickettsia africae*, *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* (Mwamuye et al., 2017; Oundo et al., 2020).

Current strategies and control of bovine ticks in Sub-Saharan Africa: chemical acaricides

Tick control in cattle in sub-Saharan Africa (SSA) has remained heavily reliant on the use of acaricides applied in various forms including sprays, dips, footbaths, and pour-on (**Figure 1**) (De Meneghi et al., 2016). Arsenics and organochlorines, especially dichlorodiphenyltrichloroethane (DDT), were the earliest acaricides used in Africa, but their use was discontinued due to high levels of toxicity to animals and the environment, as well as increasing levels of resistance by ticks (Githaka et al., 2022). Organophosphates (e.g., chlorphenvinphos, coumaphos, diazinon, dioxathion) and carbamates (e.g., carbaryl), which replaced organochlorines, are also facing the challenge of emerging resistance and contamination of milk and meat products (De Meneghi et

al., 2016; Githaka et al., 2022). Synthetic pyrethroids (e.g., permethrin, decamethrin, deltamethrin, cyhalothrin, cyfluthrin and flumethrin), alongside amidines (particularly amitraz), are highly effective groups of acaricide that are currently widely used in Africa, although also here cases of resistance to these compounds are emerging (Baron et al., 2015; Githaka et al., 2022).

Tick control in SSA, including Kenya, is characterized by several malpractices that promote resistance development in ticks including, I) exclusive reliance on chemical acaricides for tick control as opposed to integrated tick control approaches, II) inappropriate dilution of the acaricides (as recommended by manufacturers) leading to either underdosing or overdosing, III) use of substandard equipment for spraying, and IV) malpractices in acaricide rotation schedules and mixing of acaricide brands within the same active ingredient classes (Githaka et al., 2022; Mutavi et al., 2021; Vudriko et al., 2018). Tick resistance to acaricides has in fact been described as an inevitable consequence as most countries in SSA undertake minimal monitoring for emergence of resistance, experience a lack of acaricide resistance management strategies/policies, or poorly implement them (Githaka et al., 2022).

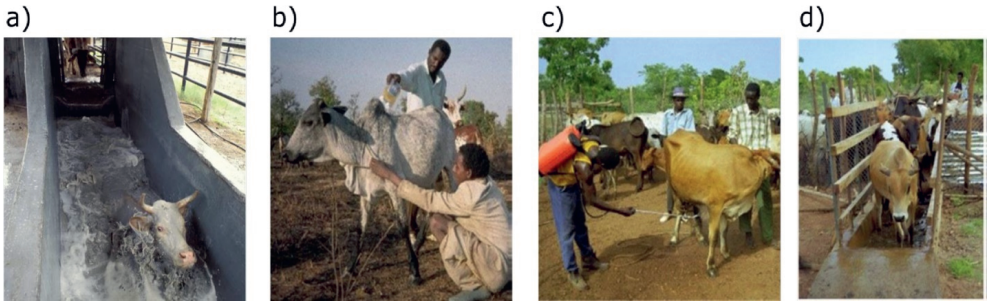

Figure 1. Methods used in the application of chemical acaricides for tick control on livestock. a) Dip-tank, b) Pour-on, c) Manual backpack sprayer, and d) Footbath. Image adapted from De Meneghi et al. (2016).

Potential for entomopathogenic fungus in bovine tick control in Africa

The entomopathogenic fungus *Metarhizium anisopliae* establishes an infection when the conidia (spores) come into contact with the surface of the tick. Once contact has been made, the conidia germinate and differentiate to form the germ tube and appressorium (penetration structure) within 24 hours (Arruda et al., 2005). The germ tube then penetrates the tick cuticle within 24–48 hours post-infection by a combination of exerting mechanical pressure (Arruda et al., 2005), mostly at

the intersegmental joints, and the action of cuticle-degrading enzymes such as lipase, protease and chitinase. Massive penetration is observed between 48 and 72 hours post-inoculation (Arruda et al., 2005). After penetration and once inside the host, the fungus develops hyphal bodies and blastopores that multiply and disseminate through the hemolymph to invade different tissues and cause death (Fernandes et al., 2012). This infection process takes several days, with the overall time to death depending mostly on fungal dose, virulence of the fungal isolate and environmental conditions. Following the death of the tick, under humid conditions, the mycelium penetrates the cuticle, again mostly at the intersegmental joints, and produces infectious conidia on the outside of the cadaver that can passively infect new ticks (Arruda et al., 2005). Under dry conditions, the fungus may survive in the hyphal stage but fail to produce conidia on the outside of the body.

The efficacy of *M. anisopliae* has been demonstrated in both *in vitro* and field settings. In laboratory experiments, *M. anisopliae* induced high mortalities in various stages of *A. variegatum, R. appendiculatus, R. evertsi,* and *R. decoloratus* (Hedimbi et al., 2011; Kaaya et al., 1996; Kaaya & Hassan, 2000; Murigu et al., 2016). Apart from the direct mortality, *M. anisopliae* increases the engorgement period, preoviposition, oviposition and postoviposition periods, and reduces tick fecundity and viability of eggs in the surviving population of treated ticks (Hedimbi et al., 2011; Kaaya et al., 1996; Kaaya & Hassan, 2000; Murigu et al., 2016). Additionally, *M. anisopliae* has been reported to be pathogenic to acaricide-resistant ticks, causing a mortality of 100% in both amitraz-resistant and amitraz-susceptible larval strains of *R. decoloratus* (Murigu et al., 2016). The observed effectiveness of *M. anisopliae* against acaricide-resistant ticks supports the idea of using fungal formulations in tick control, especially in areas where selection pressure for resistance is already threatening the efficacy of chemical acaricides.

Promising results were also obtained with topical application of entomopathogenic fungi to cattle in field settings. Application of oil-water formulation of *M. anisopliae* strain NA1 (1×10^8 conidia/ml) on cattle at triweekly intervals reduced the on-host populations of *R. evertsi* and *R. decoloratus* by 83%, 3 months after commencement of the experiment (Kaaya et al., 2011). In another study, serial applications of aqueous formulation (10^8 conidia per ml) of *M. anisopliae* once a month over 6 months onto vegetation artificially infested with adult *Rh. appendiculatus* led to the suppression of on-host tick populations by 92% (Kaaya & Hassan, 2000). Recently, oil formulation of *M. anisopliae* ICIPE 7 (10^9 conidia per ml) resulted in a significant reduction in the

on-host population of *R. decoloratus* by 69.2% when tick-infested cattle were treated once a week for four weeks (Murigu et al., 2016).

Several strategies for delivering this fungal entomopathogen to off-host ticks have been explored. This includes the use of baited traps to attract and infect ticks in the vegetation as a means of reducing the risk of cattle infestation. These traps use botanical extracts with tick-attractant properties such as *Calpurnia aurea* (Nana et al., 2012, 2015), pheromones such as attraction-aggregation-attachment pheromone (AAAP), and kairomones such as carbon dioxide (CO_2) to attract and expose off-host ticks to lethal doses of fungal conidia (Maranga et al., 2006; Nchu et al., 2009, 2010).

It has been observed that when *M. anisopliae* is incubated in organophosphate acaricides (Steladone and Supadip) for up to 120 hours, *M. anisopliae* retained its normal growth and morphological characteristics (Kaaya et al., 1996). Compatibility of *M. anisopliae* isolate ICIPE 7 and amitraz has also been demonstrated in a four-week field trial where the combination of ICIPE 7 and amitraz caused a reduction of tick counts on infested cattle by 67.1%, while the individual treatments caused a reduction of 69.2% with ICIPE 7 alone, and by 94.9% with amitraz alone (Murigu et al., 2016). These findings suggest that a combination of synthetic acaricides and entomopathogenic fungi could potentially allow for the reduction of acaricide quantities, either through sublethal doses or alternated with fungal entomopathogens, thereby curbing the emergence and spread of acaricide-resistant ticks, as well as the negative environmental impacts. The compatibility and potential synergistic interactions will also enhance the lifespan of the current chemical acaricides and slow the spread of resistance.

Despite the observed efficacies of *M. anisopliae*, its practical application in the field is limited by unfavorable environmental factors such as high temperatures, strong ultraviolet (UV) radiation, and low relative humidity (Fernandes et al., 2012). These often severely reduce the germination of conidia and thereby reduce the efficacy of fungal formulations. This, therefore, highlights the need to develop long-lasting formulations that can improve spore viability and persistence to make the intervention more practical and effective under field conditions. To date, several adjuvants have been added to fungal formulations in a bid to protect conidia against adverse conditions and enhance the shelf life and efficacy of fungal spores. These include polymerized cellulose gel (Reis

et al., 2008), UV radiation protectants (Hedimbi et al., 2008), and (micro)encapsulation (Meirelles et al., 2023) amongst others.

Although considerable progress has been made over the years in the research and development of fungal formulations, availability of a commercial product for use against ticks has been slow and inconsistent. Nonetheless, a number of biopesticides based on *M. anisopliae* have been tested for efficacy or are commercially available for use against ticks. In the USA, a formulation of *Metarhizium brunneum* strain F52 (formerly *M. anisopliae*) has been developed to kill questing ticks and is available under the tradename Met52®, formerly Tick-Ex®, (Novozymes Biological, Franklinton, NC, USA) (Sullivan et al., 2022). In Brazil, a commercial product based on *M. anisopliae* isolates ESALQ 1037 and ESALQ E9 and originally developed for pest control in agriculture under the brand name Metarril® SP Organic (Koppert® Biological Systems) has shown great potential for control of *R. microplus* in cattle (Camargo et al., 2014, 2016). In Kenya, a near-commercial formulation of *M. anisopliae* isolate ICIPE 7 (Tickoff®, Real IPM Kenya Limited) is being developed as a biopesticide for the control of ticks on cattle.

Justification of the study

While ticks and TBDs have long been recognized for their negative effects on livestock production, information on their epidemiology in coastal Kenya is inadequate and outdated. Over the years, a large part of coastal Kenya has experienced drastic effects of climate change including recurrent droughts that have led to an increase in unrestricted transboundary livestock movement for transhumance. Although such movements have been reported to cause an expansion in the range of ticks and tick-borne pathogens (TBPs) in some areas of SSA (Madder et al., 2011; Marcellino et al., 2017; Ouedraogo et al., 2021b), there has been no effort to characterize the current status of tick and TBPs diversity in the area. This study, therefore, provides an opportunity to improve our understanding of the epidemiology of bovine ticks and TBDs in this region. This information will provide support to diagnosis and in the design of effective evidence-based control strategies, and consequently improve cattle health and productivity, and ultimately the livelihoods of cattle owners.

Owing to the increasing reports of the emergence of acaricide resistance in various countries in SSA (Githaka et al., 2022), and the absence of an effective anti-tick vaccine against SSA tick strains (Kasaija et al., 2023), there is an urgent need for new tick control tools to act as alternatives

to the synthetic acaricides. Fungal formulations based on *M. anisopliae* have been put forward as a possible and valuable alternative in the management of ticks (Alonso-Díaz & Fernández-Salas, 2021; Fernandes et al., 2012). Nonetheless, there have been inconsistent reports regarding the degree of tick control under field conditions, with some studies reporting a low control efficacy (Correia et al., 1998; Leemon et al., 2008; Samish et al., 2014) while others reporting a high control efficacy (Alonso-Díaz et al., 2007; Barbieri et al., 2023; Kaaya et al., 2011; Murigu et al., 2016). Such variations in efficacy make it difficult to extrapolate conclusions from these studies to real-world conditions directly. That notwithstanding, numerous studies have been constrained by design limitations, including low statistical power and a frequent absence of data on epidemiological outcomes such as the incidence of tick-borne infections in cattle populations. Such limitations may complicate the interpretation of the study outcomes especially because the treatment effect may be overestimated or inaccurate. To this end, this study will use a combination of laboratory experiments, field trials, and modeling approaches to unravel the potential impact of entomopathogenic fungi (EPF) formulations for the control of ticks and tick-borne infections on cattle. The findings of this study will provide valuable information to inform policy decisions on tick control programs, both in the region and elsewhere.

Outline of the thesis

In view of the above, the overall objective of the study was: **to unravel the epidemiology of tick-borne diseases in cattle and the potential impact of Tickoff® biopesticide (*M. anisopliae* ICIPE 7) for the control of ticks and tick-borne infections in cattle in coastal Kenya**.

The current chapter **(Chapter 1)** provides background information of the epidemiological situation of tick-borne infections and the status of tick control strategies in cattle in SSA and Kenya, identifying gaps present in the existing knowledge and thus outlines the rationale of the study. **Chapter 2** describes the diversity, abundance, and infestation prevalence of ticks on cattle as well as the diversity and prevalence of TBPs harbored by these ticks in coastal Kenya. **Chapter 3** focuses on the diversity and prevalence of TBPs and the potential risk factors associated with TBP infections in cattle. Moreover, it provides information on the control strategies currently being implemented by livestock keepers in coastal Kenya. **Chapter 4** describes the outcome of a field trial, that was developed as part of this thesis and which specifically reports the treatment effect of Tickoff® biopesticide and comparing the treatment effect to the existing Triatix chemical and the

control (excipients of Tickoff®). It also reflects on the complexities faced during the trial period and how it might have affected the trial outcome. In **Chapter 5**, a mathematical model is developed and used to gain further insights into the potential impact of EPFs, using ECF as a case study, and to inform the implementation strategies and product properties needed to achieve a meaningful epidemiological outcome. Lastly, **Chapter 6** discusses the main findings of this thesis and integrates them with the current knowledge on epidemiology and control of bovine ticks and TBDs. This chapter concludes with future directions for research on the epidemiology and control of bovine TBDs.

2

Chapter 2

Ticks (Acari: Ixodidae) infesting cattle in coastal Kenya harbor a diverse array of tickborne pathogens

Joseph Wang'ang'a Oundo[1,2*], Shewit Kalayou[1], Quirine ten Bosch[2], Jandouwe Villinger[1], Constantianus J.M. Koenraadt[3], Daniel Masiga[1]

1 International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya,
2 Quantitative Veterinary Epidemiology, Wageningen University & Research, Wageningen, the Netherlands,
3 Laboratory of Entomology, Wageningen University & Research, Wageningen, the Netherlands

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Abstract

Ticks and the microbes they transmit have emerged in sub-Saharan Africa as a major threat to veterinary and public health. Although progress has been made in detecting and identifying tick-borne pathogens (TBPs) across vast agroecologies of Kenya, comprehensive information on tick species infesting cattle and their associated pathogens in coastal Kenya needs to be updated and expanded. Ticks infesting extensively grazed zebu cattle in 14 villages were sampled and identified based on morphology and molecular methods and tested for the presence of bacterial and protozoan TBPs using PCR with high-resolution melting analysis and gene sequencing. In total, 3,213 adult ticks were collected and identified as *Rhipicephalus appendiculatus* (15.8%), *R. evertsi* (12.8%), *R. microplus* (11.3%), *R. pulchellus* (0.1%), *Amblyomma gemma* (24.1%), *A. variegatum* (35.1%), *Hyalomma rufipes* (0.6%), and *H. albiparmatum* (0.2%). Ticks were infected with *Rickettsia africae*, *Ehrlichia ruminantium*, *E. minasensis*, *Theileria velifera* and *T. parva*. *Coxiella* sp. endosymbionts were detected in the *Rhipicephalus* and *Amblyomma* ticks. Co-infections with two and three different pathogens were identified in 6.9% (n = 95/1382) and 0.1% (n = 2/1382) of single tick samples, respectively, with the most common co-infection being *R. africae* and *E. ruminantium* (7.2%, CI: 4.6 – 10.6). All samples were negative for *Coxiella burnetii, Anaplasma* spp. and *Babesia* spp. Our study provides an overview of tick and tick-borne microbial diversities in coastal Kenya.

Introduction

Ticks (Acari: Ixodidae) are obligate blood-feeding ectoparasites that transmit a broad range of bacterial, protozoan and viral pathogens to humans and animals (de la Fuente et al., 2008). Ixodid ticks commonly infesting livestock in sub-Saharan Africa (SSA) pose enormous constraints on cattle health and productivity by acting as vectors of the etiological agents of East Coast fever (ECF), heartwater, anaplasmosis, and babesiosis (Walker et al., 2003). Besides acting as vectors, tick parasitism causes severe economic losses in the livestock sector due to weight loss, anemia, and damage to the udder, skin, and hide (Jonsson, 2006; Walker et al., 2003).

In recent decades, the geographic range of ticks has been expanded in SSA, primarily due to climate change, habitat modification, transboundary animal trade and the increased movement of animals (Githaka et al., 2021; Madder et al., 2011). These changes may potentially lead to a shift in the epidemiology of tick-borne diseases (TBDs) as tick-borne pathogens (TBPs) may spread to new areas where they were previously inexistent and thus represent a potential threat to animal health (Ouedraogo et al., 2021a; Ouedraogo et al., 2021ba). For example, previously unrecognized or emerging TBPs were recently reported in Kenya, including *Anaplasma phagocytophilum* (Mwamuye et al., 2017), *Ehrlichia minasensis* (Chiuya et al., 2021; Peter et al., 2020), *Ehrlichia chaffeensis* (Mwamuye et al., 2017) and *Candidatus* Rickettsia moyalensis (Kimita et al., 2016). These reports highlight the need for regular updating of the data on the distribution of tick species and TBPs in various geographical settings.

Available information on tick species infesting cattle and their occurrence and diversity in coastal Kenya is outdated and limited. The existing data on tick species was published over two decades ago and was based solely on phenotypic characteristics (Zulu et al., 1998). Therefore, there is a need to generate new accurate data on tick diversity, abundance and phylogenetic relationships using molecular approaches (Lv et al., 2014).

The traditional extensive system of cattle production in coastal Kenya favors the convergence of herds, mainly at grazing and watering points. This may increase the likelihood of high tick infestations among the herds and hence the risk of TBP transmission. Further, some cattle owners in the region move with their cattle during the dry season in search of water and pastures for their animals. This uncontrolled transboundary cattle movement could significantly spread ticks and TBPs to new areas (Ouedraogo et al., 2021a; Ouedraogo et al., 2021b). Therefore, there is a need

for active surveillance of ticks and TBPs to regularly update information on the presence, distribution, abundance, and prevalence of ticks and TBPs. Accordingly, we aimed to i) investigate the species composition of ticks infesting cattle and their infestation prevalence in the coastal region of Kenya, and ii) examine the prevalence and diversity of TBPs belonging to *Rickettsia* spp., *Theileria* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp. and *Coxiella burnetii* in the collected tick specimens using PCR with high-resolution melting analysis.

Materials and methods

Study area

The study was conducted in Kayafungo Ward (Kilifi County) and Kinango Ward (Kwale County) in coastal Kenya (Figure 1). Coastal Kenya is hot and dry from January to March and relatively cool from June to August. The annual temperatures range from 23–34 °C, while the average relative humidity is 60–80%. The predominant livestock kept in the region includes cattle, goats, and chickens. More details on the climate of coastal Kenya are provided in a previous study (Mwangangi et al., 2013).

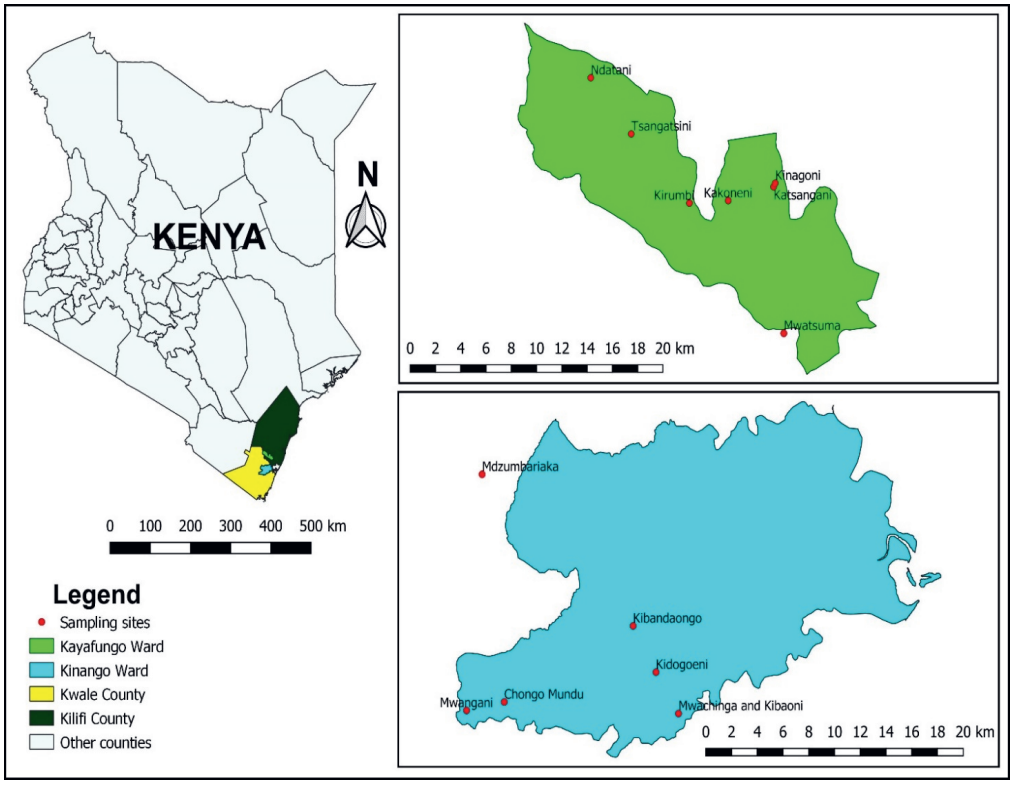

Figure 1. Map of Kayafungo and Kinango Wards in coastal Kenya showing the villages where the tick sampling took place. The map was prepared using common licensed shape files in QGIS software version 3.10 (QGIS Development Team, 2020).

Study design

The present study was conducted in 14 village clusters as a baseline survey of a more extensive operational research project that aimed to improve food and nutritional security through integrated control of tsetse and tick-borne livestock diseases (ICTLD). The two administrative wards were selected purposively based on their potential for livestock production in the study area, accessibility and the difference in access to veterinary extension services. The final listing of village clusters was made based on the cooperation of farmers and logistical feasibility (accessibility by vehicle, security, distance). The ticks were collected in two field-sampling trips, in December 2019, coinciding with the short rains, and in May 2021, at the onset of the long rains period. Attempts were made to collect samples from the same herds in both periods. However,

some herds were lost during the second sampling due to the mortality or relocation of herds, and therefore alternative herds in the same villages were included. Herd selection was made based on their location by village and the willingness of the farmers to participate in the study.

Tick collection, morphological identification and pooling

Cattle were examined for tick infestation in the following predilection sites: head, ears, neck, dewlap, belly, back, legs, udder in the case of females and testes in males, perineum region and tail. Tick-infested animals were restrained and all visible live-attached ticks were removed using blunt steel forceps. Ticks were stored in 2-ml cryovial tubes labeled with a unique sample ID, comprising the sampling site, host ID, predilection site and sampling date. Categorical data on the age, sex and breed of each cattle were recorded on predesigned forms. The collected ticks were frozen in liquid nitrogen and transported to the Martin Lüscher Emerging Infectious Disease (ML-EID) laboratory at the International Centre of Insect Physiology and Ecology (ICIPE) in Nairobi, where they were stored at -80°C before species identification and pathogen screening.

Before morphological identification, the tick samples were disinfected by immersion in 70% ethanol solution for five minutes, with occasional vortex mixing, rinsed twice with deionized water, and dried on filter paper. Ticks were then identified by developmental stage, species and sex based on published morphological descriptions (Walker et al., 2003) under a stereomicroscope (ZEISS Stemi 2000-C, Oberkochen, Germany). Representative tick species of either sex were photographed using a microscope-mounted Axio-cam ERc 5s digital camera (Zeiss). The identified ticks were sorted by sex, species and sampling site and then processed individually or in a pool of 2-5 ticks in 1.5-ml Eppendorf tubes. During extraction, partially and fully engorged ticks were discarded to reduce vertebrate host DNA.

DNA extraction

The ticks were mechanically crushed with 750 mg of 2.0-mm yttria-stabilized zirconium oxide (zirconia/yttria) beads (Glen Mills, Clifton NJ) using a Mini-Beadbeater-16 (BioSpec, Bartlesville, OK) twice for one minute. Genomic DNA was extracted from the homogenates using a previously described method (Oundo et al., 2020). The quality and quantity of extracted DNA samples were measured using a Nanodrop ND-2000 instrument (Thermo Fischer Scientific, UK). The DNA concentration was then adjusted to 50 ng/µl for all samples. The remaining stock of DNA was stored at -80°C, while diluted DNA extracts were stored at -20°C until further use.

Molecular identification of ticks

Molecular identification was carried out on two to four randomly selected ticks of each species. The PCR assays targeting the tick 16S ribosomal DNA (rDNA), cytochrome *c* oxidase subunit I (COI) and internal transcribed spacer 2 (ITS2) were carried out using a SimpliAmp™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA) as previously described (Oundo et al., 2020). The PCR products were gel-purified using QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and then sent to Macrogen Inc. (The Netherlands) for sequencing in both directions.

Molecular detection of protozoan and bacterial pathogens

The genomic DNA of ticks was screened by PCR with high-resolution melting (PCR-HRM) analyses for infection with *Rickettsia*, *Theileria, Babesia, Ehrlichia* and *Anaplasma* species. The PCR-HRM assays were conducted on a Magnetic Induction Cycler (MIC) machine (BioMolecular Systems, Australia) as previously described (Oundo et al., 2020). Positive controls containing genomic target DNA of *Rickettsia africae, Anaplasma bovis, Ehrlichia ruminantium* and *Theileria parva* and a negative control without a DNA template were included in each respective amplification run. Amplicons with unique HRM melt curves were purified for sequencing.

To re-confirm the identity of rickettsial pathogens, DNA from the samples that were positive for *Rickettsia* spp. using PCR-HRM primers were re-amplified using primers targeting rickettsial citrate synthase (*gltA*) gene, outer membrane protein A (*ompA*) gene, outer membrane protein B (*ompB*) gene, and cell surface antigen (*sca4*) gene as previously described (Mwamuye et al., 2017; Sekeyova et al., 2001). Additionally, samples positive for *Ehrlichia* spp. were further re-amplified using primers targeting the heat shock protein (*groEL*) gene (Bell and Patel, 2005). All PCR reactions were carried out using SimpliAmp™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The PCR amplicons were electrophoresed, gel-purified and sequenced as described previously.

Sequence analyses

Generated raw sequences from ticks and positive pathogen samples were edited and aligned using the MAFFT plugin (Katoh & Standley, 2013) in Geneious software version 11.1.5 (https://www.geneious.com) (Kearse et al., 2012). To confirm the identity of each species, the

sequences were compared with those available in the GenBank database using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

Raw data were entered into Microsoft® Excel 2016 and verified for missing observations and erroneous entries. Statistical analysis was performed using R software version 4.1.3 (R Core Team, 2022). Given that the two wards (i.e., Kinango and Kayafungo) are autonomous administrative units with different access to veterinary extension services, we analyzed the tick infestation prevalence at the administrative ward level. Further, we only considered the first survey data for analysis of infestation prevalence and intensity and excluded the second survey due to insufficient data to perform the analysis. The tick infestation prevalence at the animal level was calculated as the number of cattle infested with ≥ 1 tick out of the total number of cattle examined. For the herd prevalence, a herd was considered positive if at least one animal was infested with ticks. The 95% confidence intervals (95% CIs) for the infestation prevalence were estimated using the *'binom'* package (Dorai-Raj, 2014) with the exact-Clopper-Pearson interval method. The mean infestation intensity was calculated as the total number of ticks divided by the number of infested cattle. The tick infestation prevalence in the different administrative wards, sex and age groups was compared using the Chi-square test. The infestation intensity in cattle in different villages and administrative wards was compared using the Kruskal-Wallis test and the Mann-Whitney-Wilcoxon test with continuity correction, respectively. The effects of host traits (i.e., sex and age) on tick infestation were assessed using the Mann-Whitney-Wilcoxon test and the Kruskal-Wallis test, and the multiple pair-wise comparisons among age groups were done using the Tukey and Kramer (Nemenyi) test. The breed category was excluded from the analysis due to insufficient data to perform the statistical analysis.

The infection rate in tick pools was calculated using the minimum infection rate (MIR) method, with 95% CIs for unequal pool sizes, using the PooledInfRate v4.0 Excel add-in (Biggerstaff, 2009). The MIR was expressed per 100 ticks. For co-infection analyses, pools with multiple ticks were removed from the dataset as we could not confirm true co-infections in these samples. The 95% CIs for the prevalence of observed co-infection was calculated using the exact-Clopper-Pearson interval method from the R package *'binom'*. The expected coinfection prevalence was calculated by multiplying the infection rates of each of the pathogens and then multiplying by 100

(Zembsch et al., 2021). Correlation among pathogens and between pathogens and endosymbionts in single tick samples were analyzed using Spearman's rank correlation. Differences were considered statistically significant at p-values ≤ 0.05.

Ethics statement

Before sampling ticks, the cattle owners were verbally informed about the goals of the project and the sampling protocol. All owners gave their verbal informed consent to collect ticks from their animals. The study protocol was approved by the Institutional Animal Care and Use Committee of *icipe* (IACUC, Reference No. Oundo-icipeACUC-Mar2020), and the Pwani University Ethics Review (approval number ERC/EXT/002/2020). Further approval was sought from the Kenyan National Commission for Science, Technology and Innovation (NACOSTI/P/21/6726). This study did not involve endangered or protected species.

Results

Tick species composition

A total of 3,213 adult ixodid ticks (including 2,157 males and 1,056 females) were collected from 333 cattle in 14 villages in coastal Kenya. They belonged to three tick genera i.e., *Amblyomma, Hyalomma, Rhipicephalus* including *Boophilus* subgenus. *Amblyomma variegatum* (n = 1129, 35.1%) was the most abundant species, followed by *A. gemma* (n = 773, 24.1%), *R. appendiculatus* (n = 508, 15.8%), *R. evertsi* (n = 412, 12.8%), *R.* (*Bo*) spp. (n = 360, 11.2%), *H. rufipes* (n = 18, 0.6%), *H. albiparmatum* (n = 7, 0.2%) and *R. pulchellus* (n = 6, 0.2%) (Table A.1).

Sequence analysis of 16S rDNA and ITS2 sequences showed that molecular identification was consistent with morphological identification (Table A.2). The CO1 marker yielded amplicons only for *R. pulchellus* and *H. rufipes* ticks. All the collected *Rhipicephalus* (*Boophilus*) spp. were either semi-engorged or fully engorged females and thus could not be morphologically differentiated any further than the subgenus level. Analysis of ITS2 and 16S rRNA gene sequences of these species showed that they were closest to *R. microplus* sequences with 99.2-100% nucleotide sequence identities. Hence, these ticks were designated as *R. microplus*.

Tick infestation prevalence

Of the 1522 cattle examined in the first survey, 333 (21.9%, CI: 19.8 - 24.1) were infested (Table 1). Based on the number of ticks per animal, 270 cattle were infested with 1-9 ticks, while 63 cattle were infested with 10-18 ticks. There was no statistically significant difference between the proportions of male and female cattle infested with ticks (χ^2 = 0.0029851, df = 1, p = 0.96). However, the tick infestation prevalence significantly varied among the different age groups, highest in adults and lowest in calves. The tick infestation prevalence was also significantly different among the administrative wards, being highest in Kayafungo ward in Kilifi County compared to Kinango ward in Kwale County (Table 1).

Table 1. Effect of host characteristics and administrative ward on tick infestation prevalence in cattle from coastal Kenya.

Variable	Category	Number of observed cattle[a]	Infestation prevalence	
			No. of infested cattle	*P*-value
Animal sex	Male	603 (39.6%)	131 (21.7%)	χ^2 = 0.0029851, df = 1, p = 0.96
	Female	919 (60.4%)	202 (22.0%)	
Age	Calves	117 (7.7%)	14 (12.0%)	χ^2 = 7.5314, df = 2, p = 0.02*
	Juvenile	434 (28.5%)	95 (21.9%)	
	Adults	971 (63.8%)	224 (23.1%)	
Administrative ward	Kinango	799 (52.5%)	156 (19.5%)	χ^2 = 5.1701, df = 1, p = 0.03*
	Kayafungo	723 (47.5%)	177 (24.5%)	

[a] total number of observed cattle is 1,522; Calves (<6 months), Juvenile (6–24 months) and Adults (>24 months);
*statistically significant ($P \leq 0.05$)

Tick infestation intensity

A total of 333 cattle were infested with 2109 ticks (mean infestation intensity of 6.3 ticks), with the number of ticks per cattle ranging from 1 to 18 (Table A.3). The tick infestation intensity was significantly different across the study villages (H = 71.76, df = 11, $p < 0.001$) and the administrative wards (W = 17834, $p < 0.001$). The infestation intensity was also significantly different among the different age groups of cattle (H = 17.213, df = 2, p = 0.0002), with the pairwise comparison showing that calves and adults and calves and juveniles were significantly different ($p < 0.0001$). The infestation intensity was not statistically significant between male and female cattle (W = 14510, p = 0.1344).

Prevalence of tick-borne pathogens

A total of 1,382 single ticks, and 682 tick pools representing 1,831 ticks, were screened for *Rickettsia* spp., *Theileria* spp., *Ehrlichia* spp., *C. burnetii, Babesia* spp. and *Anaplasma* spp. infections. We detected *Rickettsia* spp., *Theileria* spp. and *Ehrlichia* spp. pathogen DNA, while none of the samples was positive for *Anaplasma* spp., *Babesia* spp., or *C. burnetii.* The prevalence of infection in individual ticks and the MIRs of pooled ticks are summarized in Tables 2a and b, respectively.

Table 2a. Prevalence of pathogens and *Coxiella* sp. endosymbiont identified in single ticks collected from cattle sampled in coastal Kenya

Tick species	Tested single ticks	*Ehrlichia minasensis*		*Ehrlichia ruminantium*		*Rickettsia africae*		*Theileria velifera*		*Theileria parva*		*Coxiella* sp. endosymbiont	
		+ ticks	% prevalence	+ ticks	% prevalence	+ ticks	%prevalence	+ ticks	prevalence	+ ticks	% prevalence	+ ticks	% prevalence
Rhipicephalus appendiculatus	4	0	0	0	0	2	50%	0	0	0	0	0	0
Rhipicephalus pulchellus	3	0	0	0	0	1	33.3	0	0	0	0	0	0
Rhipicephalus evertsi	27	0	0	0	0	9	33.3	0	0	0	0	12	44.4
Rhipicephalus microplus	175	4	2.3	1	0.6	55	31.4	0	0	0	0	54	30.9
Amblyomma gemma	321	2	0.6	27	8.4	273	85.0	2	0.6	0	0	26	8.1
Amblyomma variegatum	835	0	0	52	6.2	734	87.9	17	2.0	0	0	54	6.5
Hyalomma rufipes	12	0	0	0	0	5	41.2	0	0	0	0	0	0
Hyalomma albiparmatum	5	0	0	0	0	0	0	0	0	0	0	0	0
Overall	**1382**	**6**	**0.4%**	**80**	**5.8%**	**1079**	**78.1%**	**19**	**1.4%**	**0**	**0**	**146**	**10.6%**

+ ticks - Positive single ticks

Table 2b. Minimum infection rate (MIR) of pathogens and *Coxiella* sp. endosymbiont identified in pools (2-5 ticks/pool) of tick species collected from cattle sampled in coastal Kenya

Tick species	Number of ticks	Number of pools	*Ehrlichia minasensis*		*Ehrlichia ruminantium*		*Rickettsia africae*		*Theileria velifera*		*Theileria parva*		*Coxiella* sp. endosymbiont	
			+ pools	MIR per 100 ticks (95% CI)	+ pools	MIR per 100 ticks (95% CI)	+ pools	MIR per 100 ticks (95% CI)	+ pools	MIR per 100 ticks (95% CI)	+ pools	MIR per 100 ticks (95% CI)	+ pools	MIR per 100 ticks (95% CI)
Rhipicephalus appendiculatus	504	112	6	1.2 (0.2 – 2.1)	0	0	64	12.7 (9.8 – 15.6)	0	0	4	0.8 (0.0 – 1.6)	12	2.4 (1.1 – 3.7)
Rhipicephalus evertsi	385	112	4	1.0 (0.0 – 2.1)	0	0	34	8.8 (6.0 – 11.7)	0	0	0	0	8	2.1 (0.7 – 3.5)
Rhipicephalus microplus	188	81	6	3.2 (0.7 – 5.7)	0	0	31	16.5 (11.2 – 21.8)	0	0	0	0	29	15.4 (10.3 – 20.6)
Amblyomma gemma	452	226	2	0.4 (0.0 – 1.1)	0	0	217	48.0 (43.4 – 52.6)	1	0.2 (0.0 – 0.7)	0	0	1	0.2 (0.0 – 0.7)
Amblyomma variegatum	294	147	0	0	0	0	81	27.6 (22.4 – 32.7)	1	0.3 (0.0 – 1.0)	0	0	4	1.4 (0.0 – 2.7)
Hyalomma rufipes	6	3	0	0	0	0	1	16.7 (0.0 – 46.5)	0	0	0	0	0	0
Hyalomma albiparmatum	2	1	0	0	0	0	0	0	0	0	0	0	0	0
Overall	**1831**	**682**	**18**	**1.0 (0.5 – 1.4)**	**0**	**0**	**428**	**23.4 (21.4 – 25.3)**	**2**	**0.1 (0.0 – 0.3)**	**4**	**0.2 (0.0 – 0.4)**	**54**	**3.0 (2.2 – 3.7)**

+ Pools - Positive tick pools; **MIR –** minimum infection rate; **CI -** confidence Intervals

The prevalence of *Rickettsia* spp. infection in single ticks was observed to be 78.1%, while the MIR of tick pools was 23.4%. The 16S rDNA rickettsial gene sequencing confirmed *R. africae* in all positive samples, showing 100% identity with *R. africae* isolate from Uganda (Table A.4). Additional amplification of the *ompA, ompB, gltA* and *Sca4* gene fragments in the *Rickettsia*-positive samples also showed maximum identities (99.8-100%) with *R. africae* as validated species. The prevalence of infection in single ticks was highest in *A. variegatum* and lowest in *R. microplus*, while the MIR was highest in *A. variegatum* and lowest in *R. evertsi*.

Interestingly, 10.6% of single ticks and 54/1831 tick pools were positive in the 16S rRNA *Rickettsia* PCR but were negative for additional PCR amplifications targeting the *Rickettsia ompA, ompB, gltA* and *sca4* genes. Sequencing of these PCR products revealed the presence of *Coxiella* sp. endosymbionts (Table A.4). Subsequent amplification of these samples with *C. burnetii*-specific primers also yielded no amplification.

Theileria spp. DNA was identified in 1.4% of individual ticks and in six tick pools. Based on the 18S rRNA gene sequences, *T. parva* was detected in four of the *R. appendiculatus* pools with 100% identity to *T. parva* isolate from Kenya. *Theileria velifera* 18S rRNA sequences sharing 100% identity with *T. velifera* isolate from Saudi Arabia were observed in 19 single ticks and two-tick pools (Table A.4).

DNA of *Ehrlichia* spp. was detected in 86 single ticks and in 18 tick pools. Sequencing of the 16S rDNA gene revealed the presence of *E. ruminantium* in 80 single ticks and none in the pooled ticks, with the sequence showing 100% identity to *E. ruminantium* isolated from *Amblyomma hebraeum* in South Africa (Table A.4). On the other hand, *E. minasensis* 16S rDNA sequences were detected in six single ticks and 18 tick pools, and the sequences were 100% identical to the sequence of *E. minasensis* isolated from *R. microplus* from Brazil and Egypt. The identity of *E. minasensis* species was further confirmed by re-amplification of the groEL gene, which also showed 100% identity with *E. minasensis* sequences detected in cattle from Australia.

Pathogen co-infections and associations

For co-infections, we analyzed a subset of ticks limited to samples with a single tick. Out of the 1,382 single ticks, 1,138 (82.3%) ticks were infected with one TBP, while mixed infections with two and three different pathogens were observed in 6.9% (n = 95/1382) and 0.1% (n = 2/1382) of

single tick samples, respectively. The most common mixed infections were with *R. africae* and *E. ruminantium* (Table 3). Co-infection with *R. africae* and *E. ruminantium* was highest in *A. gemma* ticks with a prevalence of 7.2% (95% CI: 4.6 – 10.6), the same as the expected prevalence (7.2%).

Table 3. Prevalence of tick-borne pathogen co-infections in single ticks from coastal Kenya

Co-infection	Tick species	Ticks analyzed	Positive ticks (%)	Observed prevalence % (95% CI)	Expected prevalence %
Double co-infections					
R. africae* + *E. minasensis	*A. gemma*	321	2	0.6 (0.1 – 2.2)	0.5
	R. microplus	175	4	2.3 (0.6 – 5.7)	0.7
R. africae* + *E. ruminantium	*A. gemma*	321	23	7.2 (4.6 – 10.6)	7.2
	A. variegatum	835	47	5.6 (4.2 – 7.4)	5.5
R. africae* + *T. velifera	*A. gemma*	321	2	0.6 (0.1 – 2.2)	0.5
	A. variegatum	835	15	1.8 (1.0 – 2.9)	1.8
***E. ruminantium* + *Coxiella* sp. endosymbiont**	*A. gemma*	321	2	0.6 (0.1 – 2.2)	0.7
	A. variegatum	835	4	0.5 (0.1 – 1.2)	0.4
E. ruminantium* + *T. velifera	*A. variegatum*	835	2	0.2 (0.0 – 0.8)	0.1
***T. velifera* + *Coxiella* sp. endosymbiont**	*A. variegatum*	835	1	0.1 (0.0 – 0.7)	0.1
Triple co-infections					
R. africae* + *E. ruminantium* + *T. velifera	*A. variegatum*	835	2	0.2 (0.0 – 0.8)	0.1

Analysis of associations among pathogens and between pathogens and *Coxiella* sp. endosymbionts in single tick samples revealed a significant negative correlation between *R. africae* infection and *Coxiella* sp. endosymbionts (r = -0.64, p = 0.0133). All other combinations of pathogens were tested for their associations but showed no significant correlations (Figure 2).

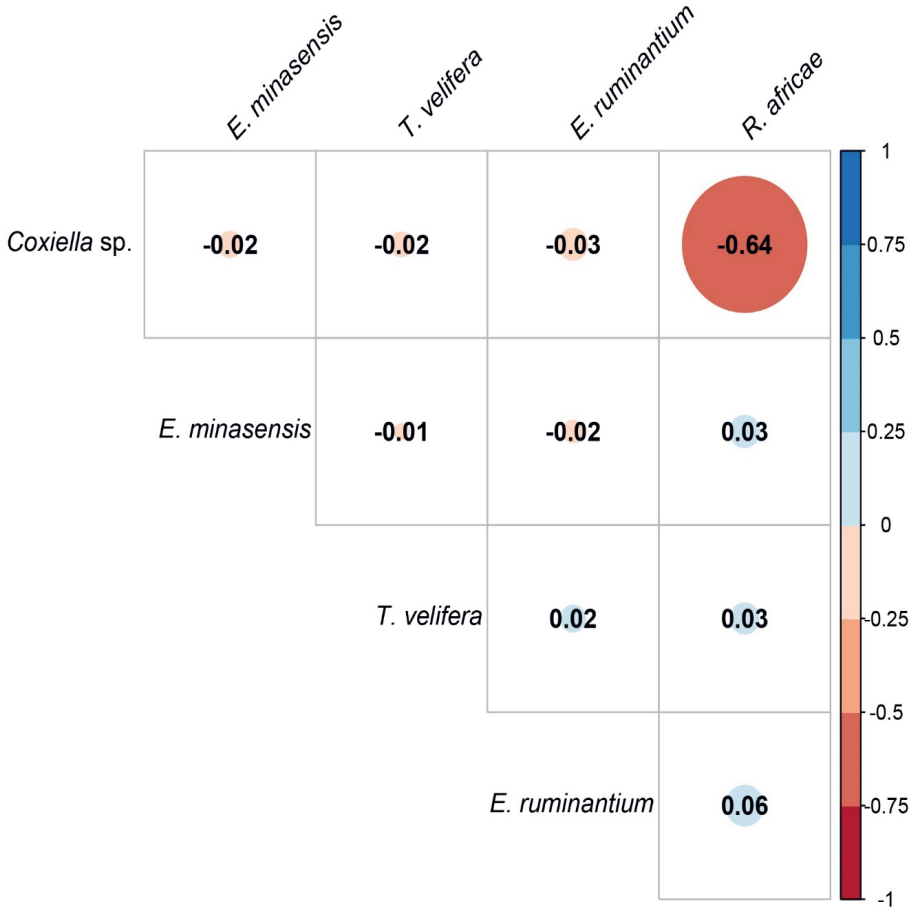

Figure 2. Correlogram showing the association between tick-borne pathogens detected in single tick samples from coastal Kenya. In the right side of the correlogram, the legend color shows the correlation coefficients and the corresponding colors. Positive correlations are displayed in blue and negative correlations in red color. Color intensity and the size of the circle are proportional to the correlation coefficients. The numbers inside are correlation coefficients. The correlation matrix is reordered according to the correlation coefficient using "hclust" method.

Discussion

This survey was conducted to assess the species diversity of ixodid ticks infesting cattle, their infestation levels and the associated TBPs in the coastal region of Kenya. We report the presence of eight tick species belonging to *Rhipicephalus* (four species), *Amblyomma* (two species), and *Hyalomma* (two species) that are infesting cattle in this region. We also provide molecular evidence showing that ticks in this region harbor a diverse array of microorganisms.

Based on morphological and genetic criteria, ticks were classified as *R. appendiculatus, R. evertsi, R. microplus*, *R. pulchellus*, *A. gemma*, *A. variegatum*, *H. rufipes* and *H. albiparmatum.* Except for *H. albiparmatum*, all these tick species have been reported to parasitize cattle in Kenya (Kariuki et al., 2012; Zulu et al., 1998). *Hyalomma albiparmatum* is a rare species that occurs only in southern Kenya and northern Tanzania (Walker et al., 2003). *Rhipicephalus decoloratus*, previously described in cattle in low numbers in some areas of coastal Kenya (Zulu et al., 1998) was not observed in our samples. However, the presence of the invasive Asian blue tick *R. microplus* in our study corroborates earlier findings, which reported this species in coastal Kenya (Kanduma et al., 2020; Zulu et al., 1998).

In the present study, the tick infestation was significantly higher in Kayafungo Ward in Kilifi County compared to Kinango Ward in Kwale County ($\chi^2 = 5.1701$, df = 1, $p = 0.03$). This could be partially explained by the presence of functional cattle dips in Mwachinga and Kibaoni villages of Kinango Ward in Kwale County. We also observed a significantly lower prevalence of tick infestation in calves than in juveniles and adults ($\chi^2 = 7.5314$, df = 2, $p = 0.02$). The lower tick infestations recorded in calves could be due to the husbandry practice of maintaining calves together close to the homesteads, separated from the adult cattle, resulting in lower tick exposure.

We report the presence of *E. minasensis* in four tick species with varying infection rates, namely *R. appendiculatus, R. evertsi, R. microplus* and *A. gemma*. This pathogen was previously reported in cattle in Kenya (Chiuya et al., 2021; Peter et al., 2020). The repeated detection in Kenya warrants further studies on their epidemiological implications for livestock health and productivity in the region. This is because *E. minasensis* has been experimentally demonstrated to cause clinical ehrlichiosis in cattle, a disease characterized by fever, lethargy, depression, thrombocytopenia, anemia, leukopenia and morulae in peripheral blood monocytes (Aguiar et al., 2014).

Rickettsia africae is the etiologic agent of African tick-bite fever (ATBF) in humans and is transmitted by *A. hebraeum* and *A. variegatum* ticks (Parola et al., 2013). We observed *R. africae* in *A. variegatum* with a prevalence of 87.9%. This high rate of *R. africae* in *A. variegatum* suggests that the risk for human infections is likely underestimated. The disease has previously been reported in international travelers returning from rural SSA, with an estimated annual incidence of 4 - 5.3% (Jensenius et al., 2003).

East Coast fever (ECF) caused by the protozoan parasite *T. parva* and transmitted by *R. appendiculatus* is the most economically important tick-borne disease of cattle in eastern, central and southern Africa, often leading to a loss in productivity and cases of mortality (Nene et al., 2016). In this study, *T. parva* was observed in *R. appendiculatus*, confirming the link between *R. appendiculatus* ticks and the epidemiology of ECF in SSA. The apparent presence of *T. parva* in its biological vector highlights the persistent risk of ECF to cattle, especially the exotic breeds, and thus the need to intensify tick control programs in this region.

Theileria velifera is non-pathogenic in cattle and is transmitted by *Amblyomma* ticks (Lawrence & Williamson, 2004). In the present study, *T. velifera* was detected in *A. gemma* and *A. variegatum* ticks and thus corroborates earlier studies that reported a close association between the distribution of *T. velifera* and *Amblyomma* ticks. Although *T. velifera* does not have any significant economic importance, its presence could complicate the specific diagnosis of the pathogenic *T. parva* in cattle and buffalo (Chaisi et al., 2013).

Coxiella sp. endosymbionts have previously been detected with varying prevalence in several tick genera, including *Rhipicephalus, Hyalomma, Ixodes*, *Amblyomma*, *Haemaphysalis* and *Dermacentor* (Oundo et al., 2020; Papa et al., 2017). This study also reports a varying prevalence of *Coxiella* sp. endosymbionts in *R. microplus, A. variegatum*, *R. appendiculatus*, *R. evertsi* and *A. gemma*. Thus, our findings add to the growing evidence of the widespread occurrence of *Coxiella* sp. endosymbionts across various tick species and geographical regions (Duron et al., 2015).

In this study, we observed that the infection frequency of *Coxiella* sp. endosymbionts was negatively correlated with the frequency of *R. africae* infection in *Amblyomma* ticks, and at no instance did we find concomitant co-infection between the pathogenic *R. africae* and *Coxiella* spp.

symbionts. A similar observation has been reported in *Rhipicephalus sanguineus* sensu lato, which was dominantly infected by either *Rickettsia* spp. or *Coxiella* spp. symbionts, but never both at the same abundance (René-Martellet et al., 2017). Thus, our finding suggests that infection with this *Coxiella* sp. symbionts may affect the colonization of *R. africae* in *Amblyomma* tick species and therefore warrants further mechanistic investigations to elucidate their interactions and their role in vector competence.

Ehrlichia ruminantium is the causative agent of heartwater disease in domestic ruminants (sheep, goats and cattle) and it is transmitted by *Amblyomma* ticks, mainly *A. hebraeum* in southern Africa and *A. variegatum* in the rest of SSA, the Caribbean and the Indian Ocean islands (Allsopp, 2010). We detected *E. ruminantium* DNA in *A. gemma* and *A. variegatum*, confirming the strong link between the distribution of *Amblyomma* ticks and heartwater disease in SSA (Allsopp, 2010). The detection of *E. ruminantium* in *R. microplus* in this study is not completely surprising since a recent study has reported the potential of *R. microplus* to transmit *E. ruminantium* in West Africa (Biguezoton et al., 2016). The presence of *E. ruminantium* in *Amblyomma* and *R. microplus* ticks in the study area suggests that the risk for heartwater infections in cattle is underestimated.

We observed co-infections in 7.0% of the analyzed single ticks and that ticks could be infected with up to three different pathogen species. The most frequent pathogen combination observed in this study was *R. africae* and *E. ruminantium*, suggesting the possibility of ticks vectoring multiple pathogens in this region. It is worth noting that co-infections of multiple pathogens can alter typical disease symptoms or enhance disease severity, thus resulting in diagnostic and treatment challenges (Diuk-Wasser et al., 2016; Moutailler et al., 2016). Therefore, it is important to continue assessing the range and frequency of co-infections occurring naturally in ticks.

Due to the nature of the cross-sectional design, this work had limited ability to investigate the typical seasonal fluctuations in tick densities, and the influence of animal movement on tick dispersal and the challenges it poses in correlating infection and infestation risk factors. As such, this study provides only one snapshot of tick diversity and infestation prevalence, as well as TBPs prevalence in ticks. Additionally, the mere presence of pathogen DNA in the collected ticks does not necessarily mean that they are biological vectors, as a tick can test positive for a pathogen if it ingests infected blood without necessarily transmitting it to a susceptible animal host during its

next blood meal. Therefore, the results of the present study should be interpreted with caution. Future studies should aim to cover a broader geographical area, allowing for a more comprehensive understanding of TBDs and the development of effective control strategies.

Conclusions

Our study provides contemporary evidence that multiple TBPs of zoonotic and veterinary importance are harbored by the bovine tick population in coastal Kenya. The observed co-infections in ticks represent a risk of acquiring multiple infections as a consequence of a single tick bite. Further studies are needed to elucidate the functional roles of *Coxiella* sp. endosymbionts in pathogen colonization and transmission in ticks. More active surveillance will help to detect the spread and potential risk of *E. minasensis* infection for the bovine population throughout coastal Kenya.

Acknowledgments

We are grateful to Dr. Raphael Nyawa, Director of Veterinary Services in Kinango sub-county (Kwale County) and Dr. Caroline Asanyo, Director of Veterinary Services in Kaloleni sub-county (Kilifi County) for facilitating our fieldwork, and local farmers who allowed us to sample their cattle. Further, we thank Peter Muasa, Richard Tumba and Philip Kolei from *icipe* for technical support. We also appreciate the administrative assistance of Caroline Muya and James Kabii.

Appendix A. Supplementary data

Table A.1. Distribution, diversity and abundance of tick species collected from cattle hosts in coastal Kenya

Ward (County)	Village name	Tick species								Total ticks collected
		R. appendiculatus	*R. evertsi*	*R. pulchellus*	*R. microplus*	*A. gemma*	*A. variegatum*	*H. rufipes*	*H. albiparmatum*	
Kinango (Kwale)	Mdzumbariaka	13	26	1	8	85	181	8	3	325
	Kibandaongo	23	79	1	3	28	0	1	0	135
	Mwangani	62	57	0	114	43	107	0	0	383
	Chongo-Mundu	44	13	0	35	3	34	0	0	129
	Kidogoeni	54	45	0	9	105	5	0	1	219
	Mwachinga	0	8	0	0	50	164	0	0	222
	Kibaoni	0	4	0	6	15	245	0	0	270
	Sub-total	**196 (11.6%)**	**232 (13.8%)**	**2 (0.1%)**	**175 (10.4%)**	**329 (19.5%)**	**736 (43.7%)**	**9 (0.5%)**	**4 (0.2%)**	**1683**
Kayafungo (Kilifi)	Mwatsuma	30	26	0	90	2	138	0	1	287
	Kakoneni	10	0	3	0	14	20	1	0	48
	Kirumbi	12	38	0	9	91	25	0	0	175
	Ndatani	20	60	1	4	161	0	4	2	252
	Tsangatsini	30	28	0	4	131	8	3	0	204
	Katsangani	115	16	0	52	16	124	1	0	324
	Kinagoni	95	12	0	26	29	78	0	0	240
	Sub-total	**312 (20.4%)**	**180 (11.8%)**	**4 (0.3%)**	**185 (12.1%)**	**444 (29.0%)**	**393 (25.7%)**	**9 (0.6%)**	**3 (0.2%)**	**1530**
	Total tick species	**508 (15.8%)**	**412 (12.8%)**	**6 (0.2%)**	**360 (11.2%)**	**773 (24.1%)**	**1129 (35.1%)**	**18 (0.6%)**	**7 (0.2%)**	**3213**

Percentages are out of the total ticks collected

Table A.2. Sequence identities and GenBank accessions of tick species identified by molecular method

Morphological identification	16S rDNA (% identity, GenBank accession)	ITS2 (% identity, GenBank accession)	COI (%identity, GenBank accession)	Consensus identification (Submitted GenBank accessions)
***Rhipicephalus* sp.**	*R. microplus* (100, MN650729)	*R. microplus* (99.2-100, MK621182)	na	***R. microplus*** **16S:** MW227415, MW227420, MW227421 **ITS2:** MW227651, MW227653, MW227654
R. appendiculatus	*R. appendiculatus* (99.5-100, MT430988)	*R. appendiculatus* (99.9, KY457500)	na	***R. appendiculatus*** **16S:** MW227410 - MW227412 **ITS2:** MW227652
R. evertsi	*R. evertsi* (99.5, KJ613642)	na	na	***R. evertsi*** **16S:** MW227413
R. pulchellus	*R. pulchellus* (99.5-99.74, MK774738)	*R. pulchellus* (99.9, AF271275)	*R. pulchellus* (98.2, KY678133)	***R. pulchellus*** **16S:** MW227414, MW227416 **ITS2:** MW227655 **CO1:** MW243657
A. gemma	*A. lepidum* (98.4-100, MK737651)	*A. gemma* (99.4, MN401350)	na	***A. gemma*** **16S:** MW227404 - MW227406 **ITS2:** MW227649
A. variegatum	*A. variegatum* (99.0-99.5, MH781753)	*A. variegatum* (99.9, MT000685)	na	***A. variegatum*** **16S:** MW227407 - MW227409 **ITS2:** MW227650
H. albiparmatum	*H. albiparmatum* (99.5, KU130412)	na	na	***H. albiparmatum*** **16S:** MW227417
H. rufipes	*H. marginatum* sensu lato (98.93-100, MK058362)	na	*H. rufipes* (99.50-99.75, KX000641)	***H. rufipes*** **16S:** MW227418, MW227419, MW227422 **CO1:** MW243658

na: no amplification

Table A.3. Cumulative mean tick intensity, median tick intensity and proportion of infested cattle during the first survey in coastal Kenya.

Ward (County)	Village name	Total ticks collected	No. of examined cattle	No. of infested cattle	Mean tick intensity (min-max)	Median tick intensity (1st and 3rd quartiles)	% of infested cattle (95% CI)
Kinango (Kwale)	Mdzumbariaka	119	116	24	5 (1-13)	4 (3-6.5)	20.7 (13.7-29.2)
	Kibandaongo	135	109	30	4.5 (1-15)	3 (3-4.8)	27.5 (19.4-36.9)
	Mwangani	320	172	54	5.9 (1-14)	6 (4-7)	31.4 (24.6-38.9)
	Chongo- Mundu	108	110	23	4.7 (2-13)	4 (3-5.5)	20.9 (13.7-29.7)
	Kidogoeni	173	91	25	6.9 (2-14)	7 (5-9)	27.5 (18.6-37.8)
	Mwachinga	0	101	0	0	0	0
	Kibaoni	0	100	0	0	0	0
	Sub-total	**855**	**799**	**156**	**5.5 (1-15)**	**5(3-7)**	**19.5 (16.8-22.4)**
Kayafungo (Kilifi)	Mwatsuma	200	124	29	6.9 (3-12)	7 (5-8)	23.4 (16.3-31.8)
	Kakoneni	48	15	6	8 (3-13)	8 (6.5-9.5)	0.4 (16.3-67.8)
	Kirumbi	130	145	31	4.2 (1-8)	4(2.5-5)	21.4 (15.0-29.0)
	Ndatani	252	110	32	7.9 (3-14)	8 (6-9)	29.1 (20.8-38.5)
	Tsangatsini	148	105	24	6.2 (2-12)	6 (4.8-7)	22.9 (15.2-32.1)
	Katsangani	266	115	30	8.9 (2-18)	8 (5-11)	26.1(18.3-35.1)
	Kinagoni	210	109	25	8.4 (2-17)	9 (5-10)	22.9 (15.4-32.0)
	Sub-total	**1,254**	**723**	**177**	**7.1 (1-18)**	**6 (5-9)**	**24.5(21.4-27.8)**
	Total	**2,109**	**1522**	**333**	**6.3 (1-18)**	**6 (4-8)**	**21.9 (19.8-24.0)**

Table A.4. Sequence identities and GenBank accessions of tick-borne pathogens and endosymbionts detected in ticks collected from coastal Kenya

Pathogen detected	Locus	Closest BLASTn hit, Reference GenBank accession numbers, Country	Sequence identity (%)	Submitted GenBank accession numbers
Ehrlichia minasensis	16S rRNA	*E. minasensis*, NR_148800 Brazil, MN372102, Egypt	99.5 - 100	MW228108 - MW228112
	groel	*E. minasensis,* MH500006, Australia	99.4 - 100	MW248709 - MW248712
Ehrlichia ruminantium	16S rRNA	*E. ruminantium*, CP040118, South Africa	100	OL410611 - OL410619
Rickettsia africae	16S rRNA	*R. africae*, MK656388, Uganda	100	MW229058 - MW229062
	ompA	*R. africae*, MH751466, South Africa	100	MW248717 - MW248721
	ompB	*R. africae*, KU721071, Austria: imported from Tanzania	99.9-100	MW248722 - MW248727
	gltA	*R. africae*, KJ645939, Madagascar	100	MW248713 - MW248716
	Sca4	*R. africae*, AF151724, France	99.8-100	MW248728 - MW248733
Theileria velifera	18S rRNA	*T. velifera,* LC431550, Saudi Arabia	100	MW241624 - MW241626 OL454896 - OL454898
Theileria parva	18S rRNA	*T. parva,* MH929322, Kenya	100	MW241627
***Coxiella* sp. endosymbiont**	16S rRNA	*Coxiella* sp. endosymbiont, MN088359, Australia	99.4-100	MW242967 - MW242972 OL411942 - OL41194

3

Chapter 3

Epidemiology of tick-borne pathogens of cattle and tick control practices in coastal Kenya

Joseph Wang'ang'a Oundo[1,2*], Daniel Masiga1, Quirine ten Bosch[2], Jandouwe Villinger[1], Constantianus J.M. Koenraadt[3], Shewit Kalayou[1]

1 International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya,
2 Quantitative Veterinary Epidemiology, Wageningen University & Research, Wageningen, the Netherlands,
3 Laboratory of Entomology, Wageningen University & Research, Wageningen, the Netherlands

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Abstract

Tick-borne diseases (TBD) are a major constraint to livestock health and productivity in sub-Saharan Africa. Nonetheless, there are relatively few robust epidemiologic studies documenting TBD and its management in different endemic settings in Kenya. Therefore, a cross-sectional study using multi-stage cluster sampling was undertaken to characterize the epidemiology of TBD and management factors among zebu cattle reared under an extensive system in coastal Kenya. Blood samples from 1486 cattle from 160 herds in 14 villages were screened for the presence of tick-borne bacterial and protozoan pathogens using PCR with high-resolution melting analysis and sequencing. Standardized questionnaires were used to collect data on herd structure and herd management practices, and a mixed-effect logistic regression model to identify risk factors for tick-borne pathogens (TBPs). The application of chemical acaricide was the primary method for tick control (96.3%, 154/160), with the amidine group (mainly Triatix®, amitraz) being the most frequently used acaricides. Respondents identified East Coast fever as the most important disease and Butalex® (buparvaquone) was the most commonly administered drug in response to perceived TBD in cattle. The overall animal- and herd-level prevalence for TBPs were 24.2% (95% confidence interval (CI): 22.0-26.4%) and 75.6% (95% CI: 68.2-82.1%), respectively. Cattle were infected with *Anaplasma marginale* (10.9%, 95% CI: 9.4 - 12.6), *Theileria parva* (9.0%, 95% CI: 7.5 - 10.5), *Anaplasma platys* (2.6%, 95% CI: 1.9 - 3.6), *Theileria velifera* (1.1%, 95% CI: 0.7 - 1.8), *Babesia bigemina* (0.5%, 95% CI: 0.2 - 1.0), and *Anaplasma* sp. (0.1%, 95% CI: 0.0 - 0.4). Moreover, 21 cattle (1.4%) were co-infected with two TBPs. None of the assessed potential risk factors for the occurrence of either *A. marginale* or *T. parva* in cattle were statistically significant. The intra-herd correlation coefficients (lCCs) computed in this study were 0.29 (*A. marginale*) and 0.14 (*T. parva*). This study provides updated molecular-based information on the epidemiological status of TBPs of cattle and herd management practices in coastal Kenya. This information can be used in designing cost-effective control strategies for combating these TBD in the region.

Introduction

Tick-borne diseases (TBD) remain among the most important livestock diseases worldwide due to their impact on livestock health and productivity which result in huge economic losses in the livestock sector (Ocaido et al., 2009). Losses due to TBD are incurred directly through decreased meat and milk production, lost draft power, morbidity and mortality, and indirectly through costly control measures and loss of cash income (Gachohi et al., 2012; Minjauw & McLeod, 2003). These economic losses disproportionately impact small-scale resource-poor households in developing countries, including Kenya, where more people depend on livestock production for financial and nutritional security (Minjauw & McLeod, 2003).

The most important TBD of cattle in Kenya include East Coast fever (ECF), caused by the protozoan *Theileria parva*, bovine anaplasmosis caused by the bacterium *Anaplasma marginale*, and bovine babesiosis caused by the protozoa *Babesia bigemina* and *Babesia bovis.* Generally, these TBD pose a greater challenge to the susceptible exotic (i.e., *Bos taurus*) and crossbred cattle (i.e., *B. taurus* × *Bos indicus*), thus representing a major constraint in the improvement of local cattle production (Gachohi et al., 2012). The clinical course of these TBD is usually subclinical in the autochthonous zebu cattle (i.e., *B. indicus*), but high tick infestation combined with other stress factors (e.g., malnutrition, pregnancy, lactation, concurrent infections, etc.) can cause clinically apparent acute disease (Kocan et al., 2010). Although the impacts of these diseases have not been comprehensively quantified, previous reports from Kenya indicate enormous losses through morbidity, mortality, and productivity losses (Gitau et al., 1999; Kiara et al., 2014; Maloo et al., 2001a; Muraguri et al., 2005; Wesonga et al., 2010).

Potential risk factors associated with TBP infection in cattle include cattle breed, age, agro-ecological zone, livestock production system (Gachohi et al., 2012), inherent resistance of cattle to ticks and TBD (Jonsson et al., 2014; Laisser et al., 2016; Robbertse et al., 2017; Shyma et al., 2013), the frequency of acaricide application (Miyama et al., 2020; Wesonga et al., 2014), tick infestation on cattle (Byaruhanga et al., 2016; Kerario et al., 2017; Wesonga et al., 2014), and distribution of tick vectors and infection rate of ticks (Norval et al., 1992). However, these potential risk factors are highly inconsistent between studies. Some studies did not find any significant association between TBP infection status in cattle and tick control practices (Gitau et al., 1997; Maloo et al., 2001a), age (Byaruhanga et al., 2016; Kerario et al., 2017), sex (Kerario et al., 2017;

Okal et al., 2020), frequency of acaricide application (Kerario et al., 2017; Kimaro et al., 2017), and presence of tick infestation among cattle (Simuunza et al., 2011). Therefore, identifying and quantifying risk factors contributing to disease occurrence and characterizing the current epidemiologic states in different endemic settings is essential in designing cost-effective control strategies for combating these TBD.

The recent climatic changes such as the extent and distribution of rainfall, in addition to anthropogenic factors such as agricultural intensification, deforestation, nomadic pastoralism and transboundary animal trade observed in recent years may further lead to a shift in the epidemiology of TBD in Kenya (Githaka et al., 2021). It is, therefore, imperative to regularly update existing epidemiological information on TBD in cattle. Currently, there are few robust epidemiologic studies of TBD in the different endemic settings in Kenya, including the coastal regions, and thus control strategies lack evidence-based guidelines. The available epidemiological studies of TBD in cattle in Kenya have traditionally been based on serological tests (Gachohi et al., 2010; Maloo et al., 2001a), microscopic examination of stained blood smears and smears of lymph node biopsies (Muraguri et al., 2005; Okuthe & Buyu, 2006) or clinical signs (Kanyari & Kagira, 2000). However, all these diagnostic techniques have considerable limitations in terms of sensitivity and specificity (Salih et al., 2015). Microscopy lacks the sensitivity required for detecting low levels of infections in carrier animals, and the pathogens are difficult to identify to species level or distinguish between closely related species. On the other hand, serological methods cannot differentiate between current infections and previous exposures in carrier animals, and reported cross-reactivity of antibodies limits specificity. Therefore, a sensitive and highly specific molecular approach is required to determine the current TBP infection status.

Little contemporary data is available on the management practices of ticks and TBD by livestock farmers following the withdrawal of government-funded veterinary services (Government of Kenya, 2008). To inform more effective and sustainable future management options, it becomes imperative to investigate management practices among cattle owners regarding ticks and TBD. These are no longer strongly informed by government policy but by farmer preferences and affordability. To improve epidemiological knowledge of TBPs in coastal Kenya and present opportunities for strategic disease prevention and control, the objectives of the present study were to 1) estimate the molecular prevalence of species of *Anaplasma, Babesia, Ehrlichia, Rickettsia*

and *Theileria* in cattle; 2) assess the potential risk factors for these TBP infections in cattle; and 3) characterize the control practices related to ticks and TBD among cattle owners in coastal Kenya.

Material and methods

Study setting

The study was conducted in Kayafungo Ward (Kilifi County) and Kinango Ward (Kwale County) in coastal Kenya (Figure 1) from November to December 2019. The two administrative wards (i.e., Kinango and Kayafungo Ward) were selected purposively based on their potential for livestock production in the region, good accessibility, and the difference in access to veterinary services. Kinango Ward has functional cattle dips sponsored by the local County government or farmer organization groups, unlike Kayafungo. Administratively, Kinango Ward is divided into 4 sub-locations (Kinango, Dumbule, Kibandaongo and Gandini sub-locations). Kayafungo Ward is divided into 6 sub-locations (Tsangatsini, Mnyenzeni, Miyani, Kinagoni, Mbalamweni, Mirimani). Sub-location is the smallest administrative unit in Kenya. The study area is characterized by a semi-arid climate with low and erratic rainfall. The rainfall pattern is bimodal, with most rains between April and June (long rains) and October to November (short rains), but some rain falls nearly every month, especially near the coastline. The average annual rainfall ranges between 500 - 600 mm in the drier hinterland, increasing to 900 - 1500 mm along the coastal belt. The mean annual temperature in the coastal region ranges between 23°C and 34°C. The area faces recurrent droughts and is characterized by extensive rangeland with sparse vegetation. The livestock production system is a predominantly traditional extensive system with the majority of households keeping chickens, cattle, goats, and sheep, which provides a source of income for families through the sale of meat and dairy. The local East African zebu breeds are the predominant cattle in the study area. They are grazed extensively on fallow or communal grazing fields in natural pastures and share watering points. The sharing of grazing land and water points exposes cattle to a high risk of tick infestation and thus increasing the likelihood of TBD outbreaks. Many cattle owners in the region also migrate with their animals, searching for pasture and water during the long dry seasons. This uncontrolled cattle movement results in mixing herds from different areas, thus increasing the risk of disease transmission between herds and new geographical areas (Ekwem et al., 2021).

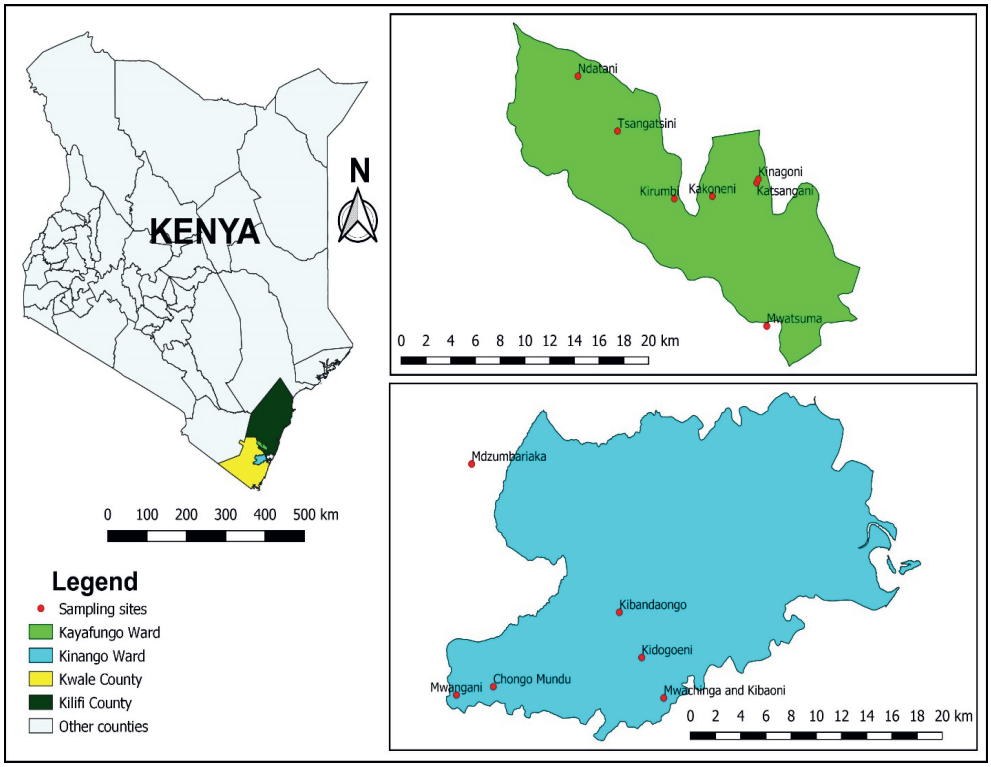

Figure 1. Map of Kayafungo and Kinango wards in coastal Kenya showing the crush sites in each village cluster where the sampling took place. The map was prepared using common-license shape files in QGIS software version 3.10 (QGIS Development Team, 2020).

Study design, sample size, and sampling strategy

The study was a baseline survey of a more extensive operational research project entitled "Improving food and nutritional security through integrated control of tsetse and tick-borne livestock diseases (ICTLD)." A cross-sectional study with multi-stage cluster sampling was used in selecting the study population (Figure 2). Cluster sampling was chosen due to the unavailability of individual animal sampling frames (Dohoo et al., 2009). All four sub-locations in Kinango Ward and four of the six sub-locations in Kayafungo Ward were purposively selected to increase the geographical spread of the study. Two spatial village clusters (each containing 3 – 6 villages) in each sub-location were then chosen by purposive sampling in collaboration with the respective sub-county's directorate veterinary personnel. The final listing of village clusters was made based

on the cooperation of farmers and logistical feasibility (accessibility by vehicle, security, distance). Since cattle from the adjacent villages share common grazing land, route, and watering point, the risk of tick infestation and hence TBPs infection prevalence within those sub-locations was assumed to be similar and therefore few spatial village clusters per sub-location were selected. From selected villages, a group of cattle owned by a household was designated as a herd and was considered the primary sampling unit, and the individual cattle within the herd were considered the secondary sampling unit. Herd selection was randomly made based on their location by village and the willingness of the farmer to participate in the study.

The sample size (n) was determined following a previously described method (Molla et al., 2018):

$$n = gc = \frac{P(100-P)D}{SE^2} \quad \text{..... (1)}$$

The seroprevalence rates for *T. parva, A. marginale* and *B. bigemina* in the region ranged from 14% - 97% (Maloo et al., 2001b). Therefore we used a 50% expected prevalence (P) and a 5% margin of error (SE) and adjusted for design effect (D), which was estimated using formula 2.

$$D = 1 + (g - 1)ICC \quad \text{..... (2)}$$

Where g is the average number of individuals sampled per cluster, and *c* is the number of clusters to be sampled. The intra-herd correlation coefficient (ICC) relates to the relatedness of clustered data.

Assuming an ICC of 0.15 and considering the possibility of collecting about 100 blood samples by a team of 4 people per day in a village cluster, *D* equals 16 (formula 2). The ICC estimate was based on the reported intra-herd correlation coefficient for exposure to *A. marginale* (Gachohi et al., 2010). Sampling 100 animals per cluster (village) with an expected disease prevalence of 50% and the desired precision of 5% gave 16 spatial village clusters and thus a total sample size of around 1600 cattle. The clusters and the total sample size were equally distributed among the two study wards. Cattle were sampled randomly, proportional to the herd size. Thus, all cattle were sampled if a herd had less than ten animals, ten were randomly selected if the herd size was up to 20 animals, and 30% were sampled in herds with more than 20 animals. Each herd was sampled with the informed consent of its owner or authorized agent. There were no sex restrictions, but

cattle were not eligible for sampling if they were less than six months of age. Due to logistical challenges, two spatial village clusters were not sampled.

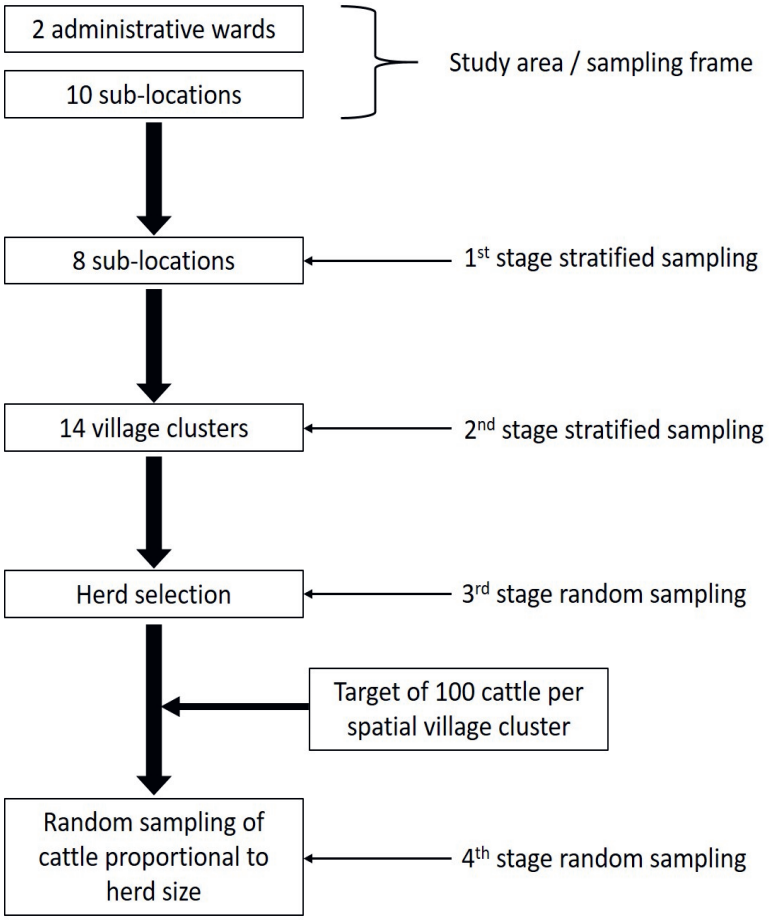

Figure 2. Scheme showing the design and sampling strategy used in this study

Sampling and data collection

Blood samples were collected from the jugular vein of each cattle using 4-ml vacutainer tubes (BD Vacutainer®) coated with ethylenediaminetetraacetic acid (EDTA). The tubes were gently inverted 4–5 times to mix the blood with the anticoagulant before being transferred to 2-ml sterile cryovials labeled with animal ID, date, and site of collection. These samples were kept in a cool box containing ice packs in the field. At the same time, approximately 125 µl of the collected blood

sample was transferred into sodium-heparinized micro-hematocrit capillary tubes to measure packed cell volume (PCV). Briefly, the blood samples in the hematocrit capillary tubes were centrifuged for 5 minutes at 12,000 rpm using a micro-hematocrit centrifuge, and the PCV was measured using a micro-hematocrit reader. A PCV below the threshold level of 24% was considered anemic. Corresponding records of each sampled animal, including location, ownership, age, sex, breed, live body weight, PCV, and ticks present on cattle, were entered onto a predesigned datasheet. The age of an animal was assessed by the dentition and farmer's information and was categorized as calves (6-12 months of age), juveniles (13–24 months) and adults (over 24 months of age). Sex was categorized as female versus male, while breed was categorized into indigenous and cross classes. The samples in the cool boxes were then transported to the field station for storage in liquid nitrogen before transportation to the Martin Lüscher Emerging Infectious Disease (ML-EID) laboratory at the International Centre of Insect Physiology and Ecology (*icipe*). The samples were stored at -80°C awaiting pathogen screening.

A pre-tested questionnaire containing both closed and open-ended questions was administered to the household head or spouse in Kiswahili or the local language. The questionnaire was designed to obtain: (i) sociodemographic information, (ii) herd management-related information, and (iii) tick and TBD-related information. The respondents were asked questions from the questionnaire without having the choices read. The trained data collectors recorded the answers given based on the listed options. This approach was preferred to avoid leading questions that could introduce bias. In cases where the expected responses were deemed not exhaustive or not in the listed choices, an option for "others: please specify" was provided. Open-ended questions were mainly used when a numerical response was expected. In some sections, the participants were allowed to provide more than one answer. For instance, the farmers were allowed to give more than one answer when asked about the type of acaricides they have used in the past 12 months, the symptoms they perceived to be associated with TBD, etc. When the brand name of drugs was the sought response, farmers were asked to verify it by producing a sales receipt or presenting the product or its used packages. The questionnaire took 30–45 minutes to administer. All this information was collected and managed using Research Electronic Data Capture (REDCap) tools (Harris et al., 2019) hosted at *icipe*.

DNA extraction

The genomic DNA was extracted from EDTA-treated blood samples according to the procedure described by (Suguna et al., 2014) with some modifications. Briefly, 300 µl of whole blood was added to a 1.5-ml Eppendorf tube containing 900 µl low salt buffer (10 mM Tris-HCl, pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 2 mM EDTA) and 50 µl of 1% Triton X-100. The samples were mixed well by vortexing and incubated for 10 minutes at 56°C to lyse the red blood cells. The cells were centrifuged at 10,000 rpm for 10 minutes, and the supernatant was discarded. This step was repeated 2-3 times with a decreasing amount of 1% Triton X-100 until a white pellet of white blood cells was obtained. After the lysis stage, 300 µl of high salt buffer (10 mM Tris-HCl, pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 2 mM EDTA, 400 mM NaCl) and 50 µl of 10% Sodium Dodecyl Sulfate (SDS)were added to the cell pellet, mixed thoroughly and incubated at 56°C for 10 minutes. At the end of incubation, 100 µl of 6M NaCl was added and vortexed to precipitate the proteins before centrifuging at 10,000 rpm for 5 minutes. The supernatant was then transferred into a new Eppendorf tube containing 500 µl of absolute isopropanol. DNA was precipitated by continuously inverting the Eppendorf tube slowly for 3 minutes before centrifuging at 10,000 rpm for 10 minutes to pellet down the DNA. The supernatant was discarded, and 500 µl of ice-cold 70% ethanol was added and mixed slowly to remove any excess salts. Finally, the tubes were centrifuged at 10,000 rpm (4°C) for 7 minutes to pellet down the DNA. The supernatant was discarded, and DNA pellets were air-dried. After thorough drying, the DNA pellets were re-suspended in 100 µl of sterile de-ionized distilled water, and the DNA was stored at -20°C until further use.

Molecular detection of tick-borne pathogens

The DNA samples were screened by PCR with high-resolution melting (PCR-HRM) analyses for the presence of species of *Anaplasma, Babesia, Ehrlichia, Rickettsia,* and *Theileria*. The PCR-HRM assays were conducted on a Magnetic Induction Cycler (MIC) machine (BioMolecular Systems, Australia) using genera-specific PCR-HRM primers listed in Table 1. The reaction mixture had a final volume of 10 µl, containing 5 µl of PCR grade water, 2 µl of 5xHOT FIREPol EvaGreen HRM mix (no ROX) (Solis BioDyne, Estonia), 0.5 µl of 10 pmol of each primer and 2 µl of the DNA extract. The PCR cycle parameters included an initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 20 sec, annealing for 30 sec at temperatures listed in Table 1, and extension at 72°C for 30 sec. This was followed by a final extension at 72°C for 7 min. The PCR cycle was directly followed by HRM analysis with an increasing temperature

from 75°C to 95°C at 0.1°C/sec. The positive controls included *Anaplasma bovis, Ehrlichia ruminantium* and *T. parva,* while a master mix without the DNA template was used as a negative control. Representative samples for each unique HRM profile were selected and purified using ExoSap-IT (USB Corporation, Cleveland, OH, USA) according to the manufacturer's protocol and then sent to Macrogen Inc. (The Netherlands) for sequencing in both directions.

Representative samples were further re-amplified for confirmation of positive *Anaplasma* samples using standard PCR primers targeting the major surface protein 4 (*msp4*). The standard PCR reaction contained 4 µl of 5 x HOT FIREPol® Blend Master Mix (Solis Biodyne, Estonia), 1 µl of 10 pmol of each forward and reverse primer, 4 µl of the DNA template, and 10 µl of PCR grade water. The cycling conditions were: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. The final extension was at 72°C for 7 min. This PCR reaction was carried out using SimpliAmp™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The PCR products were electrophoresed on a 2% agarose gel stained by ethidium bromide, and expected bands were excised and purified by the QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol before sequencing.

Table 1. PCR primer pairs and annealing temperatures used in this study

Genus	Primer	Target gene	Primer sequence (5'-3')	Annealing temperature (°C)	Amplicon size (bp)	Citations
Anaplasma	MSP45	*msp4*	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	60	851	(De La Fuente et al., 2004)
	MSP43		CCGGATCCTTAGCTGAACAGGAATCTTGC			
Anaplasma/Ehrlichia	16S8FE	*16S* rDNA	GGAATTCAGAGTTGGATCMTGGYTCAG	60.5	448	(Schouls et al., 1999)
	B-GA1B		CGGGATCCCGAGTTTGCCGGGACTTCTTCT			
Babesia/Theileria	RLB-F2	*18S* rDNA	GACACAGGGAGGTAGTGACAAG	60.5	460–500	(Georges et al., 2001)
	RLB-R2		CTAAGAATTTCACCTCTGACAGT			
Rickettsia	Rick-F1	*16S* rDNA	GAACGCTATCGGTATGCTTAACACA	55	350–400	(Nijhof et al., 2007)
	Rick-F2		CATCACTCACTCGGTATTGCTGGA			

Sequence and phylogenetic analyses

The obtained sequences were edited using Geneious software version 11.1.5 (Kearse et al., 2012). The sequences were first truncated at the 5'- and 3'-ends to remove low-quality reads and the

primer sequences. Identities of the truncated sequences were revealed by querying in the GenBank nr database using the Basic Local Alignment Search Tool (www.blast.ncbi.nlm.nih.gov/). Annotated sequences of the same genus and locus were extracted from the GenBank database and aligned with the MAFFT plugin in Geneious (Katoh & Standley, 2013). The phylogenetic analysis was inferred using the maximum likelihood (ML) approach as implemented in PhyML version 3.0 (Guindon et al., 2010) based on the Akaike information criterion (AIC) for automatic model selection. Bootstrap analysis with 1000 replications was used to estimate the confidence of the nodes and branches of the trees.

Statistical analysis

Raw data was entered into Microsoft® Excel 2016 and verified for missing observations and erroneous entries. Incomplete entries were excluded from the analysis (n = 36/1522). Statistical analysis was performed using R software version 4.1.3 (R Core Team, 2022). Descriptive statistics were calculated for all animal-, farm- and area-level variables. Since the two wards (i.e., Kinango and Kayafungo) are autonomous administrative units with different access to veterinary extension services, we calculated the descriptive statistics for demographic characteristics, awareness, perceptions and each element of the control practices at the ward administrative level (cluster of sub-locations). The outcome measure for the prevalence estimation was the presence and absence of the tested TBPs. The individual-level prevalence (proportion of infected cattle out of the total tested cattle) and herd-level prevalence (proportion of herds with at least one positive pathogen divided by the total number of herds tested) for each TBP were calculated. A herd was declared positive if at least one animal tested positive for a pathogen based on PCR-HRM and sequencing results. Only *T. parva* and *A. marginale* pathogens were considered during risk factor analysis due to their economic significance and sufficient data to perform a risk factor analysis. The breed category was also excluded from the analysis due to insufficient data. Analysis of possible risk factors related to *T. parva* and *A. marginale* infection in cattle was performed using a univariable mixed-effect logistic regression model (generalized linear mixed model with a binomial link) using the package '*lme4'* (Bates et al., 2015). Herd and villages with herds nested within villages were included as random effects to account for within-cluster correlation of infection status. The exposure variables considered were age, sex, PCV, frequency of acaricide applications, application of acaricide to other livestock species on the farm, the regular grazing area of the herds, and presence of ticks on cattle when collecting blood samples, and the administrative wards. None of

the assessed possible risk variables were statistically significant in the univariate model; therefore, we did not fit a multivariate mixed-effect logistic regression model. Variance estimates associated with the random effects (i.e., herd- and village-level clustering) were used to estimate the intra-herd correlation coefficient (ICC) of *T. parva* and *A. marginale* infections following the latent variable approach (Dohoo et al., 2009). A p-value ≤ 0.05 was considered statistically significant.

Results

Cattle owner demographics

A total of 160 respondents were interviewed across the 14 villages, and the demographic data are summarized in supplementary table 1. Sixty-seven (41.9%) respondents were from Kinango ward in Kwale county, while 93 (58.1%) were from Kayafungo ward in Kilifi county. The majority of the respondents were male (n = 137, 85.6%). All of these were household heads. A sizable portion of respondents had attained a primary level education (n = 74, 46.3%) or was illiterate (n = 60, 37.5%), practiced crop-livestock mixed farming as their primary occupation (n = 131, 81.9%), and had less than 10 years of farming experience (n = 86, 53.8%). The respondents' ages ranged from 20 to 89 years (median 51.0).

Cattle husbandry and tick control practices

Detailed aspects of cattle husbandry and tick control practices among cattle owners in coastal Kenya are shown in Table 2. All the 160 farms in the survey kept the indigenous zebu cattle, and all farmers relied exclusively on natural breeding services rather than artificial insemination. The cattle were reared for multiple purposes, including draft power, sale, and milk production. All sampled farmers in the survey area practiced extensive grazing where the cattle were left to graze free-range in the open environment. Most farmers grazed their cattle on communal land (55.6%, 89/160) and watered their cattle at a river (54.4%, 87/160). Housing was not provided on 60.0% (96/160) of the farms, with the cattle staying under a tree or next to the houses within the homestead. The frequently reported constraints of cattle production in the study area as perceived by farmers included cattle diseases (90.0%, 144/160), inadequate veterinary services (58.8%, 94/160), inadequate water for livestock (43.1%, 69/160), shortage of feed (40.6%, 65/160) and poor market for livestock products (20.6%, 33/160) (Table 2).

The majority of respondents (56.9%, 91/160) perceived an increase in tick infestation levels on cattle during the rainy season. Almost all farmers (96.3%, 154/160) used chemical acaricides for

tick control, with the amidine group (mainly Triatix®, amitraz), being the most frequently used acaricide. Most farmers regularly applied the acaricide following the recommended weekly (30.6%, 49/160) or fortnightly (35.0%, 56/160) application regime, depending on the level of tick infestation. The most commonly used method for acaricide application was spraying (88.1%, 141/160), using either a Knapsack sprayer (38.1%, 61/160) or a hand sprayer (50.0%, 80/160). Dipping was encountered in 8.1% (13/160) of the farms, and they were all in Kinango ward in Kwale County. The majority of farmers (62.5%) also applied acaricide to other animals on the farm, besides cattle. Most farms in Kinango ward (47.8%, 32/67) used bought tap water for acaricide dilution, while most farms in Kayafungo ward (49.5%, 46/93) used water pans and ponds as the main sources of water for acaricide dilution. Farmers widely used a calibrated bottle top to measure the volume of acaricide before dilution (70.6%, 113). A large proportion of farmers (92.5%) bought their acaricides from agro-veterinary shops. The agro-veterinary shop attendants were the farmers' most preferred source of advice and information for tick control (64.4%).

Table 2. Cattle husbandry and tick control practices on farms in coastal Kenya

Query/item	Response category	Administrative ward		Total (n=160 farmers)
		Kinango (n=67 farmers)	Kayafungo (n=93 farmers)	
Cattle grazing land	Communal land	48 (71.6%)	41 (44.1%)	89 (55.6%)
	Own pasture farm	1 (1.5%)	9 (9.7%)	10 (6.3%)
	Forest area	17 (25.4%)	28 (30.1%)	45 (28.1%)
	Neighbor's plot	1 (1.5%)	15 (16.1%)	16 (10.0%)
Cattle watering point	River	54, 80.6%	33 (35.5%)	87 (54.4%)
	Others (water pan, water pond, rain water)	13 (19.4%)	60 (64.5%)	73 (45.6%)
Housing infrastructure	Shaded	2 (3.0%)	4 (4.3%)	6 (3.8%),
	Open but fenced	20 (29.9%)	38 (40.9%)	58 (36.3%)
	Open and not fenced	45 (67.2%)	51 (54.8%)	96 (60.0%)
Constraints associated with cattle production	Cattle disease	58 (86.6%)	86 (92.5%)	144 (90.0%)
	Animal feeds	19 (28.4%)	46 (49.5%)	65 (40.6%)
	Water source	23 (34.3%)	46 (49.5%)	69 (43.1%)
	Animal health and extension services	34 (50.7%)	60 (64.5%)	94 (58.8%)
	Market for live animals and milk	15 (22.4%)	18 (19.4%)	33 (20.6%)
Season of high tick infestation	Dry season	22 (32.8%)	18 (19.4%)	40 (25.0%)
	Rainy season	38 (56.7%)	53 (57.0%)	91 (56.9%)
	All year	7 (10.4%)	22 (23.7%)	29 (18.1%)
Tick control practice	No tick control	0 (0.0%)	4 (4.3%)	4 (2.5%)
	Hand-picking	1 (1.1%)	1 (1.1%)	2 (1.3%)
	Chemical acaricide	66 (98.5%)	88 (94.6%)	154 (96.3%)
Frequency of acaricide application	Biweekly	0 (0.0%)	6 (6.5%)	6 (3.4%)
	Weekly	25 (37.3%)	24 (25.8%)	49 (30.6%)
	Every 2 weeks	32 (47.8%)	24 (25.8%)	56 (35.0%)
	Monthly	5 (7.5%)	11 (11.8%)	16 (10.0%)
	Depends on presence/level of tick infestation	4 (6.0%)	23 (24.7%)	27 (16.9%)
Method of applying acaricide	Spraying	53 (79.1%)	88 (94.6%)	141 (88.1%)
	Dipping	13 (19.4%)	0 (0.0%)	13 (8.1%)

Equipment used to measure the volume of acaricide before dilution	Calibrated bottle top	47 (70.1%)	66 (71.0%)	113 (70.6%)
	Acaricide bottle for dip	13 (19.4%)	0 (0.0%)	13 (8.1%)
	Non calibrated bottle top	0 (0.0%)	19 (20.4%)	19 (11.9%)
	Syringe	6 (9.0%)	3 (3.2%)	9 (5.6%)
Type of water used for diluting the acaricide	Tap water	32 (47.8%)	7 (7.5%)	39 (24.4%)
	Borehole/well water	9 (13.4%)	23 (24.7%)	32 (20%)
	River water	22 (32.9%)	11 (11.8%)	33 (20.6%)
	Water pans and ponds	4 (6.0%)	46 (49.5%)	50 (31.3%)
Application of acaricide to other farm animals apart from cattle	Yes	36 (53.7%)	64 (68.8%)	100 (62.5%)
	No	30 (44.8%)	24 (25.8%)	54 (33.8%)
Brand name of acaricides used in the farm in the past 12 months*	**Trade name (Active ingredient)**			
	Synthetic pyrethroids group			
	Dominex (Alpha-cypermethrin)	14 (20.9%)	3 (3.2%)	17 (10.6%)
	Decatix (Deltamethrin)	0 (0.0%)	1 (1.1%)	1 (0.6%)
	Sypertix (Alpha-cypermethrin)	30 (44.8%)	10 (10.8%)	40 (25.0%)
	Bayticol (Flumethrin)	1 (1.5%)	5 (5.4%)	6 (3.4%)
	Ectomin (Cypermethrin)	3 (4.5%)	2 (2.2%)	5 (3.1%)
	Delete (Deltamethrin)	0 (0.0%)	1 (1.1%)	1 (0.6%)
	Co-formulation			
	Duodip (Chlorpyrifos 50% + Cypermethrin 5%)	5 (7.4%)	6 (6.5%)	11 (6.9%)
	Amidine group			
	Taktic (Amitraz)	15 (22.4%)	3 (3.2%)	18 (11.3%)
	Triatix (Amitraz)	30 (44.8%)	53 (57.0%)	83 (51.9%)
	Norotraz (Amitraz)	13 (19.4%)	34 (36.6%)	47 (29.4%)
	Almatix (Amitraz)	1 (1.5%)	6 (6.5%)	7 (4.4%)
	Bimatraz (Amitraz)	1 (1.5%)	1 (1.1%)	2 (1.3%)
	Actraz (Amitraz)	5 (7.5%)	17 (18.3%)	22 (13.8%)
Where do you buy your acaricide*	Agroveterinary store	62 (92.5%)	86 (92.5%)	148 (92.5%)
	Veterinary office	13 (19.4%)	0 (0.0%)	13 (8.1%)
	Unofficial source (e.g., market, Dips/crush center, fellow farmer)	8 (11.9%)	2 (2.2%)	10 (6.3%)
Source of information/ advice on tick control*	Agroveterinary shop attendant	36 (53.7%)	67 (72.0%)	103 (64.4%)
	Fellow farmers	31 (46.3%)	58 (62.4%)	89 (55.6%)
	Veterinary officer	34 (50.7%)	8 (8.6%)	42 (26.3%)
	Radio/TV	2 (3.0%)	1 (1.1%)	3 (1.9%)
	Social media	1 (1.5%)	1 (1.1%)	2 (1.3%)
	Farmer group organization	0 (0.0%)	4 (4.3%)	4 (2.5%)
	Personal judgement/decision	0 (0.0%)	6 (6.5%)	6 (3.4%)

* More than one answer was allowed. The frequency of mention for a given answer response is the percentage of total respondents.

Tick-borne disease control practices

Although all the respondents (n = 160) had heard of TBD, about half (48.1%, 77/160) could correctly name at least one TBD (Table 3). East Coast fever (locally known as "*ngai*") was the most frequently named TBD and was associated with cattle infections and losses (46.3%), followed by anaplasmosis (3.1%), babesiosis (2.5%), and heartwater (0.6%). A total of 127 (79.4%) respondents perceived TBD as having ever occurred on their farm, while 71 (44.4%) had perceived TBD cases in the past 12 months. A quarter of the farmers seld-diagnosed the TBD when they occurred on their farms (25%, 40/160), and 6.9% (11/160) of the farmers sought the

diagnostic services of a veterinary officer. The frequently mentioned drugs used to treat cases of TBD on the farm included the antibiotic adacycline (19.4%, 31/160) and antiprotozoal Butalex® (20.6%, 33/160). Five percent (8/160) could not remember the name of the drug used. The most common source of awareness regarding TBD was the agro-veterinary shop attendant (70.0%, 112/160), followed by fellow farmers (56.9%, 91/160). Only 2 (1.3%) respondents could not describe the perceived clinical signs and symptoms of TBD on cattle. The most commonly cited symptoms suggestive of TBD were enlarged lymph nodes (53.1%), loss of appetite (49.4%), cough (48.8%), and fever (40.6%) (Supplementary Table 2).

Table 3. Tick-borne disease management practices on farms in coastal Kenya

Query/item	Response	Administrative ward		Total (n=160 farmers)
		Kinango ward (n=67 farmers)	Kayafungo ward (n=93 farmers)	
Ability to name a tick-borne diseases	Yes	20 (29.9%)	57 (61.3%)	77 (48.1%)
	No	47 (70.1%)	36 (38.7%)	83 (51.9%)
Named tick-borne diseases frequently associated with cattle infection or losses*	East Coast fever (ECF)	20 (29.9%)	54 (58.1%)	74 (46.3%)
	Babesiosis/redwater	2 (3.0%)	2 (2.2%)	4 (2.5%)
	Anaplasmosis	0 (0.0%)	5 (5.4%)	5 (3.1%)
	Heartwater	0 (0.0%)	1 (1.1%)	1 (0.6%)
Occurrence of tick-borne diseases in the farm	Yes	56 (83.6%)	71 (76.3%)	127 (79.4%)
	No	11 (16.4%)	22 (23.7%)	33 (20.6%)
Occurrence of tick-borne diseases in the farm in the past 12 months	Yes	26 (38.8%)	45 (48.4%)	71 (44.4%)
	No	41 (61.2%)	48 (51.6%)	89 (55.6%)
Personnel who confirmed the diagnosis when the disease occurred in the farm in the past 12 months	Veterinary personnel (Vet officers, animal health officers, etc.)	3 (4.5%)	8 (8.6%)	11 (6.9%)
	Self/ family member judgment	21 (31.3%)	19 (20.4%)	40 (25.0%)
	Herdsman/ employee on the farm	0 (0.0%)	1 (1.1%)	1 (0.6%)
	Para veterinarians (non-professional but possess the knowledge for drug and vaccine delivery)	1 (1.5%)	5 (5.4%)	6 (3.4%)
	Agro veterinary dealer	1 (1.5%)	0 (0.0%)	1 (0.6%)
	Fellow farmer	0 (0.0%)	12 (12.9%)	12 (7.5%)
Drugs used to treat the animal when they fell ill in the past 12 months*	Butalex	6 (9.0%)	27 (29.0%)	33 (20.6%)
	Parvexon	4 (6.0%)	0 (0.0%)	4 (2.5%)
	Buperquine	2 (3.0%)	0 (0.0%)	2 (1.3%)
	Adacycline LA 20%	7 (10.4%)	24 (25.8%)	31 (19.4%)
	Alamycin LA 20%	0 (0.0%)	15 (16.1%)	15 (9.4%)
	Bimahistamine	0 (0.0%)	2 (2.2%)	2 (1.3%)
	Imochem	1 (1.5%)	0 (0.0%)	1 (0.6%)
	Imizol	3 (4.5%)	0 (0.0%)	3 (1.9%)
	Diminakel	0 (0.0%)	4 (4.3%)	4 (2.5%)
	Veriben (plain)	4 (6.0%)	9 (9.7%)	13 (8.1%)
	Veriben + B12 vitamin	13 (19.4%)	14 (15.0%)	27 (16.9%)
	Epsom salt	1 (1.5%)	0 (0.0%)	1 (0.6%)
	Not sure/ Don't know	3 (4.5%)	5 (5.4%)	8 (5.0%)
Source of information/ advice on tick-borne disease control*	Agrovet shop attendant	39 (58.2%)	73 (78.5%)	112 (70.0%)
	Fellow farmers	29 (43.3%)	62 (66.7%)	91 (56.9%)
	Veterinary personnel (Vet officers, animal health assistants, etc.)	43 (64.1%)	14 (15.1%)	57 (35.6%)
	Local/ traditional healers	1 (1.5%)	0 (0.0%)	1 (0.6%)
	Paravets (on-professional but trained for drug and vaccine delivery)	16 (23.9%)	1 (1.1%)	17 (10.6%)
	Radio/TV/newspaper/magazines	2 (3.0%)	0 (0.0%)	2 (1.3%)
	Social media	1 (1.5%)	0 (0.0%)	1 (0.6%)
	Farmer co-op/union/group	0 (0.0%)	3 (3.2%)	3 (1.9%)

*This was a multi-response question. The frequency of mention is expressed as the percentage of group-specific respondents.

Diversity and identity of tick-borne pathogens detected

We detected *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. by PCR-HRM. *Ehrlichia* spp. or *Rickettsia* spp. were not detected. The *Anaplasma* 16S rDNA sequences detected in this study were identical to reference *Anaplasma* sp., *Anaplasma platys* and *A. marginale* sequences

(Supplementary Table 3). Sequencing of the amplified *msp4* gene validated the identity of *Anaplasma* sp. and *A. marginale*. The maximum likelihood phylogenetic analysis of the 16S rDNA and *msp4* sequences from this study showed that all the *Anaplasma* spp. from coastal Kenya clustered together in the same clade with related species found in other parts of the world (Figure 3). Blast analysis of *Babesia/Theileria* spp. 18S rRNA sequences identified the presence of *T. velifera*, *T. parva*, and *B. bigemina*.

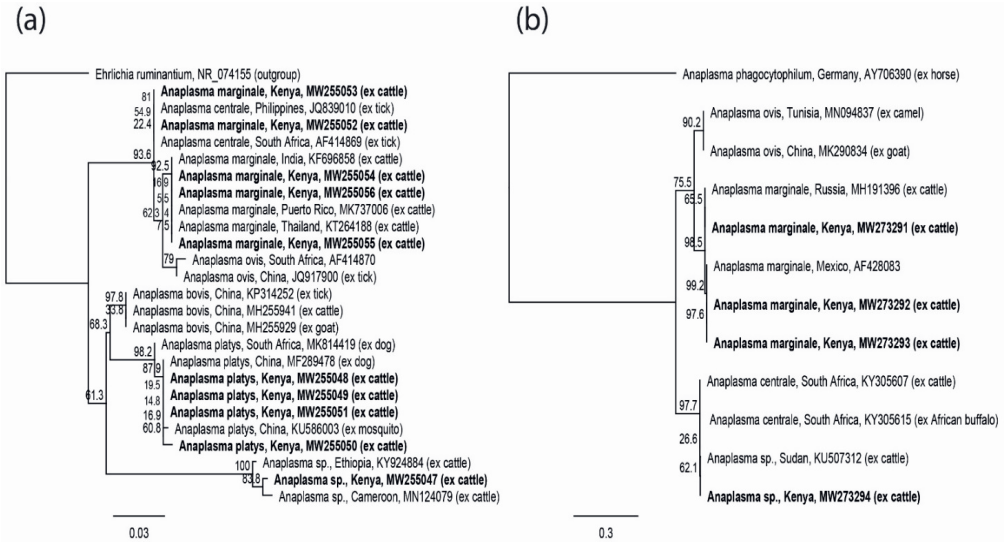

Figure 3. Maximum-likelihood phylogenetic analysis of *Anaplasma* spp. using (a). 16S rRNA sequences and (b) *msp4* sequences constructed using the Tamura Nei evolutionary model (TN93). The sequences obtained in the present study are highlighted in bold. Numbers on the nodes indicate percentages of 1000 bootstrap replicates. The scale bars represent substitutions per site.

Prevalence and risk factor analysis of tick-borne pathogens detected

Of the 1486 cattle tested, 359 (24.2%, 95% confidence interval (CI): 22.0–26.4) were positive for at least one TBP (Table 4 and Supplementary table 4). The overall herd levels prevalence was 75.6% (95% CI: 68.1–81.9). The most prevalent pathogen was *A. marginale*, followed by *T. parva, A. platys, T. velifera, B. bigemina,* and *Anaplasma* sp.. Dual infections were detected in 1.4% (95% CI: 0.9–2.2) cattle, and the highest frequency of co-infection was recorded for *T. parva* and *A.*

marginale (1.0%), followed by *T. parva* and *A. platys* (0.3%), *A. marginale* and *T. velifera* (0.1%), and *A. marginale* and *B. bigemina* (0.1%) (Table 4).

Table 4. Individual animal- and herd-level prevalence of tick-borne pathogens in cattle from coastal Kenya

Pathogen	Animal-level prevalence		Herd-level prevalence	
	No. of positive cattle[a]	% prevalence (95% CI)	No. of positive herds[b]	% prevalence (95% CI)
Single pathogen infections				
Anaplasma marginale	162	10.9 (9.4 – 12.6)	76	47.5 (39.6 – 55.5)
Anaplasma platys	39	2.6 (1.9 – 3.6)	27	16.9 (11.6 – 23.8)
***Anaplasma* spp.**	1	0.1 (0.0 – 0.4)	1	0.6 (0.0 – 4.0)
Babesia bigemina	7	0.5 (0.2 – 1.0)	6	3.8 (1.5 – 8.3)
Theileria parva	133	9.0 (7.6 – 10.5)	79	49.4 (41.4 – 57.4)
Theileria velifera	17	1.1 (0.7 – 1.9)	12	7.5 (4.1 – 13.0)
Overall	**359**	**24.2 (22.0 – 26.4)**	**121**	**75.6 (68.1 – 81.9)**
Co-infections				
A. marginale* + *B. bigemina	1	0.1 (0.0 – 0.4)	1	0.6 (0.0 – 3.4)
A. marginale* + *T. parva	15	1.0 (0.6 – 1.7)	12	7.5(3.9 – 12.7)
A. marginale* + *T. velifera	1	0.1 (0.0 – 0.4)	1	0.6 (0.0 – 3.4)
A. platys* + *T. parva	4	0.3 (0.0 – 0.7)	4	2.5 (0.7 – 6.3)
Overall	**21**	**1.4 (0.9 – 2.2)**	**14**	**8.8 (4.9 – 14.2)**

[a] Total of individual cattle tested positive out of 1486, [b] total number of herds tested positive out of 160 herds.

Results from the univariate mixed-effect logistic regression model showed that none of the assessed risk variables were statistically significant for TBP infection in cattle (Table 5). Estimations of ICC values found a substantially higher value for *A. marginale* infection (0.29) compared to *T. parva* infection (0.14).

Table 5. Descriptive statistics of cattle (n = 1,486) from coastal Kenya and univariable analysis of potential risk factors associated with *T. parva* and *A. marginale* infections using mixed effect logistic

Risk factor	Category	Total No. (%)	*A. marginale*			*T. parva*		
			No. + ve (%)	*P*-value	OR (95% CI)	No. + ve (%)	*P*-value	OR (95% CI)
Animal variables								
Sex	Female	898 (60.4)	89 (9.9)	-	1.0	87 (9.7)	-	1.0
	Male	588 (39.6)	73 (12.4)	0.33	1.2 (0.8 - 1.7)	46 (7.8)	0.114	0.7 (0.5 - 1.1)
Age	Calf	115 (7.7)	11 (9.6)	-	1.0	9 (7.8)	-	1.0
	Juvenile	426 (28.7)	53 (12.4)	0.547	1.3 (0.6 - 2.6)	38 (8.9)	0.474	1.3 (0.6 - 2.9)
	Adult	945 (63.6)	98 (10.4)	0.772	1.1 (0.6 - 2.2)	86 (9.1)	0.548	1.3 (0.6 - 2.9)
PCV	≤ 23	240 (16.2)	30 (12.5)	-	1.0	24 (10.0)	-	1.0
	≥ 24	1246 (83.8)	132 (10.6)	0.636	0.9 (0.6 - 1.4)	109 (8.7)	0.705	0.9 (0.6 - 1.5)
Farm variables								
Frequency of acaricide application	None	31 (2.1)	3 (9.7)	-	1.0	2 (6.5)	-	1.0
	Irregular	242 (16.3)	21 (8.7)	0.555	0.6 (0.1 - 3.0)	14 (5.8)	0.813	0.8 (0.2 - 4.0)
	Regular	1213 (81.6)	138 (11.4)	0.593	0.7 (0.1 - 3.0)	117 (9.6)	0.916	1.1 (0.2 - 5.0)
Presence of ticks on cattle when	No	1162 (78.2)	123 (10.6)	-	1.0	105 (9.0)	-	1.0
collecting blood samples	Yes	324 (21.8)	39 (12.0)	0.758	1.0 (0.6 - 1.5)	28 (8.6)	0.758	0.9 (0.6 - 1.5)
Application of the acaricide to other farm	No	521 (35.1)	56 (10.7)	-	1.0	47 (9.0)	-	1.0
animals other than cattle	Yes	965 (64.9)	106 (11.0)	0.879	1.1 (0.6 - 1.6)	86 (8.9)	0.783	1.1 (0.7 - 1.6)
Grazing field	Own pasture farm	70 (4.7)	5 (7.1)	-	1.0	5 (7.1)	-	1.0
	Shared/common land	1416 (95.3)	157 (11.1)	0.201	2.2 (0.6 - 7.8)	128 (9.0)	0.815	1.1(0.4 – 3.1)
Area variables								
Administrative ward	Kinango	781 (52.6)	66 (8.5)	-	1.0	85 (10.9)	-	1.0
	Kayafungo	705 (47.4)	96 (13.6)	0.381	1.5 (0.6 - 3.7)	48 (6.8)	0.272	0.7 (0.3 - 1.4)

OR: odds ratio, CI: confidence interval, No.: Number, + ve: Positive

Discussion

The current study provides molecular evidence of the diversity of TBP in cattle and information on the management practices relating to ticks and tick-borne diseases among cattle owners in coastal Kenya. The use of chemical acaricide was the primary method for tick control, with the amidine group (mainly Triatix®) being the most frequently used acaricide. East Coast fever was the most important disease and Butalex® was the most commonly administered drug in response to perceived TBD in cattle. The present study detected *A. marginale, B. bigemina* and *T. parva,* which are economically important in livestock production in Kenya. Additionally, the study reported *Anaplasma* sp., *A. platys* and *T. velifera,* whose epidemiology and association with clinical disease in cattle in Kenya are still unclear.

Tick and tick-borne disease control practices

As perceived by farmers, the most important constraints to cattle production in the study area included cattle diseases (mainly ECF), inadequate veterinary services, inadequate water for livestock, and shortage of feed, and a poor market for livestock products. Similar constraints have been identified in other cattle production systems in Kenya (Mugambi et al., 2012; Ohaga et al., 2007; Wesonga et al., 2010), Uganda (Byaruhanga et al., 2015), and Tanzania (Swai et al., 2005). There is, therefore, a need to improve access to veterinary extension services in the region to mitigate the impact of these constraints on cattle production.

In the current study, the respondents exhibited a high level of awareness of ticks, as 96.3% of the farmers use chemical acaricides for tick control. Nevertheless, we identified a few malpractices associated with acaricide use, including farmers' failure to adhere to the manufacturer's instructions on the correct acaricide dilution and frequency of application. Indeed, 11.9% of the farms used non-calibrated materials to measure the volume of acaricide for dilution, while another 3.4% of the farms had adopted a shorter acaricide application interval (twice a week) as opposed to the recommended weekly or fortnightly interval. Such malpractices pose a serious threat to public and environmental health and could lead to the emergence and spread of acaricide resistance in the region (De Meneghi et al., 2016; Vudriko et al., 2016).

We also identified several malpractices associated with the diagnosis and treatment of cattle infection on the farms. The farmers treated the sick cattle based on clinical signs without seeking accurate diagnostic services from the local veterinary office that guided rational prescriptions.

Instead, most farmers relied on the advice given by local agro-veterinary shop attendants and fellow cattle farmers on the choice of drugs to use. Such malpractices may complicate the control of TBD in the region especially when the wrong information is spread, or an incorrect dosage is prescribed (Irungu et al., 2008).

Tick-borne pathogens identified in cattle

Anaplasma marginale was the most prevalent pathogen (10.9%). This bacterium can be transmitted biologically to cattle by infected hard ticks (*Rhipicephalus* spp.) and mechanically by infected biting flies (*Stomoxys* spp., *Tabanus* spp.) and by blood-contaminated fomites (such as needles, ear tagging, and dehorning) (Aubry & Geale, 2011). This multitude of transmission routes may be responsible for the high prevalence of *A. marginale* in this region. The high molecular prevalence of 10.9% in the present study was not surprising, based on the similarly high seroprevalences reported in similar settings in Eastern Kenya (58.3%) (Gachohi et al., 2010) and coastal Kenya (81-97%) (Maloo et al., 2001b).

Babesia bigemina is the causative agent for bovine babesiosis and is transmitted by *Rhipicephalus* ticks (Bock et al., 2004). Only 0.5% of cattle sampled were positive for the protozoan *B. bigemina,* which is consistent with previous molecular-based study from western Kenya (Njiiri et al., 2015). The low prevalence of *B. bigemina* reported in this study is in agreement with the apparent absence of the *B. bigemina* pathogen in ticks in this region, as found in an earlier study (**Chapter 2**).

East Coast fever (ECF), caused by *T. parva*, is the most economically important TBD in Kenya, causing high morbidity and mortality in cattle (Gachohi et al., 2012; Wesonga et al., 2010). The prevalence of *T. parva* (9.0%) recorded here is comparable to the previous molecular finding reported on farms in western Kenya (12.9%) (Njiiri et al., 2015). The low prevalence of *T. parva* in this study area are in agreement with the low infection rates in ticks in this region, as found in an earlier study (**Chapter 2**).

This study also confirms the occurrence of an uncharacterized *Anaplasma* sp., *Anaplasma platys* and *Theileria velifera* in cattle. Although their epidemiology and association with clinical disease in cattle in Kenya are still unclear, *T. velifera* is generally non-pathogenic to cattle, while *A. platys* causes canine cyclic thrombocytopenia (Harvey et al., 1978). Therefore, further detailed epidemiological investigations are required to determine their potential pathogenicity on cattle production in coastal Kenya.

Ehrlichia minasensis, *Ehrlichia ruminantium* and *Rickettsia africae* were recently detected in *Rhipicephalus* and *Amblyomma* ticks in the same study area (**Chapter 2**). However, the present study did not detect any species of *Ehrlichia* or *Rickettsia* in any of the cattle samples analyzed. The absence of *Ehrlichia* in our samples may be attributed to the biology of *Ehrlichia* species, as it mainly resides in endothelial cells and is only periodically found in the bloodstream during the febrile stage of infection (Andrew & Norval, 1989; Steyn et al., 2008). The absence of *R. africae* corroborates previous studies in western Kenya that recorded no evidence of pathogenic rickettsial species in blood samples collected from livestock (Chiuya et al., 2021; Maina et al., 2014; Okal et al., 2020).

Co-infections were detected in 21 blood samples (1.4%) and were mainly due to double infections. The overall co-infection prevalence reported in the present study is lower than in previous studies in Lambwe Valley in Kenya (31.6%) (Okal et al., 2020) and in western Kenya (87.1%) (Njiiri et al., 2015). The most frequent co-occurrences included *A. marginale* and *T. parva,* followed by *A. platys* and *T. parva, A. marginale* and *T. velifera,* and *A. marginale* and *B. bigemina*. These co-infections may have consequences on TBD management in the region as it may complicate the clinical presentation, diagnosis and treatment in cattle with multiple pathogen infections than those with single infections (Diuk-Wasser et al., 2016; Hofmann-Lehmann et al., 2004; Moutailler et al., 2016). Therefore, veterinary practitioners should be aware of co-infections in cattle from coastal Kenya as this may warrant different clinical management strategies.

The epidemiology of TBPs in cattle varies depending on the agro-ecological zone, livestock production system, and individual animal traits such as sex, breed and age (Gachohi et al., 2012). In this study, we did not find any significant association between TBP infection status and potential risk factors in cattle. This general lack of significant risk factors for TBP positivity in cattle may suggest a relatively uniform distribution of the infections across the study area and that the study population was possibly too uniform in terms of herd management practices, and therefore difficult to detect clear differences in the classical risk factors. Further research is therefore needed to better understand the risk of TBP transmission in other extensive livestock systems of Kenya and to address the potential of control options.

The computed lCCs in this study were 0.29 (*A. marginale*) and 0.14 (*T. parva*), and these were within the previously reported ranges of 0 to 0.6 for five TBD (i.e., *A. marginale, B. bigemina, E.*

ruminantium, T. mutans and *T. parva*) (Deem et al., 1993; Gachohi et al., 2010; Otte & Gumm, 1997). Our computed ICC estimates can inform the design effects needed to adjust for cluster sampling in future TBD surveys in areas with similar agro-climatic and ecological conditions and production systems.

Conclusions

This study identified several malpractices in the management of ticks and TBD among cattle owners in the coastal region. These included inappropriate acaricide dilution and frequency of acaricide application, and overreliance on unprofessional sources rather than the veterinarians regarding diagnosis and treatment of sick cases on their farms. This study also provides molecular evidence of the existence of highly pathogenic *A. marginale*, *B. bigemina* and *T. parva,* as well as other pathogens, including uncharacterized *Anaplasma* sp., *A. platys*, and *T. velifera* in cattle from coastal Kenya. The general lack of association between the prevalence of *A. marginale* or *T. parva* with the animal-, farm- and area-level variables suggests that the study population was possibly too uniform in terms of herd management practices. There is a need to intensify integrated tick control programs to reduce the risk and burden of disease in the area.

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Chapter 4

A randomized controlled trial of Tickoff® (*Metarhizium anisopliae* ICIPE 7) for control of tick infestations and transmission of tickborne infections in extensively grazed zebu cattle in coastal Kenya

Joseph Wang'ang'a Oundo[1,2*], Shewit Kalayou[1], Gerrit Gort[3], Gebbiena M. Bron[2], Constantianus J.M. Koenraadt[4], Quirine ten Bosch[2$], Daniel Masiga[1$]

1 International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya,
2 Quantitative Veterinary Epidemiology, Wageningen University & Research, Wageningen, the Netherlands,
3 Biometris, Wageningen University & Research, Wageningen, the Netherlands,
4 Laboratory of Entomology, Wageningen University & Research, Wageningen, the Netherlands
$ Equal senior contributions

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Abstract

The entomopathogenic fungus *Metarhizium anisopliae* isolate ICIPE 7 is being developed as an eco-friendly alternative to chemical acaricides in managing natural tick infestation on livestock. Its impact on tick infestation and tick-borne infections in cattle under natural conditions are yet unclear. We conducted a randomized controlled field trial to assess the safety and effects of Tickoff® (a formulation of *M. anisopliae* isolate ICIPE 7) and the chemical acaricide Triatix® on tick infestation and incidence of *Anaplasma marginale* and *Theileria parva* in extensively grazed zebu cattle in coastal Kenya. A total of 217 eligible herds comprising 1,459 intent-to-treat zebu cattle were enrolled from 12 villages. The herds were randomly assigned in a 1:1:1 ratio to Tickoff®, Triatix®, or Tickoff® excipients. Tick counts, treatment administrations, and adverse events were registered every two weeks for seven months. The mortality of ticks collected from treated cattle was monitored *in vitro*. Infections with *A. marginale* and *T. parva* were monitored every two months. No adverse events were reported in either treatment group. Tickoff® did not significantly affect tick infestation ($p=0.869$) or infection incidence ($p>0.05$) compared to excipients. Triatix® significantly reduced tick infestation ($p<0.001$) and incidence of *T. parva* ($p=0.042$), but not *A. marginale* ($p=0.509$) compared to the reference Tickoff®. In ticks that were removed from cattle, Tickoff® demonstrated significant pathogenicity *in vitro* relative to excipients (hazard ratio: 8.50, 95% CI: 4.67 – 15.47). Fungus growth and sporulation were also observed on tick cadavers from Tickoff®, but not from excipients. While Tickoff® did not impact tick counts, its delayed, but significant effect on tick mortality may hinder onward pathogen transmission and give rise to indirect (i.e., to untreated animals) epidemiological effects, that were not picked up with this study design. Additionally, adverse environmental conditions resulted in low tick abundance and pathogen circulation towards the end of the study period, reducing the power of the study. This work re-emphasizes the challenges of randomized controlled field trials and the complexity of assessing the impact of vector control products on both direct and indirect impacts on pathogen transmission.

Introduction

Ticks (Acari: Ixodidae) are responsible for significant economic losses to the livestock industry. This is the result of direct effects through reductions in meat and milk yields, damage to teats, skins and hides, blood loss, and even anemia (Jongejan & Uilenberg, 2004). In addition, ticks have indirect effects through their role as vectors of viral, bacterial, and protozoal agents that cause tick-borne diseases (TBDs) in livestock and humans (Walker et al., 2003). In vast areas of Kenya, zebu cattle are kept under a traditional extensive management system which is characterized by a constant high risk of tick infestations and TBD transmission (Gachohi et al., 2012). Therefore, sustainable strategies are needed to control tick infestations on cattle and reduce tick-borne pathogen transmission.

East Coast fever (ECF) and bovine anaplasmosis are among the most economically important TBDs of cattle in Kenya (Gachohi et al., 2012; Moumouni et al., 2015). East Coast fever is caused by the protozoan *Theileria parva* and transmitted by the three-host tick *Rhipicephalus appendiculatus*. The Cape buffalo (*Syncerus caffer*) is the natural reservoir host for *T. parva* (Gachohi et al., 2012; Nene et al., 2016). The disease is endemic in eastern, central, and southern Africa where it causes considerable economic losses, especially to resource-poor smallholder farmers and pastoralists. Infected cattle can exhibit a mild, moderate, or severe clinical disease, and those that recover following treatment or spontaneous recovery become long-term asymptomatic carriers and can infect ticks (Baylis et al., 1992; Kariuki et al., 1995; Olds et al., 2018).

Bovine anaplasmosis is caused by the intra-erythrocytic bacteria *Anaplasma marginale,* and occurs mainly in tropical and subtropical areas, causing high morbidity and mortality in susceptible animals (Aubry & Geale, 2011). The pathogen is transmitted biologically by approximately 20 different tick species and mechanically by biting flies or blood-contaminated fomites (Aubry & Geale, 2011). In sub-Saharan Africa, the main tick vectors are *Hyalomma rufipes, Rhipicephalus annulatus, Rhipicephalus decoloratus, Rhipicephalus microplus, Rhipicephalus evertsi,* and *Rhipicephalus simus* (Walker et al., 2003). The severity of *A. marginale* infection in cattle is age dependent. The disease is acute and often fatal in adult cattle over two years of age. Animals between one and two years of age suffer from acute but rarely fatal disease, while animals aged between six to twelve months usually develop mild disease. Calves are less susceptible to clinical

disease and the illness is rare under six months of age (Aubry & Geale, 2011). Recovered animals remain persistently infected carriers for life and act as a source of infection to ticks.

Whether sustained transmission of tick-borne pathogens occurs in a susceptible population is determined by several factors, including the ratio of ticks to cattle and the related number of tick bites per day per animal. These factors affect the basic reproduction number R_0 (Hartemink et al., 2008), a metric for transmission efficiency. Only if R_0 is above one a pathogen can persist in a population. Control of the tick population on cattle could potentially reduce the risk of exposure to tick-borne infections and disrupt pathogen transmission cycles (Hoch et al., 2012; Medley et al., 1993). Control of tick populations on cattle has relied heavily on the use of chemical acaricides, but its long-term sustainability is threatened by the widespread emergence of resistance in ticks (Abbas et al., 2014; Githaka et al., 2022) as well as by contamination of the environment and meat and milk products with toxic residues when withdrawal periods are not respected (De Meneghi et al., 2016). This highlights the need to develop tick control strategies that are safe and efficacious and provide sustainable options for controlling tick infestations on livestock.

Biological control of ticks with entomopathogenic fungi is a promising alternative to chemical acaricides. *In vitro* studies have shown that the pathogenic effects of the fungus *Metarhizium anisopliae* are not limited to the direct effect on tick mortality, but also continue in female ticks by reducing fecundity, egg hatchability and engorgement weight, and increasing engorgement duration, pre-oviposition, oviposition and post-oviposition periods (Camargo et al., 2012; Nana et al., 2015). However, the existing data supporting *M. anisopliae* efficacy under field conditions are limited to small-scale and short-term studies, and with inconsistent reports on control levels (Alonso-Díaz et al., 2007; Correia et al., 1998; Murigu et al., 2016). Additionally, these studies are often limited by low statistical power and did not include data on epidemiological outcomes, such as the incidence of tick-borne infections in cattle populations. Robust large-scale randomized controlled trials that are the gold standard for providing empirical evidence of efficacy are therefore needed to establish the efficacy of formulations of entomopathogenic fungi under natural field conditions.

The biopesticide Tickoff® is a product based on the entomopathogenic fungus *M. anisopliae* ICIPE 7 and is being developed as an alternative to chemical acaricides for the control of tick

infestation on livestock. The effect of this biopesticide in reducing natural tick infestation on cattle has not yet been established in a large-scale field trial. Knowledge on the effectiveness of this biopesticide in reducing the incidence of tickborne infections in cattle is limited. We therefore conducted a randomized controlled field trial to evaluate the safety and effects of Tickoff® in reducing (1) natural tick infestations and (2) the incidence of *A. marginale* and *T. parva* infections in indigenous zebu cattle (*Bos indicus*) managed under an extensive grazing system in coastal Kenya. We included the synthetic acaricide Triatix® as a positive comparator and the excipients of Tickoff® as a placebo control.

Study design

We followed a previously published protocol (Oundo et al., 2022) with some modifications, which included adjustments to the laboratory experiments (Appendix A). This randomized controlled trial was conducted during the dry and rainy seasons, from December 2021 to July 2022, in twelve villages in Kayafungo ward, Kaloleni sub-county in Kilifi County in coastal Kenya (Figure 1). The selected villages were easily accessible by vehicle, practiced livestock farming and the region is a known hotspot for tick-borne diseases (Maloo et al., 2001a). Herds composed of local zebu cattle and managed under an extensive grazing system were enrolled in the study based on evidence of infestation with live attached ticks, generally in good health, and the owners' willingness to participate in the study. Qualifying herds were stratified by village, herd size, and tick infestation level and randomly allocated in a 1:1:1 ratio to either Tickoff® (Real IPM Ltd, Kenya), Triatix® (12.5% EC amitraz, CKL Africa Ltd, Kenya), and excipient of Tickoff® (Real IPM Ltd, Kenya). Tickoff® formulation was prepared using *M. anisopliae* ICIPE 7 (4×10^9 conidia/mL) as the active ingredient, mixed with canola oil (95%), 0.05% Triton X-100 (1.5%) and Kerosene (3.5%). The excipient contains the formulation of Tickoff® without *M. anisopliae* ICIPE 7. The excipient was chosen as a control treatment to ensure blinding of the experiment: the smell of the treatment is similar to that of Tickoff®. However, owing to the color of the products, it was not possible to fully blind the study. Treatment was allocated at the herd level to ensure adequate protection of all cattle in a herd, with measurements of effectiveness conducted at the individual cattle level. Cattle received treatment on day 0, and thereafter every two weeks until the end of the study. Day 0 was defined individually as the day an animal received the first treatment. Whole-body tick counts were also done on Day 0, and thereafter at two-week intervals until the end of the study. Blood sampling was done on days 0, 60, 120 and 180 to determine the presence of *A. marginale* and *T.*

parva in cattle. The study was conducted in compliance with the study authorizations issued by Kenya's Veterinary Medicines Directorate (Approval reference: MOALF/SDL/VMD/TRIALS/VOL1/14), Directorate of Veterinary Services (no objection ref: MOALF/SDL/DVS/DS/RES/74), the National Commission for Science, Technology and Innovation (NACOSTI/P/21/6726), and the Pwani University Ethics Review (approval number ERC/EXT/002/2020). The use of trade, brand, or corporation names in this publication is for information and convenience of the reader, and should not be misinterpreted as an endorsement, promotion, or demotion of such products based on their efficacy result.

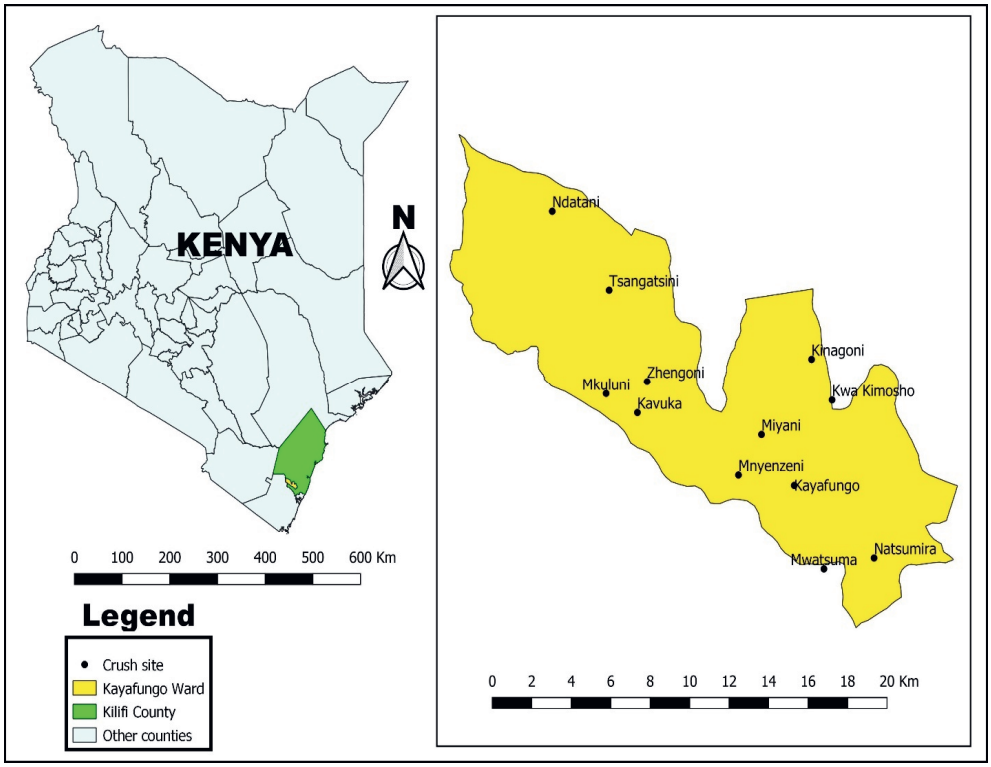

Figure 1. Map of Kayafungo Ward in Kilifi County in coastal Kenya showing the trial sites. The map was prepared using common-license shape files in QGIS software version 3.10 (QGIS Development Team, 2020).

Sample size determination

This trial aims to assess the performance of Tickoff® in reducing on-host tick counts compared to an existing synthetic chemical acaricide. A weekly application of Triatix® acaricide on tick-infested cattle for four weeks caused a reduction in on-host tick counts by 94.9% (Murigu et al., 2016). In semi-field experiments, the efficacy of oil-based formulations of *M. anisopliae* (10^8 - 10^9 conidia/ml) on the reduction of tick counts on cattle ranged between 65–92% depending on the application intervals, study duration, tick species, and life cycle stage (Kaaya et al., 2011; Kaaya & Hassan, 2000; Kaaya & Hedimbi, 2012; Murigu et al., 2016). Similarly, our previous pilot study showed that Tickoff® produced an efficacy of 86.1% in treated cattle compared to untreated cattle (https://patents.google.com/patent/WO2017216752A1/en). Given the expected variation in the efficacy of oil-based formulations of *M. anisopliae* against ticks, we considered the minimal worthwhile difference in efficacy between the conventional Triatix® acaricide and Tickoff® to be 15%. An efficacy margin of 15% below which the fungal formulation would not offer a viable alternative to existing chemical acaricide was considered acceptable given the limited availability of alternatives for tick control and the added advantages of *M. anisopliae* in biological control of ticks, i.e., it being selective and virulent against all tick stages (Hedimbi et al., 2011; Kaaya et al., 1996, 2000, 2011; Kaaya & Hassan, 2000; Kaaya & Hedimbi, 2012), pathogenic to acaricide-resistant ticks (Murigu et al., 2016), and it being safe for humans, animals and the environment (Zimmermann, 2007). The significance level and power of the study were set at 5% and 80%, respectively. Assuming a 94.9% (~95%) efficacy in the conventional Triatix® acaricide (Murigu et al., 2016), a minimum sample size of 73 zebu cattle per intervention arm was calculated as follows (Sakpal, 2010):

$$n = [(Z_{\alpha/2} + Z_{\beta})^2 \times \{(p1\,(1-p1) + (p2\,(1-p2))\}] / (p1 - p2)^2$$

Where:

n = sample size required in each intervention arm,

p1 = protection efficacy of Triatix® = 0.95,

p2 = protection efficacy of Tickoff® = 0.80,

p1 - p2 = minimal worthwhile difference = 0.15,

$Z_{\alpha/2}$: for 5% level of significance = 1.96,

Z_{β}: for 80% power = 0.84.

Herd-level treatments and repeated measurements are likely to enhance the intra-herd clustering of measurements estimated at individual animal levels. Given the variation that may occur among herds, i.e., the clustering effect, inflating the sample size by two- to four folds can account for the potentially large variation among clusters (Thrusfield et al., 2018). We, therefore, inflated the sample size threefold and obtained a total of 219 zebu cattle per intervention arm. A dropout rate of 30% was included in the calculation to account for potential dropouts during the trial, bringing the total number of cattle per treatment group to 285 and thus 855 zebu cattle in total.

Data analysis

Data analysis was performed using the R software version 4.2.2. The individual cow in each herd was the observational unit, repeatedly measured over time, and the primary endpoint was the live-attached tick count and incidence of tick-borne infections in each intervention arm. The secondary endpoints were the number, type, and severity of adverse events in cattle in each intervention arm. Descriptive statistics were completed for baseline demographic variables of cattle (age, sex, and body weight), herd size, and tick infestation. *P*-values ≤ 0.05 were considered significant for all statistical tests.

Tick count analysis

The analysis of the percent reduction in tick infestation was based on the Intention-to-Treat (ITT) population, comprising all cattle that were randomized to a treatment group and that received at least one dose of either study product. For cattle withdrawn before the final day of the trial, data up to the time of removal were included in statistical summaries and analyses. The percentage reduction in tick infestation was calculated for each post-treatment day as the reduction in live-attached tick counts compared to the pre-treatment counts (recorded on Day 0). Day 0 was defined individually as the day a cow received its first treatment. The percent reduction at each time point was calculated as follows:

$$\%\ \text{reduction in tick infestation} = \frac{\text{tick count (Day 0)} - \text{tick count (post} - \text{treatment)}}{\text{tick count (Day 0)}} \times 100$$

A generalized linear mixed model (GLMM) with a negative binomial distribution (log-link function), fitted with the R-package glmmTMB, was used to compare live tick counts post-

treatment among the treatment groups. The fixed part of the model contained the linear and quadratic trends of time point per treatment, occurrence of rainfall since previous treatment, cattle age group and number of days since previous treatment. Rain was included in the model as a covariate since rain may wash off the treatments from cattle skin and thus reduce treatment persistence and efficacy. Moreover, rain has been associated with increased tick activity and abundance (Chepkwony et al., 2021). The age group of cattle was included as a covariate in the model because tick counts are expected to differ among cattle of different age groups, while the age distribution was significantly different among the three treatment arms at baseline. As some cattle or herds occasionally skipped the biweekly interval spraying and tick counting session, we added time since previous treatment (i.e., the extra time beyond 14 days) in the model to evaluate if this had a significant association with the outcome measure. The random part of the model included random effects for village, herd within village, and time points within herd. This part was included to respect characteristics of the study design: strata (i.e., villages) and experimental units (i.e., herds which were used to randomize the treatments), and time points per herd. The random part also contained random intercepts and slopes per cattle for the linear and quadratics terms of time, in order to handle the repeated measurements per cattle over time. Finally, there were data collector random effects that may capture the observer bias among the tick assessors. Testing was two-sided at the significance level of $\alpha = 0.05$.

Survival analysis of treated ticks

We carried out *in vitro* experiments using both lab-reared and field-collected ticks to monitor mortality rates in the Tickoff®, excipients, and untreated treatment groups (Appendix A). Cox Proportional Hazard analysis was used to estimate the hazard ratios (HR) and their 95% confidence intervals (CI).

Epidemiological analysis

To estimate the epidemiological impact of the treatments, we followed a cohort of recruited cattle for six months at bimonthly intervals, during which their infection status with *A. marginale* and *T. parva* were recorded. Interval-censored survival analysis with left censoring was used to estimate the probability of cattle remaining free of infection. Cox's proportional hazard regression models were fitted to identify the significant predictors of infection occurrence. The model included

treatment and age as covariates. Frailty terms for village and herd within the village were included in the model to adjust for clustering within herd and village.

Results

Cattle demographics

The ITT population comprised 1,459 zebu cattle from 217 herds that were randomized to either Tickoff® (n = 541, 37.1%), Triatix® (n = 473, 32.4%) or excipient (n = 445, 30.5%) groups and received at least one dose of either treatment (Table 1). Most of the cattle were adults (n = 896, 61.4%) followed by juveniles (n = 410, 28.1%) and calves (n = 153, 10.5%). There were 900 (61.7%) female and 559 (38.3%) male cattle. All treatment groups from the ITT population showed reasonable homogeneity for sex, body weight, herd size, and median tick counts at baseline. However, there were small yet significant differences in the baseline distribution of age groups among the treatment groups (Table 1).

Table 1. Demographics and baseline characteristics of recruited and treated cattle that were (ITT population)

Demographics	Mazao Tickoff® (n = 541, 37.1%)	Triatix® (n = 473, 32.4%)	Excipient (n = 445, 30.5%)	Homogeneity
Age				
Calf (6 months-1 year)	45 (8.3%)	50 (10.6%)	58 (13.0%)	χ^2 = 9.521, df = 4, p = **0.049**
Juvenile (1-2 years)	141 (26.1 %)	136 (28.8%)	133 (29.9%)	
Adult (above 2 years)	355 (65.6 %)	287 (60.7%)	254 (57.1%)	
Sex				
Male	199 (36.8%)	196 (41.4%)	164 (36.9%)	χ^2 = 5.080, df = 2, p = 0.279
Female	342 (63.2%)	277 (58.6%)	281 (63.1%)	
Body weight (kg)				
Arithmetic mean ± SD	153.7 ± 54.4	149.3 ± 53.1	151.8 ± 55.6	χ^2 of a Kruskal-Wallis test = 2.764, df = 2, p = 0.251
Range	28 - 375	36 - 370	26 - 257	
Herd size				
1-10	60	60	59	χ^2 of a Wald test = 1.431, df = 2, p = 0.489
11-20	12	10	10	
21-25	3	2	1	
Day 0 tick count (live attached)				
Median (1st and 3rd quartiles)	10 (4 – 21)	10 (3 – 26)	9 (3 – 18)	χ^2 of a Wald test = 0.814, df = 2, p = 0.666

Abbreviations: Df Degree of freedom, *SD* standard deviation, ITT Intention-to-Treat population

Tick infestation, relative reductions in tick counts, and safety of treatments

A total of 91,741 ticks were observed from the 12,222 cattle inspections (Figure 2, Supplementary Table 1). Compared to baseline, the averages of mean percent reductions in tick counts across all post-day 0 assessments were 72.5% (range 40.3 – 93.9%) in the Tickoff® group, 87.4% (76.1 – 94.5%) in the Triatix® group and 72.7% (range 28.9 – 92.7%) in the excipient group. The animals were healthy throughout the trial period and no physical, behavioral, or physiological change that could be interpreted as an adverse reaction to experimental treatments was observed.

During the entire trial (December 2021 to July 2022), some animals did not have any post-treatment evaluation data (Supplementary Table 1) due to several reasons including loss of contact with the farmer, migration of herds due to prolonged drought, loss of ear-tags, cattle disappearance from home or lost in the forest, withdrawal of consent by the farmer, cattle sold, and death of the animal (because of prolonged drought). In addition, some cattle or herds occasionally skipped treatment sessions and sampling and therefore did not have tick count and epidemiological data generated. Animals received an average of 7.8 treatments out of the 13 treatment rounds.

Multivariable analysis

We fitted a GLMM to the infestation counts (Figure 2) and found that, even though substantial reductions in tick infestations were observed, there was no significant difference in tick infestation in cattle treated with Tickoff® compared to the animals in the excipient group (Table 2a). When comparing the excipient group to the reference Tickoff® group, the mean tick count (at log scale) at the average time point did not differ (p=0.427), and the change in counts between the two groups over enrolled time was also not significant (interaction term time point linear p=0.932, and interaction term time point quadratic p=0.869). On the other hand, the Triatix® group did have a significantly lower mean tick count (at log scale) at the average time point compared to the reference Tickoff® group (p< 0.001) (Table 2a). Additionally, the change in tick counts between the Triatix® and the reference Tickoff® groups over the enrolled time was (close to) significantly different, as indicated by the interaction term time point linear (p=0.055) and the interaction term time point quadratic (p< 0.001). Younger animals (calf and juvenile) had significantly lower tick infestation when compared to adult cattle (Table 2a). Calves have tick counts estimated to be 0.5 times (exp(-0.682)) the values for adults, while juveniles 0.8 times (exp(-0.263)) the values for adults. The effect of rain on tick counts was non-significant (p=0.144). The Wald tests for the main

effects and interactions of treatments and post-treatment time points are presented in Table 2b. Comparing mean tick counts at biweekly time points (at 2 to 26 weeks) showed significantly lower tick counts for the Triatix® group at 4 weeks up until 22 weeks, while the Tickoff® and excipient groups were never significantly different (Supplementary Table 2).

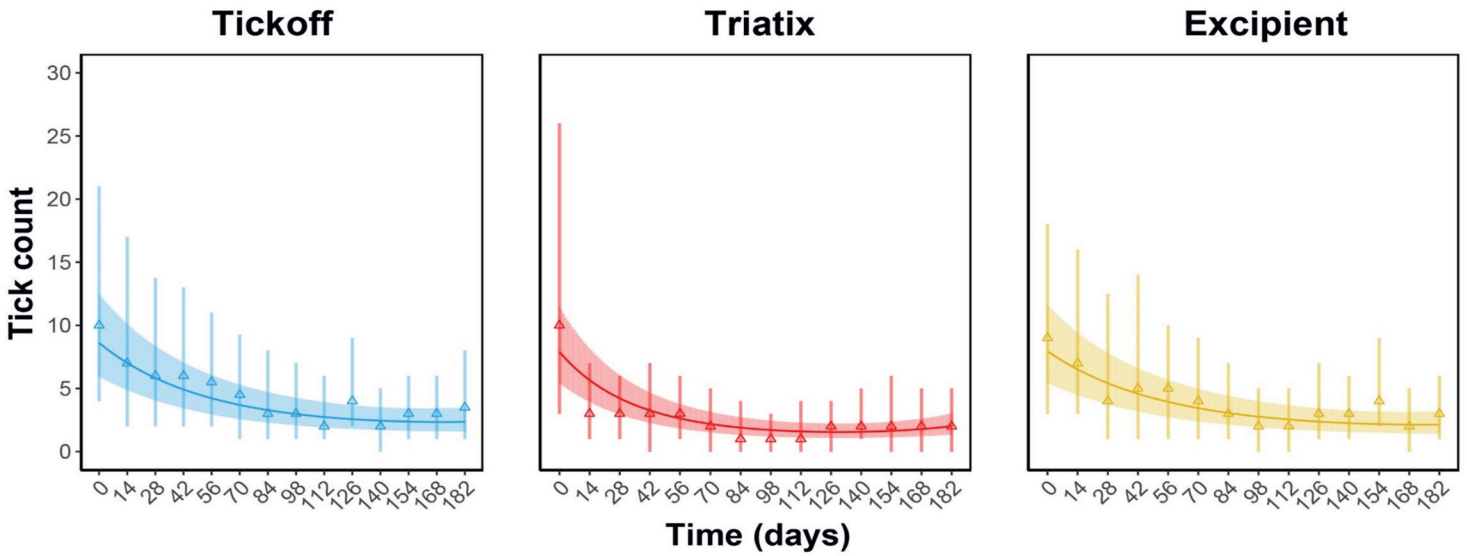

Figure 2: Observed and predicted tick counts during the trial period based on the fitted model. The continuous lines represent the predicted tick counts (with 95% CI), while the triangle point-up symbol represents the observed median tick count (with interquartile range) for each treatmen

Table 2a. Fixed-effect coefficients for the negative binomial mixed model for tick counts on cattle as the response variable.

Parameters	Estimate	Std. Error	Z value	*P*-value
(Intercept)	1.724	0.180	9.530	**< 0.001**
Triatix®	-0.384	0.095	-4.057	**< 0.001**
Excipient	-0.076	0.096	-0.794	0.427
Time-point (linear term)	-45.73	3.369	-13.571	**< 0.001**
Time-point (quadratic term)	12.95	2.880	4.495	**< 0.001**
Time delay in treatment	-0.0002112	0.002	-0.121	0.904
Triatix®: Time-point (linear term)	-8.59	4.472	-1.921	0.055
Triatix®: Time-point (quadratic term)	15.29	4.097	3.731	**< 0.001**
Excipient: Time-point (linear term)	-0.392	4.588	-0.085	0.932
Excipient: Time-point (quadratic term)	-0.687	4.158	-0.165	0.869
Rain	0.049	0.034	1.460	0.144
Age (calf)	-0.682	0.058	-11.741	**< 0.001**
Age (juvenile)	-0.263	0.038	-6.969	**< 0.001**

Abbreviation: Std. Error standard error
Day 0, Tickoff® treatment, no rain and adult cattle were used as references for analysis

Table 2b. Wald-tests (type II) for main effects and interactions.

Parameters	Chisquare	Df	*P*-value
Treatment	16.104	2	**< 0.001**
Time-point (linear and quadratic terms)	545.787	2	**< 0.001**
Treatment: Time-point (linear and quadratic terms)	23.526	4	**< 0.001**
Time delay in treatment	0.015	1	0.904
Rain	2.133	1	0.144
Age	158.126	2	**< 0.001**

Abbreviation: Df. Degrees of freedom
Day 0, Tickoff® treatment, no rain and adult cattle were used as references for statistical analysis

Survival analysis and mycosis

Ticks collected after treatment from Tickoff®-treated cattle in the field and maintained in the laboratory had a median survival time of 13 days (95% CI: 12 – 14 days), which was shorter than that of the ticks collected from the excipient group (>21 days) (Figure 3). The Cox regression model showed that Tickoff® treatment was associated with a significantly higher mortality rate compared to the excipient treatment (HR=8.50, 95% CI: 4.67 – 15.47, p<0.001). Ticks collected from cattle treated with Triatix® and transported to the lab were either dead on

arrival, or could not exhibit leg movement or response to external stimuli (e.g. touching with a pen or exhaling air on a tick) and hence were considered dead.

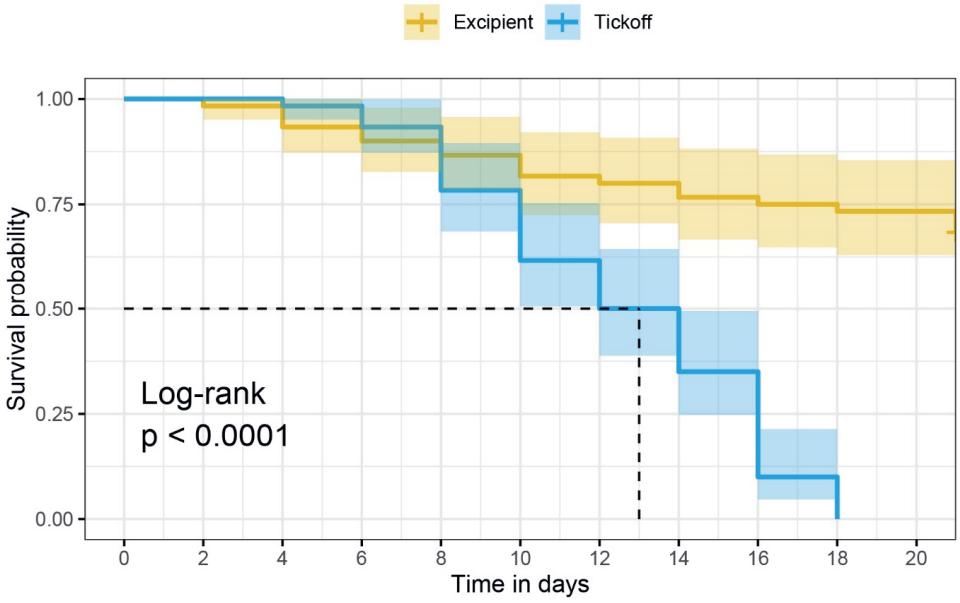

Figure 3. Kaplan-Meier survival curves for *Rhipicephalus appendiculatus* ticks collected from cattle treated with Tickoff® and excipient.

Monitoring of mycosis development on dead ticks

Fungal growth was observed in over 90% of ticks that were collected from cattle at three to six hours after Tickoff® application (Figure 4). No mycosis developed on tick cadavers from excipient and Triatix® treatments.

Figure 4. Fungal growth on ticks collected from cattle treated with Tickoff® and maintained in the humidity chamber (26 ± 1°C and 80 ± 5% RH) in the laboratory.

Epidemiological impact (Survival times to infection)

Blood samples were taken from the cohort of 1,488 zebu cattle at 2-month intervals for a duration of six months and tested for the presence of *A. marginale* and *T. parva* infections. Of these, 6.2% (n = 92/1488) and 4.8% (n = 71/1488) had *A. marginale* and *T. parva* infections at baseline, respectively, and were excluded from the analysis as no new infection could be observed. However, cattle positive for *A. marginale* were included in the *T. parva* analysis and vice-versa. During the six months, a total of 69 (4.9%) new cases of *A. marginale* infection and 51 (3.6%) new cases of *T. parva* infection were detected in cattle (Figure 5). Neither treatment nor age group had a significant effect on the incidence of *A. marginale* infections in cattle. Triatix® significantly reduced the incidence of *T. parva* (HR=0.44, 95% CI: 0.20-0.97, *p*=0.0415) (Table 3).

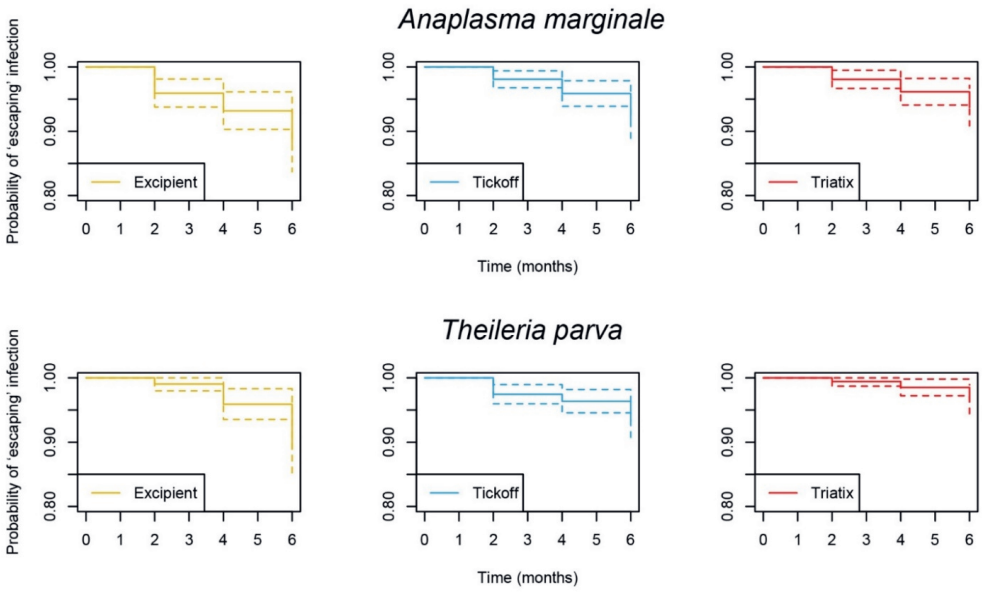

Figure 5. Estimated probabilities of 'escaping' infection with *Anaplasma marginale* and *Theileria parva* in cattle in different treatment groups.

Table 3. Hazard ratios and significance levels from Cox' proportional hazards regression model for infection incidence

	Anaplasma marginale		*Theileria parva*	
Variable	**HR (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-value**
Treatment				
Excipient	1.59 (0.91 – 2.75)	0.101	1.37 (0.75 – 2.50)	0.311
Triatix®	0.81 (0.44 – 1.50)	0.509	0.44 (0.20 – 0.97)	**0.042**
Age				
Adult	1.25 (0.53 – 2.93)	0.613	2.24 (0.54 – 9.41)	0.269
Juvenile	1.04 (0.41 – 2.61)	0.941	3.28 (0.76 – 14.02)	0.110

Abbreviation: HR hazard ratio. CI confidence interval.
Tickoff® treatment and calf were used as references for analysis

Discussion

We conducted a large-scale randomized controlled trial and coupled it with laboratory experiments to evaluate the safety and effects of Tickoff® biopesticide on tick infestation, tick mortality, and incidence of two tick-borne pathogens in zebu cattle managed under an extensive system. Overall, tick counts in all treatment groups dramatically decreased. The reduction in the Tickoff® group did not, however, differ significantly from that of excipient-treated cattle.

The Triatix® group did show a significantly greater reduction in tick counts compared to Tickoff® and excipient. Ticks exposed to Tickoff® biopesticide collected from animals treated with Tickoff® had a significantly shorter survival time compared to ticks exposed to excipient. This increased mortality did not result in a significant effect on the incidence of tick-borne pathogens in cattle in this setting. This was contrary to the effects of the Triatix® acaricide, which was associated with significant reductions in incidence of *T. parva* infection in cattle but not *A. marginale* infection.

Entomological impact

Tickoff® did not impact tick infestation, in line with some literature

Tickoff® biopesticide showed no significant reduction in tick infestation on cattle when compared to the excipient group. Additionally, the effect of Tickoff® did not change through time as revealed by the non-significant effect of the interaction of time with the treatment. Our findings corroborate earlier studies which also reported a lack of significant effect of fungal formulations (vs. controls) on tick infestation (Correia et al., 1998; Samish et al., 2014). This is, however, in contrast with other studies which reported a significant effect of fungal formulations on the reduction of tick infestation when compared to the respective control groups (Alonso-Díaz et al., 2007; Murigu et al., 2016). Whereas no significant effect of Tickoff® on tick reduction was observed in our field trial, *in vitro* experiments using both lab-exposed and field-collected ticks showed a clear pathogenic effect of Tickoff® biopesticide on the tick population when compared to the excipient group. In addition, fungus growth and sporulation were observed in all lab-exposed ticks and over 90% of ticks that were collected from cattle at three to six hours after fungal application. This indicates that Tickoff® could induce elevated mortality in ticks, albeit delayed as compared to Triatix® acaracide. This delayed mortality effect is expected to result in death after the ticks have detached from their treated host animal. This could explain the limited effect on tick infestation of treated cattle. Additionally, the ability of *M. anisopliae* to reduce tick fecundity and egg hatchability as reported in other studies (Camargo et al., 2012; Nana et al., 2015; Rot et al., 2013) may result in a reduction in progeny of ticks. Combined, these effects could still contribute to tick control provided a high enough coverage among tick host animals is achieved.

Effect of Triatix treatment

Cattle in the Triatix® group had significantly lower tick infestation than cattle in the Tickoff® group throughout the study period. This significantly higher impact of Triatix® on tick infestation is consistent with earlier studies reporting the efficacy of amitraz-based acaricides

when applied as a spray or dip for the control of ticks on cattle (George et al., 1998; Murigu et al., 2016). This effect may be due to their combined lethal and sublethal effects, such as rapid detachment or clearance of attached ticks within 6-30 hours after application, and immobilization and killing of detached ticks before they have the chance to lay eggs or molt (Barry Haigh & Gichang, 1980; Davey et al., 1984; Kagaruki, 1996). Despite its effectiveness in this study, this synthetic acaricide should be used with caution for tick management due to its toxicity to humans, its potential to increase contamination of the environment as well as milk and meat products (De Meneghi et al., 2016), and development of tick resistance (Githaka et al., 2022).

Effect of excipient treatment

Survival analysis on experiments in the laboratory showed that the excipient treatment, which contains canola oil (95%), 0.05% Triton X-100 (1.5%) and Kerosene (3.5%) and without the *M. anisopliae* ICIPE 7 (active ingredient of Tickoff®), produced mortality effects on ticks when compared to the untreated control (Appendix C). Such toxic effects of kerosene have been reported before in ticks (George et al., 2004), sand fleas (Enwemiwe et al., 2020) and immature stages of mosquitoes (Djouaka et al., 2007; Ojianwuna & Enwemiwe, 2022). It is believed that kerosene interferes with the physiology of arthropods, by penetrating tissues, causing inflammation and hypoxia, interfering with breathing, suppressing the insect immune system, and causing imbalances in hormones and enzymes (Maiyoh et al., 2015). This could play a role in the observed reductions in tick infestation in cattle in the excipient group. However, in the absence of water as a control treatment, we could not disentangle to what extent these reductions in tick infestation were due to the excipient or were reflective of natural, drought-induced, fluctuations. Additional research is required to determine the extent of tick repellency achieved with low concentrations of kerosene.

Epidemiological impact

Tickoff® had no effect on infection incidence, Triatix did

There was no significant effect of Tickoff® treatment on the incidence of both *A. marginale* and *T. parva* infections in cattle, relative to excipient treatment. Cattle treated with Triatix® did show significantly lower incidence of *T. parva* infection, but not *A. marginale*. This is in line with earlier studies, for instance using Spot-on® (a 10 % deltamethrin pour-on) (Muraguri et al., 2003). Other studies using Vectoid® (an emulsifiable deltamethrin concentrate) failed to show a significant effect on incidence of *T. parva* (Muhanguzi et al., 2014). The attack rates for *A. marginale* and *T. parva* infections were 4.9% and 3.6%, respectively, indicating that both

pathogens circulated at lower levels. This was likely due to the prolonged drought experienced during the study period and the consequential drop in tick counts. Consequently, we had limited statistical power to detect the epidemiological effects of the treatments on either pathogen.

The observed differences in levels of protection of Triatix® against *T. parva* and *A. marginale* infection could be related to the transmission dynamics of these pathogens. For instance, while *T. parva* is transmitted by *R. appendiculatus* ticks only, *A. marginale* can be transmitted biologically by several tick species in the *Rhipicephalus* and *Hyalomma* genera, but also mechanically by hematophagous arthropods or blood-contaminated fomites if they are not properly sterilized (Aubry & Geale, 2011). The latter transmission routes are not affected by tick control treatments and could therefore result in a smaller potential treatment effect. Indeed, hematophagous arthropods such as the stable flies (*Stomoxys calcitrans*) as well as biological vectors namely, *H. rufipes, R. decoloratus R. microplus* and *R. evertsi* are present in the region (**Chapter 2**). Besides, livestock farmers often used disposable needles on more than one animal, while livestock vaccination drives conducted by county government often used the same set of injection guns on all the animals presented for the vaccination.

Safety of Tickoff®

In this study, no physical or behavioral abnormalities were observed in the Tickoff®-treated cattle at any time during the trial. These results are in agreement with previous studies that reported no adverse reactions in cattle sprayed with fungal formulations of *M. anisopliae* (Alonso-Díaz et al., 2007; Kaaya et al., 2011). This is further exemplified by reports indicating that *M. anisopliae* poses minimal risk to mammals, humans, and non-target organisms (Fischhoff et al., 2017; Zimmermann, 2007).

Challenges during the study

Low power due to prolonged dry season

During the second half of the trial period, a prolonged dry season occurred. This may have contributed to a natural decline in the tick population in the environment and thus resulting in a low infestation abundance. Indeed, such climatic conditions have been associated with decreased survival, development, and questing activity of ticks in the environment (Brown et al., 2014; Jones & Kitron, 2000). The low tick population might have resulted in low circulation of tick-borne pathogens, reducing the power to detect a difference between the treatment groups.

Unfavorable environmental conditions

Fungal conidia are sensitive to unfavorable environmental factors such as high temperatures, low relative humidity, and direct ultraviolet (UV) radiation which reduce the germination, viability, and persistence of conidial spores (Fernandes et al., 2012). With the effect of drought, we suspect that these environmental factors might have adversely affected the germination of conidial spores thus affecting the treatment effect of Tickoff®.

Direct versus indirect effects of Tickoff® biopesticide

Tick control products have varying active ingredients with different modes of action. Depending on the mode(s) of action, these tick control products may produce a range of direct and indirect effects on transmission of tick-borne pathogens. Direct effects are those that protect the treated animal by killing the ticks before feeding thereby preventing pathogen transmission from ticks to tick-infested animals. In contrast, indirect effects protect both treated and untreated animals by killing the ticks after feeding thereby preventing onward transmission to other animals (treated or untreated). *Metarhizium anisopliae* isolate ICIPE 7 (the active ingredient of Tickoff®) kills ticks after feeding and thus may mostly provide community protection and limited direct individual protection. Additionally, *M. anisopliae* ICIPE 7 reduces the reproduction potential of ticks by reducing the tick fecundity and increasing the preoviposition, oviposition, and post-oviposition periods (Maranga et al., 2006; Nana et al., 2015; Nchu et al., 2010). Combined these indirect effects cannot directly protect treated cattle from getting infected by an already attached infectious tick but could result in a reduction in the next generation of ticks which would otherwise become infected and infectious. *M. anisopliae* ICIPE 7 will also kill the newly infected ticks before they molt and become infectious. Such reductions in tick population may reduce the risk of pathogen transmission and thereby reduce the incidence of tick-borne infections in the cattle population, including cattle that were not treated. The impact of these indirect effects will depend on the coverage level in the cattle population, the frequency and duration of treatment application, abundance of alternative hosts for ticks and pathogens that would not be reached by the treatment program, cattle mobility, and alternative pathogen transmission routes other than by an infectious tick bite. All these factors intertwine and make it complex to understand and study indirect effects of this biological control agent. Models can help to improve our understanding of how indirect effects of *M. anisopliae* ICIPE 7 formulation can protect the overall cattle population from tick-borne infections, including those that may not directly receive treatment.

Conclusion

Our study demonstrated the significant pathogenic effects of Tickoff® biopesticide on ticks removed from treated cattle and followed up for survival. While delayed mortality may be less effective at conferring direct protection of treated cattle, such effects may disrupt the life cycle of ticks and prevent onward pathogen transmission and reduced tick population sizes. The observed pathogenic effect is promising but was insufficient to result in significant effects of Tickoff® on tick infestation levels or incidence of infection with two tick-borne pathogens. This study was not designed to detect indirect treatment effects and had limited power due to a prolonged drought that occurred during the study period. Before subsequent trials will be rolled out, further efforts on the optimization of the Tickoff® formulation are needed, exploring suitable UV protectants to protect the conidia from damage by UV radiation, and continue with searching for thermo-tolerant strains of the fungi. Thereby, this work presents a next step towards the development of environmentally friendly tick and tick-borne disease control tools.

Acknowledgments

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Appendix A. Supplementary methods

Selection of the study herds

A herd was enrolled based on the following criteria: (i) at least one cattle in the herd had to be naturally infested with live attached ticks as evidence that the herd visits tick-infested areas, (ii) all cattle in the herd were to be of local zebu breed managed under an extensive grazing system, (iii) cattle in the herd were to be healthy or with conditions judged not likely to interfere with the objectives of the study, and (iv) herds had a maximum of twenty-five cattle. There were no sex restrictions but cattle were not eligible for enrolment if they were less than 6 months of age, pregnant, or had pre-existing medical conditions at the time of recruitment. Each herd was enrolled with the written informed consent of its owner or authorized agent. Every recruited cow was ear tagged with a unique identification number containing information about the village, herd number and individual animal number. Data on the owner and their cattle (sex, age, weight) were recorded for each cattle. The age of an animal was assessed by the dentition and farmer's information and was categorized as a calf (6-12 months), juvenile (13–24 months) and adult (over 24 months).

Herds would be removed from the study at any time at the discretion of the investigator for reasons that included (a) protocol non-compliance that was likely to compromise the integrity of the study or interpretation of study results e.g. treatment with other acaricidal products; (b) the appearance of concomitant disease that was incompatible with continuation in the study; (c) if an owner withdrew consent, and (d) loss of the herd to follow-up while still on the trial. Treatment of recruited cattle with any acaricidal formulations was not permitted within two weeks before the start of the study in order to minimize the residual effect which could potentially impact the day 0 tick counts. All cattle were kept with their owners under their usual husbandry practices throughout the trial.

Randomization

Enrolled herds were stratified (blocked) with respect to village, herd size, and tick infestation level and randomly allocated to either Tickoff® (Real IPM Ltd, Kenya), Triatix® (CKL Africa Ltd, Kenya), or excipient (Real IPM Ltd, Kenya) in a 1:1:1 ratio. All cattle from the same herd were allocated to the same treatment.

Treatment administration

The treatments i.e., Tickoff®, Triatix® and the excipient, were diluted as recommended by the manufacturer before being applied to the cattle. A designated unmasked dispenser at each

village was solely responsible for dispensing the experimental products. Treatments were topically applied to the skin using a hand rocker sprayer with a cone-type nozzle and a pressure of 6 kg/cm. The cattle were restrained in a crush and then sprayed from the bottom up and in the opposite direction to how the hair lies, giving greater attention to the areas most affected by ticks, such as the inner thighs, dewlap, tail, belly, inside ears, legs, and perineum. Each animal was sprayed with approximately 4 liters of the corresponding treatment, which was enough to wet the entire body surface. All cattle from the same herd received the same product every two weeks from day 0 till the end of the study on day 182. Day 0 was defined individually as the day a cow received the first treatment. Cattle were sprayed in the morning (6-8 a.m.) to avoid the adverse effects of sunlight and ultraviolet (UV-A and UV-B) radiation, which interfere with the germination of the fungus (Polar et al., 2005; Rangel et al., 2004).

Monitoring of unintended adverse effects

To demonstrate that the use of Tickoff® biopesticide is not associated with any adverse outcome, every effort was made to examine all the herds throughout the study period. During the tick counting exercise, another personnel physically examined each cattle for any suspected adverse events that may be associated with the topical application of trial products. These adverse events were mainly skin disorders i.e., pruritus, skin lesions, alopecia, dermatitis and eczema. Cattle owners were also instructed to observe their cattle for any suspected adverse events while at home and to document such observations and report them as soon as they occurred or at the next scheduled assessment.

Tick counts

Whole body tick counts were conducted, before treatment, on day 0, and thereafter at bi-weekly intervals until day 182. The cattle were restrained in a crush and the entire body was physically examined systematically. Counting began at zone 1 (head, ears, neck and dewlap to the point of the sternum), and proceeded to zone 2 (back and loin), zone 3 (forelegs, shoulders and ribs), zone 4 (belly, rear legs, udder/scrotum, fore and rear flank) and zone 5 (perineum, rump and tail) with the cattle in a standing position. The hair was pushed manually against its natural lie to expose the skin and attached ticks. Any tick observed was classified based on status (e.g., attached or non-attached, engorged or non-engorged) and counted. Data on tick counts were entered directly into a predesigned clinical form.

Monitoring mortality rate and development of mycosis on field collected ticks

A maximum of 3–4 live-attached ticks per animal were collected from randomly treated cattle in each group (three to six hours after spraying) and held for the development of mycosis as a check on fungal activity in the Tickoff® group, and possible fungal contamination in the Triatix® and excipient groups. Fungal contamination was a concern because all three treatments were administered (per herd) in each crush. An effort was made to collect representatives of each species and engorgement status. The collected samples were placed in vials labeled with the treatment group, village and dates. The samples were sent to the laboratory at the Regional Veterinary Investigations Laboratories (RVIL) in Mariakani, Kilifi County, for speciation under a stereomicroscope using previously described morphological characteristics (Walker et al., 2003). The identified ticks were not separated based on sex and the location of attachment on the body of an animal. The identified tick samples were placed in a sterile Petri dish (at most 10 ticks per dish) lined with Whatman No. 1 filter paper (Whatman, Maidstone, England) and kept in a humidity chamber maintained at a temperature of 26 ± 1°C and 85 ± 5% relative humidity (RH) for 21 days. Mortality was recorded and dead ticks were removed and transferred to another sterile Petri dish lined with filter paper. The Petri dishes were labeled and kept in an incubator under controlled conditions (26 ± 1°C and 80 ± 5% RH) to allow fungal growth (mycosis) on the cadaver.

Monitoring mortality rate and development of mycosis on lab-reared ticks

Unfed adult *R. appendiculatus* ticks were obtained from the Animal Rearing and Containment Unit of the International Centre of Insect Physiology and Ecology (*icipe*). The bioassays were composed of three groups: a non-treated control group, Tickoff® (Real IPM Ltd, Kenya) group, and excipient (Real IPM Ltd, Kenya) group. Ticks were infected using the adult immersion test (AIT) (Food and Agriculture Organization, 2004). The treated ticks were placed in sterile Petri dishes lined with filter paper, labelled and kept in an incubator under controlled conditions (26 ± 1°C and RH 85 ± 5%). Mortality was recorded for a maximum of 21 days. Dead ticks were removed and transferred to another sterile Petri dish lined with filter paper to allow mycosis on the cadavers. Treatments consisted of 30 ticks each and each bioassay was repeated three times.

Molecular detection of *Anaplasma marginale* and *Theileria parva* in cattle

A longitudinal survey to determine the impact of Tickoff® on the incidence of *A. marginale* and *T. parva* on cattle was done at two-month intervals. Approximately 4 ml of blood samples were collected from the jugular vein of each cattle using vacutainer tubes (BD Vacutainer®) coated with ethylenediaminetetraacetic acid (EDTA). Total genomic DNA was isolated from

whole blood samples and then screened for the presence of *A. marginale* and *T. parva* by PCR with high-resolution melting analysis as previously described (**Chapter 3**). All PCR reactions were carried out using Magnetic Induction Cycler (MIC) machine (BioMolecular Systems, Australia). The positive controls included *A. marginale* and *T. parva,* while a master mix without the DNA template was used as a negative control. Amplicons with unique melt curves were purified for sequencing to confirm species identity.

Generated raw sequences from ticks and positive pathogen samples were edited and aligned using the MAFFT plugin (Katoh & Standley, 2013) in Geneious software version 11.1.5 (https://www.geneious.com) (Kearse et al., 2012). To confirm the identity of each species, the sequences were compared with those available in the GenBank database using the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Blinding

Owing to the nature of the interventions i.e., color, viscosity and smell of the products, it was impossible to blind the study. However, non-blinded trained personnel were responsible for dispensing treatments to cattle and did not participate in outcome assessment. Outcome assessors did not have access to the tick records or a preview of the previous tick count. The study personnel involved in safety assessments were blinded to treatment assignments.

Appendix B. Supplementary table

Supplementary Table 1. Cumulative tick infestation, median tick infestation on cattle and percent reduction of tick counts relative to baseline at each time-point.

Days	Total cattle examined for tick infestation			Cumulative live-attached tick counts			Tick infestation per cattle[a]			% reduction in tick counts relative to baseline		
	Mazao Tickoff®	Triatix®	excipient	Mazao Tickoff®	Triatix®	excipient	Mazao Tickoff®	Triatix®	excipient	Mazao Tickoff®	Triatix®	excipient
0	541	471	445	8544	8766	6484	10 (4 – 21)	10 (3 – 26)	9 (3 – 18)	0	0	0
14	431	418	389	5099	2097	4613	7 (2 – 17)	3 (1 – 7)	7 (3 – 16)	40.3	76.1	28.9
28	454	404	375	4719	1900	3609	6 (2 – 13.6)	3 (1 – 6)	4 (1 – 12.5)	44.8	78.3	44.3
42	447	388	319	4891	1967	3174	6 (2 – 13)	3 (0 – 7)	5 (1 – 14)	42.8	77.6	51
56	388	358	325	3625	1584	2574	5.5 (2 – 11)	3 (1 – 6)	5 (1 – 10)	57.6	81.9	60.3
70	388	367	297	2830	1273	2074	4.5 (1 – 9.25)	2 (0 – 5)	4 (1 – 9)	66.9	85.5	68.0
84	342	317	294	1988	840	1674	3 (1 – 8)	1 (0 – 4)	3 (1 – 7)	76.7	90.4	74.2
98	361	334	275	1705	774	993	3 (1 – 7)	1 (0 – 3)	2 (0 – 5)	80.0	91.2	84.7
112	326	285	210	1266	948	912	2 (1 – 6)	1 (0 – 4)	2 (0 – 5)	85.2	89.2	85.9
126	243	227	196	1514	772	952	4 (2 – 9)	2 (0 – 4)	3 (1 – 7)	82.3	91.2	85.3
140	133	157	103	527	551	535	2 (0 – 5)	2 (1 – 5)	3 (1 – 6)	93.8	93.7	91.7
154	197	178	127	930	633	818	3 (1 – 6)	2 (0 – 6)	4 (2 – 9)	89.1	92.8	87.4
168	119	153	120	517	600	473	3 (1 – 6)	2 (0 – 5)	2 (0 – 5)	93.9	93.2	92.7
182	126	105	89	920	483	593	3.5 (1 – 8)	2 (0 – 5)	3 (1 – 6)	89.2	94.5	90.9

[a]Values for tick infestation are presented as median (1st and 3rd quartiles)

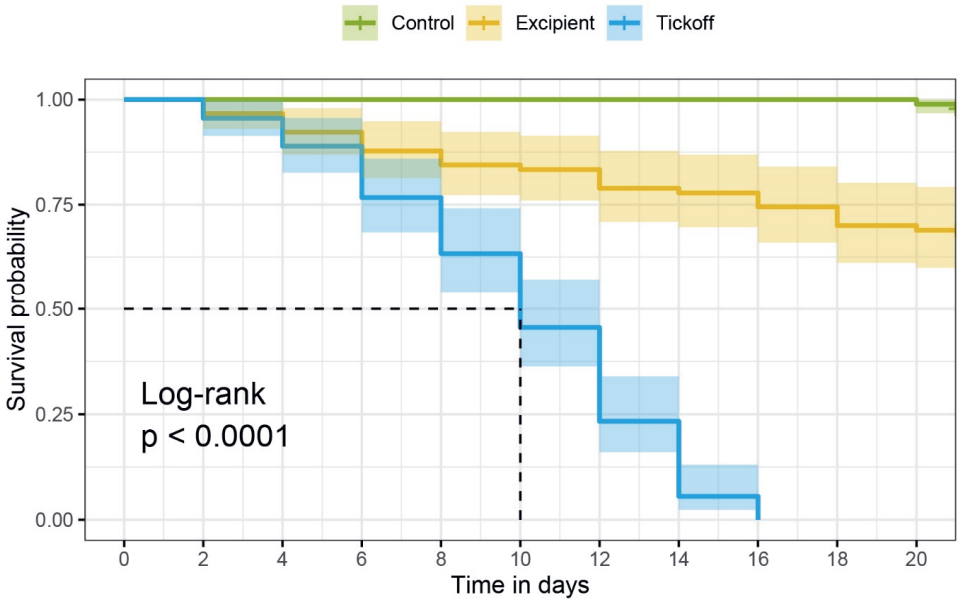
Supplementary Table 2. Pairwise comparisons of tick counts at biweekly timepoints.

Day (week)	Contrast	Estimate	SE	Z ratio	p-value
0 (week 0)	Tickoff® – Triatix	0.08626	0.121	0.716	0.7542
	Tickoff® – excipient	0.0801	0.122	0.658	0.7878
	Triatix – excipient	-0.00616	0.125	-0.049	0.9987
14 (week 2)	Tickoff® – Triatix	0.21494	0.108	1.982	0.1166
	Tickoff® – excipient	0.07625	0.110	0.696	0.766
	Triatix – excipient	-0.13869	0.113	-1.231	0.4349
28 (week 4)	Tickoff® – Triatix	0.32307	0.103	3.122	**0.0051**
	Tickoff® – excipient	0.07331	0.105	0.700	0.7632
	Triatix – excipient	-0.24975	0.108	-2.316	0.0536
42 (week 6)	Tickoff® – Triatix	0.41065	0.103	3.987	**0.0002**
	Tickoff® – excipient	0.07131	0.104	0.684	0.7729
	Triatix – excipient	-0.33935	0.108	-3.153	**0.0046**
56 (week 8)	Tickoff® – Triatix	0.47769	0.104	4.580	**<.0001**
	Tickoff® – excipient	0.07022	0.106	0.665	0.7841
	Triatix – excipient	-0.40747	0.109	-3.729	**0.0006**
70 (week 10)	Tickoff® – Triatix	0.52419	0.105	4.970	**<.0001**
	Tickoff® – excipient	0.07006	0.107	0.655	0.7894
	Triatix – excipient	-0.45413	0.111	-4.100	**0.0001**
84 (week 12)	Tickoff® – Triatix	0.55014	0.105	5.215	**<.0001**
	Tickoff® – excipient	0.07082	0.107	0.662	0.7858
	Triatix – excipient	-0.47932	0.111	-4.316	**<.0001**
98 (week 14)	Tickoff® – Triatix	0.55554	0.104	5.331	**<.0001**
	Tickoff® – excipient	0.0725	0.106	0.685	0.7724
	Triatix – excipient	-0.48305	0.110	-4.390	**<.0001**
112 (week 16)	Tickoff® – Triatix	0.5404	0.102	5.284	**<.0001**
	Tickoff® – excipient	0.0751	0.104	0.721	0.751
	Triatix – excipient	-0.4653	0.108	-4.294	**0.0001**
126 (week 18)	Tickoff® – Triatix	0.50471	0.101	4.982	**<.0001**
	Tickoff® – excipient	0.07863	0.103	0.760	0.7277
	Triatix – excipient	-0.42609	0.108	-3.958	**0.0002**
140 (week 20)	Tickoff® – Triatix	0.44848	0.104	4.319	**<.0001**
	Tickoff® – excipient	0.08308	0.106	0.780	0.715
	Triatix – excipient	-0.3654	0.110	-3.308	**0.0027**
154 (week 22)	Tickoff® – Triatix	0.3717	0.113	3.298	**0.0028**
	Tickoff® – excipient	0.08845	0.116	0.763	0.7256
	Triatix – excipient	-0.28325	0.12	-2.367	0.0471
168 (week 24)	Tickoff® – Triatix	0.27438	0.13	2.110	0.0878
	Tickoff® – excipient	0.09474	0.134	0.708	0.7588
	Triatix – excipient	-0.17964	0.137	-1.307	0.3911
182 (week 26)	Tickoff® – Triatix	0.15651	0.156	1.001	0.5764
	Tickoff® – excipient	0.10196	0.161	0.634	0.8015
	Triatix – excipient	-0.05455	0.164	-0.332	0.9411

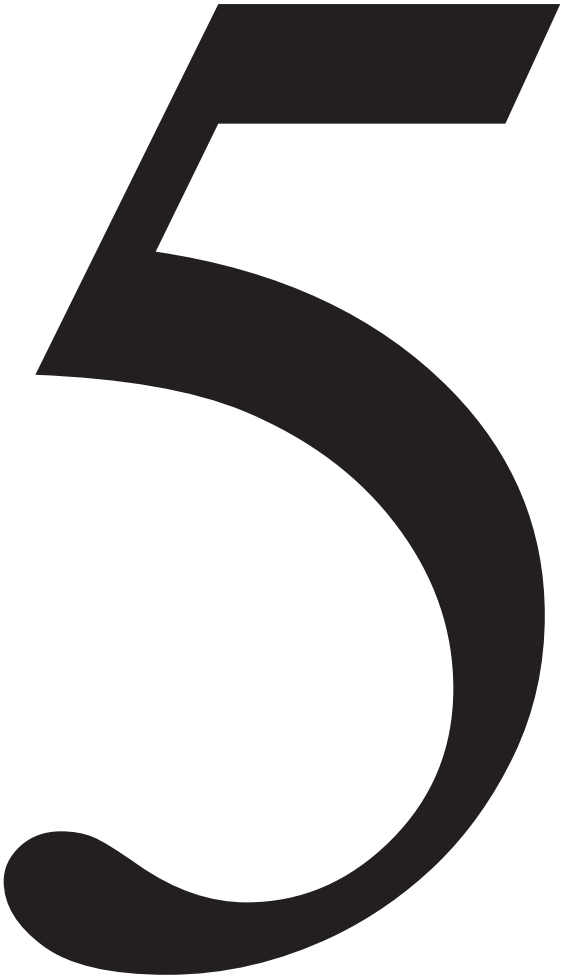
Appendix C. Supplementary results

Supplementary result: laboratory-exposed ticks

In the laboratory bioassays, the median survival time of lab-reared ticks exposed to Tickoff® treatment was 10 days (95% CI: 10 – 12 days) (Supplementary Figure 1). The hazard ratio (HR) was 197.38 (95% CI: 46.94 – 829.96; $p<0.001$) in Tickoff® treatment and 17.83 (95% CI: 4.25 – 74.85; $p<0.001$) in excipient treatment, indicating a significantly higher mortality rate for treated ticks compared to untreated controls.



Supplementary Figure 1. Kaplan-Meier survival curves for lab-reared *Rhipicephalus appendiculatus* ticks exposed to different treatment groups via the adult immersion test.



Chapter 5

Biological tick control: modeling the potential impact of entomopathogenic fungi on the transmission of East Coast fever in cattle

Joseph Wang'ang'a Oundo[1,2*], Nienke Hartemink[2], Mart C.M de Jong[2], Constantianus J.M. Koenraadt[3], Shewit Kalayou[1], Daniel Masiga[1], Quirine ten Bosch[2]

1 International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya,
2 Quantitative Veterinary Epidemiology, Wageningen University & Research, Wageningen, the Netherlands,
3 Laboratory of Entomology, Wageningen University & Research, Wageningen, the Netherlands

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Abstract

Biological control of ticks using entomopathogenic fungi (EPF) is a promising alternative to chemical acaricides for the control of tick-borne pathogens. For *Metarhizium anisopliae* isolate ICIPE 7, one of these EPFs, efficacy against multiple tick species has been demonstrated in laboratory and field settings. However, we currently have little quantitative understanding of how EPFs, through the control of tick population sizes, can impact transmission. We developed a deterministic model of tick–host–pathogen interactions to explore how the effects of EPF on *Rhipicephalus appendiculatus* ticks may impact the transmission dynamics of East Coast fever (ECF) in cattle populations. We parameterized the multi-faceted effects of EPFs on tick dynamics using experimental data on Tickoff® biopesticide (a novel formulation of *M. anisopliae* ICIPE 7) and related EPFs. The epidemiological impact of EPF was evaluated across a range of product profiles and implementation strategies. Model results indicate that, for the explored product profiles, EPF derives most of its epidemiological impact through the delayed mortality effect. This EPF-induced mortality would not only reduce the onward *Theileria parva* transmission to cattle (both treated and untreated) but will also cause a reduction in the tick-to-host ratio and thus cattle exposure to ticks. The effects of EPF on reproduction fitness and engorgement of ticks elicit negligible impact. High levels of population coverage and treatment frequency are needed to reduce the tick population size and reach meaningful epidemiological impact in cattle populations. Additionally, increasing the persistence time of fungal conidia on cattle skin – through technological improvements to the EPF formulation – can lead to a substantial reduction in acute infections when combined with appreciable population coverage levels, treatment frequency, and efficient spraying techniques. Our model analysis provides insights into the potential impact of EPF when deployed at a population level, and thus lends support to further research and development of this biological tick control tool.

Introduction

East Coast fever (ECF) disease is a major constraint to cattle health and productivity in 11 countries in eastern, central, and southern Africa, including Kenya (Gachohi et al., 2012; Nene et al., 2016). The disease is caused by the protozoan parasite *Theileria parva,* and is transmitted by the three-host ixodid tick *Rhipicephalus appendiculatus*. The African Cape buffalo (*Syncerus caffer*) is the natural reservoir host for *T. parva* (Nene et al., 2016). The economic impact of ECF includes reduced meat and milk production, cattle morbidity and mortality, and control measure costs against both ticks and the disease (Gachohi et al., 2012; Nene et al., 2016). These economic losses tend to affect resource-poor households disproportionately. In recent years, the geographic range of *T. parva* has expanded, for example to non-endemic countries of Comoros island (De Deken et al., 2007) and Cameroon (Silatsa et al., 2020).

Control of tick-borne diseases in cattle has traditionally relied on the use of chemical acaricides to kill the tick vector. The effectiveness of chemical acaricides for the control of tick infestation in cattle has been demonstrated in several field trials (Muraguri et al., 2003; Murigu et al., 2016; Nonga et al., 2012). However, the long-term sustainability of this tick control method is threatened by the emergence of acaricide resistance in ticks, including *R. appendiculatus* ticks (Githaka et al., 2022; Ntondini et al., 2008; Vudriko et al., 2016), and concerns regarding contamination of the environment and milk and meat products (De Meneghi et al., 2016). The intensive and frequent use, as well as inappropriate use of these chemicals, has been associated with the development of acaricide resistance in ticks (Githaka et al., 2022; Vudriko et al., 2018). There is, therefore, a need for new and environmentally friendly alternatives for tick control.

Biological control of ticks using entomopathogenic fungi (EPF), especially *Metarhizium anisopliae* sensu lato (s.l.) and *Beauveria bassiana* s.l., has attracted much interest as a possible and valuable alternative to conventional chemical acaricides. A range of laboratory studies has demonstrated the ability of EPFs to cause high mortality in the larva, nymph, and adult stages of various tick species (Hedimbi et al., 2011; Kaaya et al., 1996; Kaaya & Hedimbi, 2012). However, the success of tick control under field conditions has had variable results, in that, some studies reported a substantial efficacy (Alonso-Díaz et al., 2007; Barbieri et al., 2023; Murigu et al., 2016) while others reported a lack of significant efficacy when compared to the respective controls (**Chapter 4**; Correia et al., 1998; Leemon et al., 2008). The success of EPFs in the field is influenced by environmental factors such as temperature, relative humidity, and solar ultraviolet (UV) radiation (Fernandes et al., 2012). Therefore, this discrepancy in the

outcomes underscores the need for further research and development of EPFs before their adoption can be recommended and implemented at wider scale.

Beyond increasing the mortality rate of ticks, EPFs also elicit a multitude of effects on the infected ticks including a reduction in engorgement weight, fecundity (egg mass weight), and egg hatchability. Besides, increases in periods of engorgement, preoviposition, oviposition, and post-oviposition have also been observed (Nana et al., 2012, 2015). However, we currently lack a comprehensive quantitative understanding of how these effects interact to impact ECF transmission in cattle. While EPFs do not cause instantaneous tick mortality, the hallmark of chemical acaricides, they will still kill the infected ticks before they can molt to become infectious and thereof transmit the infection to the next host. This slow mortality rate implies that EPFs have limited potential to provide direct protection from infective tick bites at the individual animal level, but may still offer community-level protection. However, the level of coverage within the population, the duration of persistence on treated cattle, and the frequency and duration of treatment application required to achieve a maximum impact are unknown. Mathematical models can help to improve our quantitative understanding of how EPFs can indirectly protect the overall cattle population from tick-borne infections.

Mathematical models have been used to evaluate different control strategies for ECF in cattle populations. The study by Walker et al. (2014), for example, illustrated that the elimination of *T. parva* infection in cattle is unlikely to be accomplished solely by frequent acaricide use on cattle when grazing land is shared with the reservoir host Cape buffalo. This work builds on the earlier recognition by Medley et al. (1993) that the interruption of transmission of *T. parva* infection through tick control requires drastic reductions in tick infestations. Their modeling approaches did not incorporate the infection dynamics within the tick population and did not explicitly include the development stages (egg, larva, nymph, adult) of the tick vector. As the transmission cycle of ECF and other tick-borne pathogens encompasses several tick development stages, each of which may be affected differently by EPFs, these frameworks may not be suitable for investigating the multifaceted effects of EPFs.

Here, we introduce a detailed deterministic model of tick–pathogen–host interactions developed to estimate the impact of EPFs on the transmission of ECF in the cattle population managed under an extensive grazing system. We used the model to explore the implementation strategies and product properties needed to achieve a meaningful epidemiological impact. This model simulation is not intended for prediction of a specific product, but rather to provide an

illustrative framework to improve our understanding of the potential benefits that can be accrued when EPF is deployed at a population level.

Model development

Study setting/system

We are simulating the impact of EPF on a cattle population managed under an extensive grazing system where cattle are allowed to graze on natural pasture on fallow or communal grazing land. There is no controlled rotational grazing and animals have access to the entire grazing area. Cattle in this grazing system are exposed to tick reinfestation from the environment throughout the year and hence are at a constant risk of tick-borne infections. The tick control practice consists mostly of regular biweekly treatments and the treatment coverage level is limited, reaching a maximum of 40% of the population. This study setting allows us to assess the practical impact of EPF within the context of this herd management system and tick control efforts.

We constructed a tick–pathogen–host interaction model, describing the transitions between infection and treatment states for cattle and the different life stages of ticks (Figure 1; Appendix A.1. Times to events are assumed to be exponentially distributed, so that the average duration of a state/stage is the reciprocal of the rate of leaving that state/stage. The ordinary differential equations (ODEs) describing the full model are presented in the supplementary material (Appendix A.2). In this continuous-time, stage-structured deterministic model, we describe the population dynamics of *R. appendiculatus* ticks and cattle, the transmission of *T. parva* within the tick vector and between the tick and host population, and the application and decay of the treatment.

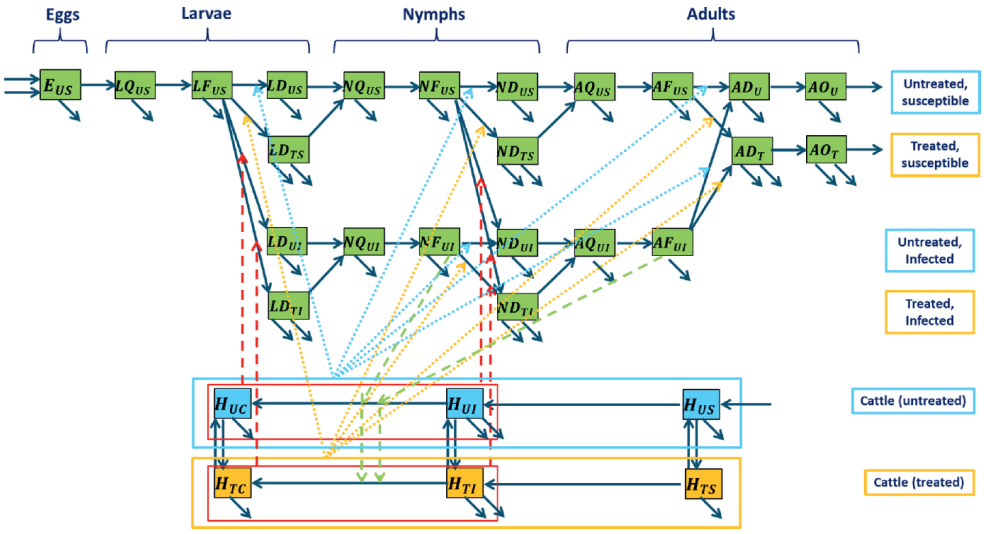

Figure 1. Conceptual model representing the tick–pathogen–host–treatment interactions. For the tick compartments, the capital letters indicate the developmental stage (E-egg, L-larva, N-nymph, A-adult) and the physiological phase (Q-questing, F-feeding, D-development, O-ovipositing). Host compartments are denoted with an H. Subscripts represent the treatment status (U – untreated or T – treated) and infection status (S – susceptible and I – infected/infectious, for hosts and ticks, and C – for carrier hosts). Solid navy-blue arrows denote demography, developmental, treatment, or pathogen-state transitions; green dashed arrows denote tick-to-host transmission routes; dashed red arrows denote host-to-tick pathogen transmission and/or biopesticide treatment; dotted arrows denote contact of the tick with EPF (light blue: no tick contact with the treatment, yellow: tick contact with the treatment).

Tick and host population dynamics

The life cycle of *R. appendiculatus* involves four successive development stages, namely egg (E), larva (L), nymph (N), and adult (A). Except for the egg stage, each tick could either be in the questing (Q), feeding (F), or interstadial development (D) phase. Adult females can also be in the oviposition (O) phase. A female tick that survives to reproduce will consume three blood meals in its lifetime. Each of these blood meals will occur on a different host individual. In this model, we assume that the ticks feed only on cattle hosts. This assumption is based on the notion that *R. appendiculatus* is well adapted to the presence of domestic cattle and can be maintained by all stages feeding on cattle (Walker et al., 2003). In the study area of interst, wildlife such as buffaloes, elands, waterbucks, nyalas, greater kudus, and sable antelopes, which are alternative hosts for this tick (Walker et al., 2003), are not present.

After taking a blood meal from the cattle host, the female ticks will detach and find a suitable location in the environment to lay their eggs (E). A fully engorged adult female *R. appendiculatus* will lay 3,000 to 5,000 eggs and then die (Walker et al., 2003). The production of eggs is assumed to be proportional to the total number of ovipositing adult female ticks (AO_U) and the egg-laying rate:

$$AO_{U(t)}\left(\frac{E_{total}}{t_o}\right),$$

which is the reciprocal of the average time between each oviposition event. The eggs produced will hatch and develop into the questing larval stage at a constant developmental rate k_E. Questing larvae will attach to cattle hosts at a constant rate α_L to feed. The feeding larvae will engorge with a mean duration d_L then detach from the host and enter the development phase. Larvae in the development phase will molt to the questing nymphal stage at a developmental rate k_L. The same process of questing (α_N, α_A), feeding (d_N, d_A), and development (k_N, k_o) is repeated for nymphs and adults. After this development phase, adult ticks mate and only the females proceed to the oviposition phase (through a sex proportion ζ). The ovipositing females will lay eggs for a duration t_o and the depleted female will then die. The natural mortality rates are represented by μ_{ij} where i and j represents the tick developmental stages and phases respectively.

For the cattle, we assume a constant population size, which is obtained by keeping the birth rate and the death rate the same (μ_H). Since there is no vertical transmission of the disease, all newborn cows are assumed to be susceptible and enter the H_{US} class.

Theileria parva transmission dynamics

Host to tick transmission

Theileria parva infection is acquired from an infectious host by larvae or nymphs, maintained trans-stadially through the tick's development and molting processes, and transmitted to a susceptible host by the next tick stage (nymph or adult). Infection acquired by adult ticks cannot be transmitted transovarially via the eggs to larvae of the next generation (Nene et al., 2016). Our model therefore ignores the infection acquired by the adult ticks. The model also assumes that ticks do not die of the *T. parva* infection and remain infectious for the remainder of their lives with 100% transstadial transmission. The probability of acquiring *T. parva* infection from the infectious cattle by larvae and nymph depends on whether it feeds on the acutely infectious host (p_{LI}, p_{NI}) or the persistent carrier host (P_{LC}, P_{NC}). The tick population is sequestered into

a susceptible class and an infected/infectious class (second index subscripts: S – susceptible, I – infected/infectious).

Tick to host transmission

The total cattle host population (H_{total}) is divided into sub-categories depending on treatment status (using subscript U for untreated or T for animals treated with EPF). This is further divided into susceptible (H_{US} and H_{TS}), symptomatic infectious (H_{UI} and H_{TI}), and carrier (H_{UC} and H_{TC}) compartments. Individuals move between compartments when their disease status and/or treatment status changes.

The susceptible host moves to the symptomatic infectious class after getting a bite from an infectious nymph or adult tick. The force of infection in the susceptible cattle (i.e., the rate at which the susceptible cattle become infected) is determined by the cattle exposure rate to ticks, the probability that the bite is by an infectious tick, and the probability of transmission per bite (P_{HN} and P_{HA}, for nymphs and adults, respectively). The cattle exposure rate to ticks, defined here as the average number of tick bites per host per day, is calculated as the ratio between the number of questing ticks and the number of hosts $\left(\frac{AQ_{UI}+AQ_{US}}{H_{Total}}\right)$ or $\left(\frac{NQ_{UI}+NQ_{US}}{H_{Total}}\right)$, representing the number of questing adults or nymphs available per host, multiplied by the attachment rate of questing ticks (α_A and α_N for adults and nymphs).

For the probability that the bite is taken by an infectious tick, we use the proportion of feeding ticks that are infectious $\left(\frac{AF_{UI}}{AF_{UI}+AF_{US}}\right)$ and $\left(\frac{NF_{UI}}{NF_{UI}+NF_{US}}\right)$, for adults and nymphs respectively.

The transmission probability per bite is P_{HN} for infectious nymphs and P_{HA} for infectious adults. A proportion (P_I) of symptomatically infectious cattle may experience disease-induced death at a constant rate (μ_I), while surviving individuals progress to the carrier compartment at the rate σ_H. Cattle in this compartment develop solid immunity against re-infection with similar strains (Nene et al., 2016) and remain persistent carriers of tick-transmissible infection (Kariuki et al., 1995; Olds et al., 2018).

Treatment with entomopathogenic fungi

Spraying of untreated cattle host (H_{US}, H_{UI} and H_{UC}) with EPF at the rate φ will produce a treated host population (H_{TS}, H_{TI} and H_{TC}). The treated host population also loses the treatment status over time, due to the decay of the conidial spores; we assume a constant decay rate (δ)

(i.e. the rate of losing treatment status). In the absence of concrete data, we assumed a conservative estimate for δ of 1.0 per day. Thus, the EPF biopesticide is presumed to exhibit an effective acaricidal activity lasting one day, on average. Ticks attached to the cattle at the time of treatment will contact the EPF with varying probabilities for larvae (P_{LT}), nymphs (P_{NT}) and adult ticks (P_{AT}). The on-host ticks that will contact the treatment will progress to treated status while those that escape treatment will remain in the untreated status (first index subscripts: U – untreated or T – treated with EPF).

Effects of entomopathogenic fungi

The entomopathogenic fungi *Metarhizium anisopliae* ICIPE 7 elicits multifaceted effects on *R. appendiculatus* ticks, including increasing the overall tick mortality rate, prolonging the engorgement period, and interfering with the reproductive fitness of engorged female ticks by reducing fecundity (egg mass), and increasing pre-oviposition, oviposition and post-oviposition periods (Nana et al., 2015).

Increased mortality

Unlike chemical acaricides which cause rapid death of exposed ticks, EPFs will take several days to kill a tick after exposure. We included this delayed lethality in our model by setting EPF-induced mortality rate ϑ_{iDF} (where i = L , N, A) at the interstadial development phase instead of the feeding phase. The EPF-induced mortality ϑ_{iDF} was derived by fitting a Weibull model to tick mortality data (**Chapter 4**). The death rate increased with a factor 10 .

Increased engorgement duration

In addition to increasing mortality, *M. anisopliae* ICIPE 7 may prolong the engorgement duration in treated *R. appendiculatus* ticks by 37.6% (Nana et al., 2015). We explore this in our model by increasing the engorgement duration (d_i where i = L, N, A) by τ_i days, resulting in an overall delay in detachment from the host. Thus, the rate of leaving the feeding compartment for treated ticks is assumed to be $\left(\frac{1}{d_i+\tau_i}\right)$.

Decreasing reproduction fitness

Fungal infection can reduce the reproductive fitness of engorged females of *R. appendiculatus* ticks through a reduction in the fecundity rate by 36.9%, and an increase in the pre-oviposition, oviposition, and post-oviposition periods by 38.9%, 24.4% and 37.9% respectively (Nana et al., 2015). We explore this by increasing the pre-oviposition period (k_o, i.e., the interval that elapses between the detachment of an engorged female and the first appearance of eggs) by k_τ

days. Thus, the rate at which treated ticks leave the preoviposition phase is expressed as $\left(\frac{1}{k_o+k_\tau}\right)$. The fecundity (i.e., the average number of eggs laid per female tick) is reduced by a factor TFR (treatment fecundity reduction), while the oviposition duration is increased by τ_o days. This means that the rate of leaving the oviposition phase is assumed to be $\left(\frac{1}{t_o+\tau_o}\right)$, and the expression for the egg laying rate becomes:

$$\left(\frac{E_{total}(1-TFR)}{t_o+\tau_o}\right)$$

Where E is the number of eggs laid per ovipositing female, TFR is the treatment fecundity reduction, t_o is the normal oviposition duration in the untreated adult females, and τ_o is the increased oviposition duration.

Parameterization

The tick model is based on the assumption that, without treatment, the *R. appendiculatus* population is at equilibrium, that is, there is neither exponential growth nor decline of the tick population over time. We calibrated the model to achieve an equilibrium state where one egg-laying female tick replaces herself per generation (Randolph, 1998, 2004). If each female lays approximately 3000 eggs, the tick population equilibrium requires 3.9% survival from eggs to larvae, 9% survival from larvae to nymphs, and 19% survival from nymphs to fully reproductive adults (Randolph, 1998). We also calibrated the model such that the lifecycle duration of one generation of tick lasts for 276 days as observed in a previous field observation study (Branagan, 1973a). Besides, the equilibrium prevalence of *T. parva* in the cattle population has been calibrated to range between 7-10% as observed in the earlier study (**Chapter 3**).

The growth rate of the tick population within an ecosystem is assumed to be density-dependent, meaning the tick population growth rate increases when the population is low but slows down when the population approaches the carrying capacity. This density-dependent growth rate of tick population follows a characteristic logistic model depending on the environment's carrying capacity K_T (i.e., the maximum number of ticks an environment can sustain for an indefinite period given resource availability), and is expressed as:

$$\left(1-\frac{N_{T(t)}}{K_T}\right)$$

Where:

N_T – is the total tick population size

K_T – environmental carrying capacity for the tick population

The carrying capacity for the environment was set to be seven times larger than the total tick population at the equilibrium state.

Model simulations of implementation strategy and product profile

The model simulations were implemented using the package 'deSolve' (Soetaert et al., 2010) in R version 4.3.1. Parameter values used to simulate the model are summarized in Table 1. We evaluated the potential impact of EPF by simulating different implementation strategies and product properties: (1) the application of biopesticides to cattle population at varying coverage levels (defined here as the proportion of cattle within the population that is treated with EPF) and treatment intervals for one year, (2) efficiency of treatment application (spraying) technique, (3) the duration of persistence of the EPF on cattle skin post-treatment, and (4) the different combinations of the effects of *M. anisopliae* isolate ICIPE 7 on the tick life cycle. Simulations (1) and (2) are implementation strategies while (3) and (4) are product properties. The impact of EPF was assessed based on the number of susceptible cattle that get infected.

Table 1. Parameter estimates used in the model

Parameter	Description	Value	Unit	References
E	Maximum number of eggs laid per ovipositing female	3500	eggs	(Walker et al., 2003)
k_o	Preoviposition period of the engorged female tick	6	days	(Branagan, 1973b)
t_o	Oviposition period of the engorged female tick	24	days	(Branagan, 1973b)
k_E	Interstadial development rate of egg	0.01098901	day^{-1}	(Branagan, 1973a)
k_L	Interstadial development rate of larva	0.03225806	day^{-1}	(Branagan, 1973a)
k_N	Interstadial development rate of nymph	0.02222222	day^{-1}	(Branagan, 1973a)
μ_E	Egg mortality rate	0.1	day^{-1}	This study
μ_{LQ}	Mortality rate of questing larva	0.05	day^{-1}	(Randolph & Rogers, 1997)
μ_{NQ}	Mortality rate of questing nymph	0.03	day^{-1}	(Randolph & Rogers, 1997)
μ_{AQ}	Mortality rate of questing adult	0.01	day^{-1}	(Randolph & Rogers, 1997)
μ_{LF}	Natural larval tick mortality	0.005714286	day^{-1}	(Walker et al., 2014)
μ_{NF}	Natural nymphal tick mortality	0.003703704	day^{-1}	(Walker et al., 2014)
μ_{AF}	Natural adult tick mortality rate	0.0025	day^{-1}	(Walker et al., 2014)
μ_{LD}	Mortality rate of developing larva	0.177388	day^{-1}	This study
μ_{ND}	Mortality rate of developing nymph	0.065	day^{-1}	This study
μ_{AD}	Mortality rate of developing adult	0.02	day^{-1}	(Randolph & Rogers, 1997)
μ_{AO}	Mortality rate of egg-laying female adult	0.02	day^{-1}	This study
α_L	Attachment rate by larva	0.08333333	day^{-1}	(Branagan, 1973a)
α_N	Attachment rate by nymph	0.05	day^{-1}	(Branagan, 1973a)
α_A	Attachment rate by adult	0.03571429	day^{-1}	(Branagan, 1973a)
d_L	Feeding duration of larval tick	5	days	(Branagan, 1973a)
d_N	Feeding duration of nymphal tick	6	days	(Branagan, 1973a)
d_A	Feeding duration of adult tick	8	days	(Branagan, 1973a)
P_{HN}	Probability nymph infects susceptible host	0.09	No unit	(Walker et al., 2014)
P_{HA}	Probability adult tick infects susceptible host	0.9	No unit	(Walker et al., 2014)
P_{LI}, P_{NI}	probability of a larva and nymph tick becoming infected when feeding on an acutely infectious host	0.118656	No unit	(Medley et al., 1993)
P_{LC}, P_{NC}	probability of a larva and nymph tick becoming infected when feeding on an infectious carrier host	0.023	No unit	(Medley et al., 1993)
P_{LT}	Probability of a feeding larva coming into contact with treatment during spray	0.7	No unit	This study
P_{NT}	Probability of a feeding nymph coming into contact with treatment during spray	0.8	No unit	This study
P_{AT}	Probability of a feeding adult tick coming into contact with treatment during spray	0.9	No unit	This study
ζ	Proportion females	0.5	No unit	This study
μ_H	Natural host mortality	0.0006859604	day^{-1}	(Medley et al., 1993)
μ_I	Mortality due to East Coast fever	0.25	day^{-1}	(Medley et al., 1993)
P_I	Proportion of hosts dying from East Coast fever	0.05	No unit	(Medley et al., 1993)
σ_H	Rate of host recovery from disease	0.06666667	day^{-1}	(Medley et al., 1993)
ϑ_{iDF}	Mortality rate of ticks due to treatment with EPF	9.886674	No unit	**Chapter 4**
τ_L	Increased feeding duration of larva due to EPF treatment	1.9	days	(Nana et al., 2015)
τ_N	Increased feeding duration of nymph due to EPF treatment	2.3	days	(Nana et al., 2015)
τ_A	Increased feeding duration of adult tick due to EPF treatment	3	days	(Nana et al., 2015)
k_τ	Increased pre-oviposition period of the engorged female tick due to EPF treatment	2.3	days	(Nana et al., 2015)
τ_o	Increased oviposition duration	6	days	(Nana et al., 2015)
TFR	treatment fecundity reduction	0.369	No unit	(Nana et al., 2015)
K_T	Environment's carrying capacity for the tick population	35514150		This study

Results

The results shown here portray the conservative estimate of the impact of EPF. Unless explicitly stated, the default treatment strategy involves treating the cattle every two weeks for one year, and a maximum of 40% of the population receives the treatment. The duration of effective acaricidal activity of EPF is one day. The default parameter values are listed in Table 1.

Entomopathogenic fungi marginally reduce the transmission of ECF in the cattle population

The simulation projects a slight reduction of acute infections by 11% relative to the baseline equilibrium infections after one year of treatment (Figure 2A). The EPF contributes to the observed decrease in acute infections in the cattle population by reducing the ratio of feeding ticks to cattle (Figure 2B) and limiting exposure to ticks in both treated and untreated cattle (Figures 2C and 2D), thus offering community-level protection. This is however insufficient to break the transmission cycle. Discontinuation of treatment will cause a resurgence of ECF cases in the population, an increase in the tick-to-host ratio, and an increase in cattle exposure rate to ticks.

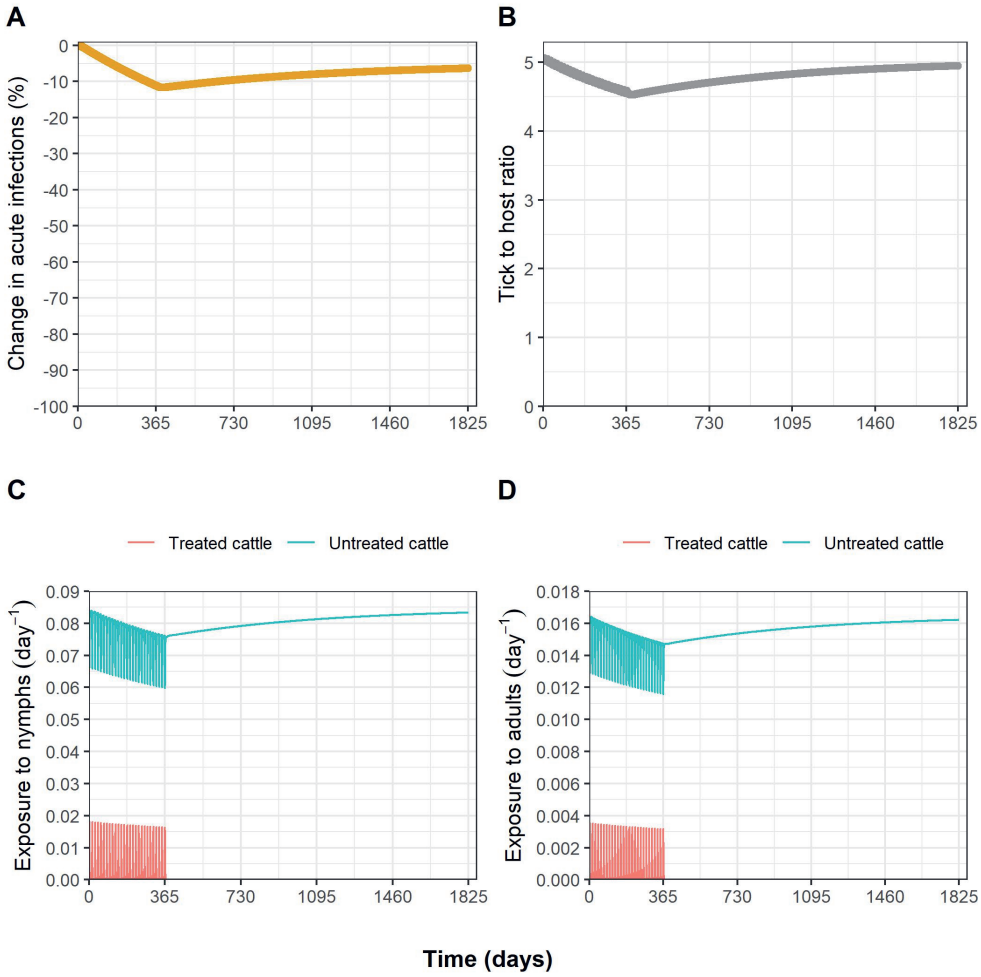

Figure 2. Epidemiological and entomological effects of entomopathogenic fungi. (A) Effect on acute *T. parva* infection in cattle population relative to the baseline equilibrium cases, **(B)** Effect on tick to host ratio, **(C)** Effect on cattle exposure to nymphal ticks, **(D)** Effect on cattle exposure to adult ticks. The treatment was applied to 40% of the host population at biweekly intervals for one year. The total simulation duration is five years and the duration of effective acaricidal activity in the EPF is one day.

The population-level impact of entomopathogenic fungi depends on the implementation strategy

The estimated community-level impact on relative reductions of acute infections in the host population was projected to be minimal to modest depending on the coverage level and treatment frequency (Figure 3). When considering the conservative scenario of the best

coverage of 40% with the biweekly treatment interval, the simulation projects a marginal decrease of 11% within one year in equilibrium acute infections. In comparison, the weekly treatment regimen with EPF emerges as the most effective, resulting in a 21% reduction in acute infections relative to the baseline equilibrium. Increasing the population coverage level and treatment frequency will result in a further reduction in acute infections within the cattle population. Nevertheless, none of the treatment strategies in the current product profile can cause a 50% reduction in cases of acute infections in the host population.

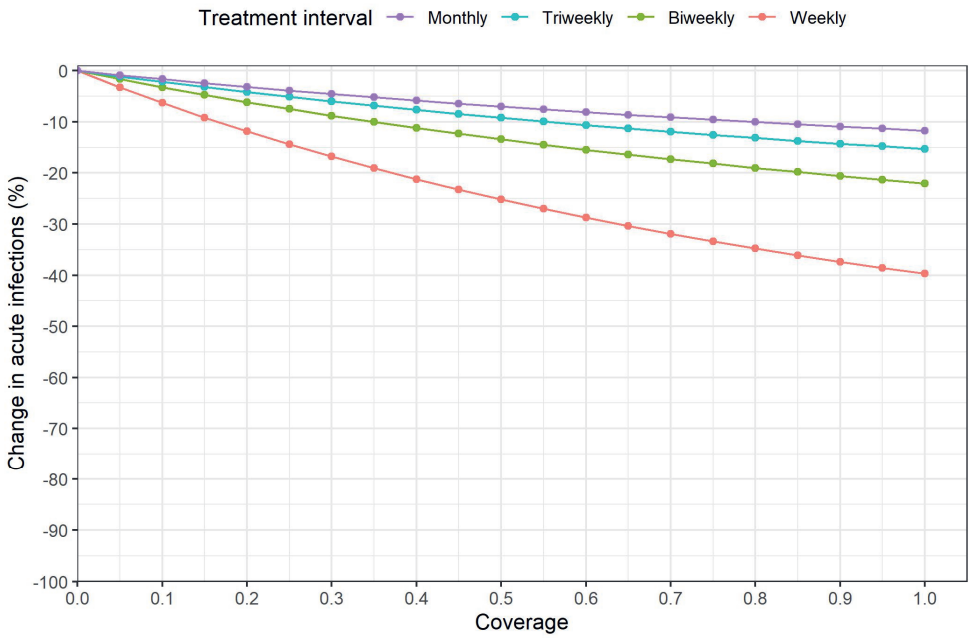

Figure 3. Effects of different treatment implementation strategies on the reduction of acute *T. parva* infections in the cattle population relative to the baseline equilibrium cases. Effects were assessed as a function of population coverage for different treatment intervals. The simulation period is one year and the duration of effective acaricidal activity in the EPF is one day

Extending fungal decay time enhances the impact of entomopathogenic fungi

The model output suggests that optimizing the EPF formulation by increasing the fungal decay time will greatly improve the population-level impact of EPF, even at low coverage levels (Figure 4). For example, in the case of the most conservative estimate, which entails attaining

40% coverage through treatments administered every two weeks, and assuming a conservative estimate of effective acaricidal activity lasting one day, the simulation forecasts a slight reduction of 11% in acute infections compared to the baseline equilibrium (Figure 4B). However, extending the fungal decay time to three days leads to a 30% reduction, while a five-day decay period results in a 44% decrease. A fungal decay time of seven days causes a moderate 55% decline, and a ten-day decay period yields a substantial 66% reduction in acute infections relative to the baseline (Figure 4B). Nonetheless, the model projects that in the event it is not feasible to achieve a decay period of more than one day, then the population-level impact of EPF is maximized by high population coverage and higher treatment frequency (Figure 4A). Decreasing treatment frequency to monthly intervals will offset the impact of EPF (Figure 4C). A 50% reduction in cases of acute infections is achievable with an increase in fungal decay time.

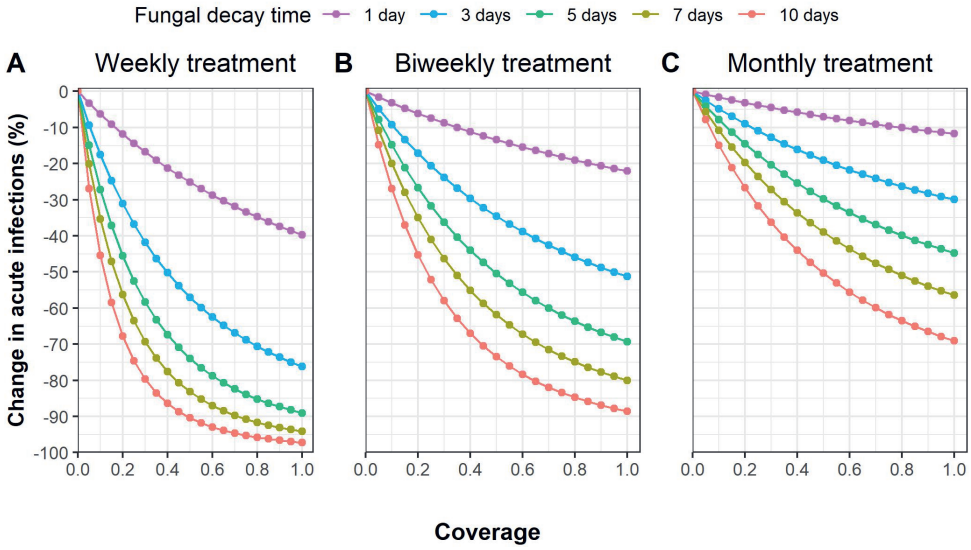

Figure 4. Effects of extending fungal decay time on the reduction of acute *T. parva* infections in the cattle population relative to the baseline equilibrium cases. Effects were assessed as a function of population coverage for different fungal decay times and treatment intervals. The simulation period is one year.

The impact of entomopathogenic fungi on ECF transmission is expected to derive from the fungal mortality effect

The model simulations show that fungal-induced mortality of ticks accounts for the majority of the EPF's impact (Figure 5). The extent of this effect will depend on the coverage and treatment frequency. In the absence of the fungal mortality effect on ticks, EPF will elicit a negligible reduction of acute infections in the cattle population (Figure 5B). This shows that the other effects of fungal infection on ticks, even when they act simultaneously, have limited potential to reduce cases of acute infections within the cattle population (Figure 5B). Notably, the model output also shows that the EPF effect of prolonging tick engorgement duration on the host is, in fact, increasing the transmission of infection in cattle, and thus exerting a minor deleterious effect on the performance of EPF (Figure 5E).

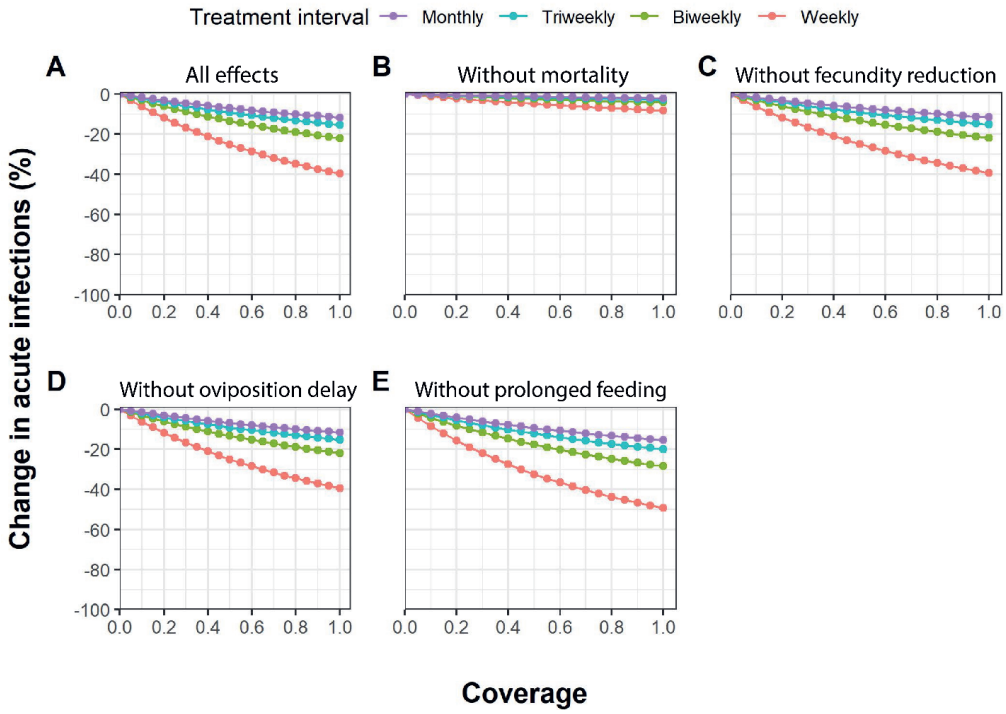

Figure 5. Composite effects of *Metarhizium anisopliae* ICIPE 7 product properties on the impact of entomopathogenic fungi on the acute *T. parva* infections in cattle population. Effects were assessed as a function of population coverage for different modes of action and treatment intervals. **(A)** all effects, i.e., increased mortality, reduced fecundity, delayed

oviposition period, and prolonged engorgement duration, **(B)** as A without mortality effect, **(C)** as A without reduced fecundity effect, **(D)** as A without delayed oviposition effect, and **(E)** as A without prolonged engorgement effect. The treatment was applied to 40% of the host population (coverage level). The simulation period is one year and the duration of effective acaricidal activity of the EPF is one day.

The impact of entomopathogenic fungi on ECF transmission is partly dependent on the efficiency of the spraying technique

Our simulation results indicate that enhancing the probability of tick contact with EPF, achieved through an efficient spraying technique, will result in varying degrees of effectiveness for the EPF. This impact is contingent upon the treatment frequency and partly on the coverage level (Figure 6). At a higher treatment frequency (weekly), increasing the probability that a tick contacts the treatment will result in a considerable reduction in cases of acute infections in the host population, signifying a higher impact of EPF (Figure 6A). Conversely, a lower treatment frequency (monthly), even with a higher coverage level, will only result in a marginal reduction in the cases of acute infections in the population, signifying a lower impact of EPF (Figure 6C).

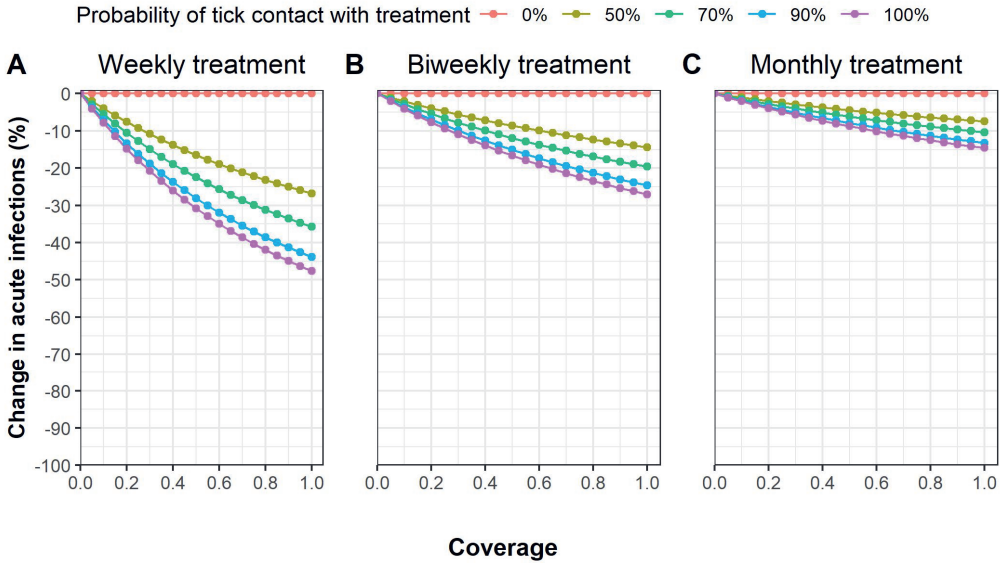

Figure 6. Effects of treatment efficiency and coverage level on the percentage reduction of acute infections in cattle. Effects were assessed as a function of population coverage for different treatment intervals and probabilities of tick contact with the EPF. The simulation

period is one year. The probability of tick contact with EPF (P_{LT}, P_{NT}, P_{AT}) are all the same for a given value.

Discussion

In this study, we have developed a tick-pathogen-host interaction model to examine the potential impact of entomopathogenic fungi (EPF) on the transmission dynamics of ECF in cattle populations in an endemic context. The model was parameterized based on our results with Tickoff® biopesticide. We highlight that under the assumed product profile, EPF derives most of its impact on ECF through indirect protection: it does not prevent feeding ticks from picking up or transmitting ECF to treated animals, but it does reduce onward transmission to other animals (treated or untreated) due to delayed lethal effects. High levels of population coverage and frequent applications are needed to reduce the tick population and reach meaningful impact in cattle populations. Substantial improvements can be obtained by improving the stability of EPFs on the cattle skin: an increase in the decay time of EPF from 1 to 7 days leads to a considerable reduction in acute infections when combined with appreciable population coverage levels, treatment intervals, and efficient spraying technique. Whether sufficient coverage levels can be reached, is also determined by the relative importance of wildlife in maintaining tick-reservoir hosts and may vary between settings.

Mechanisms by which entomopathogenic fungi offer indirect protection against ECF in the cattle population

The rate at which susceptible cattle become infected with *T. parva* depends on several factors, including the rate of cattle exposure to host-seeking ticks, the prevalence of infection among ticks and the probability that cattle become infected after being bitten by an infectious tick. The prevalence of infection among ticks depends further on the probability that a tick becomes infected upon biting infectious cattle, the prevalence of infection in cattle, the daily tick mortality rate, and the rate of development in ticks (i.e., molting rate). Whether these factors accumulate to cause outbreaks, can be informed by the basic reproductive number, R_0 (defined as the average number of newly infected cattle that arise from a single infected cattle over the course of its infectious period, in a fully susceptible cattle population). Major outbreaks may occur in naive populations only if R_0 is greater than 1, whereas transmission will certainly die out if R_0 is less than 1. Calculating the R_0 for this complex system, where the epidemiological potential of a tick depends on the life stage at which it became infected, will require the construction of a next-generation matrix (NGM) model (Diekmann et al., 1990, 2010;

Hartemink et al., 2008). One key component of the NGM, and part of the definition of R_0 for this system, is the expected number of ticks feeding on a cow. A threshold for the number of ticks per cow could be calculated, above which the R_0 would be higher than 1, and below which the transmission would stop. This threshold could give insights in how much (ongoing) treatment would be required to eradicate the pathogen, without necessarily eradicating the ticks, which may prove difficult and perhaps not even desirable as ticks have a role in (natural) ecosystems.

Whereas the rapid killing effect of synthetic acaricides can offer direct protection to treated cattle against pathogen transmission from infectious ticks, the slow-acting EPFs can only provide indirect protection to other animals (treated or untreated) by killing the infected and infectious ticks after feeding. Our model experiment indicates that EPF will indirectly reduce onward transmission of *T. parva* to other cattle by increasing the mortality rate of ticks, thus reducing the probability of surviving until the next feed and therefore transmitting the pathogen. Further, the killing of ticks by EPFs leads to reductions in the ratio of ticks to cattle and hence reduced probability of the cattle (both treated and untreated) of encountering ticks to transmit to, or acquire infection from.

Transmission of *T. parva* from *R. appendiculatus* ticks to host animal begins at 72 hours post-tick attachment (Konnai et al., 2007). Therefore, EPF can only offer direct protection to treated cattle if it kills the infectious tick before it starts transmitting the pathogen to cattle. Although attempts have been made to enhance the virulence (i.e., the average length of time it takes to kill the tick) of EPFs through genetic manipulations (St. Leger et al., 1996; St. Leger & Wang, 2010), there is a need for further studies to investigate if these genetically modified EPFs can offer direct protection to the treated cattle against ECF.

Although laboratory experiments have demonstrated the multifaceted effects of EPFs including increased mortality, increased engorgement duration, decreased engorgement weight, and reduced reproductive fitness (Nana et al., 2015), our model simulations indicate that the impact of EPF derives most strongly from the fungus' mortality effect. The relative contribution of this mortality effect depends on the proportion of cattle treated in the population and the frequency of re-treatment. In the absence of mortality effects, the model predicts an inconsequential impact from other modes of action.

Product properties needed for entomopathogenic fungi to achieve a maximum epidemiological effect

Our model framework can also be used as a tool to inform what product properties are desired to obtain a better epidemiological outcome. A significant challenge in deploying fungal formulations in the field is the rapid inactivation of the conidia, and a delay in the germination process of the surviving conidia due to environmental factors such as ultraviolet (UV) radiation, low humidity, and extreme temperatures (Braga et al., 2001; Fernandes et al., 2012; Rangel et al., 2004). Conflicting findings exist regarding the average duration of persistence of *M. anisopliae* conidia on cattle skin post-treatment, ranging from up to three weeks (Kaaya et al., 1996) to up to 72 hours after application (Polar et al., 2008). Our model result shows that even at the most conservative parameter value of 40% coverage, a longer fungal decay time from 3-10 days will result in a 30%–66% reduction in the incidence of ECF infection in the cattle population compared to 11% for a decay time of 1 day. To date, techniques that have been explored for improving the persistence of EPF on treated surfaces include encapsulation of fungal conidia (Meirelles et al., 2023), incorporation of UV protectants in the formulation (Hedimbi et al., 2008), and use of thermotolerant strains of EPF (Gava et al., 2022). This may not only protect conidia from environmentally adverse conditions but also potentially increase the effectiveness of fungal formulations in natural field conditions. Further studies would be needed to ascertain the net effect of these advanced formulations of EPF.

Implementation strategy needed to achieve a maximum epidemiological effect

Our model indicates that the projected epidemiological impact of EPF will depend on the context of their deployment strategy i.e., the treatment frequency, the population coverage level, and the efficiency of spraying. At sufficient coverage levels and treatment frequency, a large proportion of on-host *R. appendiculatus* ticks are likely to come into contact with the treatment and in doing so experience the mortality effect of EPF (Nana et al., 2015). At present, there is no standardized guideline on the optimal frequency for the application of EPFs. Previous trials have used either weekly (Murigu et al., 2016), biweekly (**Chapter 4**; Alonso-Díaz et al., 2007), or triweekly (Kaaya et al., 2011) treatment intervals. Our model shows an unqualified benefit of weekly (or shorter) treatment intervals for each coverage level. This strategy may, however, be logistically undesirable, especially for large herds in resource-poor settings. The biweekly treatment interval offers the best alternative.

Poor application techniques of EPF on cattle, such as improper dilution, insufficient fungal concentrations, and low spraying pressure can limit the probability of attached ticks

encountering the EPF. This can further be reduced due to failure to reach the hard-to-reach predilection sites for *R. appendiculatus* such as the ear pinna, tail brush, and perianal region (Walker et al., 2003). Our model demonstrates that increasing the efficiency of the spraying technique could result in a considerable improvement of epidemiological impact of EPFs, particularly in scenarios with high treatment frequencies. This finding of our model underscores the importance of adhering to best practices in biopesticide application, emphasizing the need to enhance spraying efficiency while maintaining a sufficient treatment frequency.

Model limitations and future directions

Our model framework explicitly models the life cycle of ticks by incorporating the different development stages and phases, including the infection dynamics within the tick. Further, all the effects of EPF on ticks at different phases are modeled explicitly. Nevertheless, some parameter values were poorly known, especially the mortality and development rates for some immature stages. These parameters were chosen such that a population equilibrium was achieved.

The variations between dry and rainy seasons impact the population dynamics of *R. appendiculatus* ticks, by influencing their questing activity, development rate, survival rate, and thereby overall seasonal abundance (Randolph, 1994). Additionally, seasonal variations may affect the performance of the EPFs, with the highest efficacy occurring during the rainy season and soon thereafter (Kaaya et al., 2011; Maranga et al., 2005). Future studies could extend our model framework to incorporate such seasonality aspects.

Further, the current model is implemented in the context of a domestic environment where there is no interaction between cattle and wildlife hosts. However, *T. parva* is a multi-host pathogen with a transmission cycle involving domestic cattle and/or the Cape buffalo (*Syncerus caffer*), the wildlife reservoir host (Nene et al., 2016). The presence of this wild reservoir host may reduce the effectiveness of treatment programs, by reducing the effective coverage that can be reached. Similarly, alternative hosts such as rodents, hares, and other small mammals that may feed immature tick stages (Walker et al., 2003), and so act as tick amplifiers, are not included in the model. Their presence may, too, result in a lower chance of ticks coming into contact with EPFs. Future studies could therefore extrapolate our model framework to the wildlife-livestock interfaces.

We investigated several treatment effects of EPF on ticks. The effect of the kerosene component in the Tickoff® formulation is however not explicitly modeled due to the current lack of knowledge regarding the effects of low kerosene concentration. Nevertheless, formulations containing kerosene have elicited mortality effects on ticks (**Chapter 4**; George et al., 2004), sand fleas (Enwemiwe et al., 2020) and immature stages of mosquitoes (Djouaka et al., 2007; Ojianwuna & Enwemiwe, 2022). It is therefore likely that our model results are underestimating the impact of EPF formulated in kerosene.

Despite the various assumptions, our model captures the most essential components of the biology of tick-pathogen-host interactions relevant to the transmission dynamics of ECF, and this allowed for a more realistic assessment of the epidemiological impact of EPF. The model results should however not be interpreted as predictive, but rather a demonstration of how EPF could potentially contribute to the control of ECF when deployed at a population level.

Conclusion

The model developed here can enhance our comprehension of both the direct and indirect effects of treatments with entomopathogenic fungi, which are difficult to assess in RCTs (Reiner et al., 2016). While the model is developed for EPFs and placed in the context of the pathogen *T. parva* and *R. appendiculatus* ticks, the model can be readily adapted to other tick species, tick-borne pathogens, tick control tools, and vaccination strategies. The results from our model framework are encouraging and can be used as a basis to advocate for increased financial support towards further development of this novel tool for tick control. Further cost projections are also needed to evaluate the economic impact and cost-effectiveness of different deployment strategies.

Acknowledgments

We are grateful to Mariken de Wit and You Chang of the Quantitative Veterinary Epidemiology group who offered support during the coding of the modeling script in R.

Appendix A. Supplementary data

Appendix A.1: Variables in the model

Variable	Description
E_{US}	Oviposited eggs from female tick
LQ_{US}	Untreated and susceptible questing larva
LF_{US}	Untreated and susceptible feeding larva
LD_{US}	Untreated and susceptible developing larva
LD_{TS}	Treated and susceptible developing larva
LD_{UI}	Untreated and infected developing larva
LD_{TI}	Treated and infected developing larva
NQ_{US}	Untreated and susceptible questing nymph
NQ_{UI}	Untreated and infectious questing nymph
NF_{US}	Untreated and susceptible feeding nymph
NF_{UI}	Untreated and infectious feeding nymph
ND_{US}	Untreated and susceptible developing nymph
ND_{TS}	Treated and susceptible developing nymph
ND_{UI}	Untreated and infectious (and newly infected) developing nymph
ND_{TI}	Treated and infectious (and newly infected) developing nymph
AQ_{US}	Untreated and susceptible questing adult
AQ_{UI}	Untreated and infectious questing adult
AF_{US}	Untreated and susceptible feeding adult
AF_{UI}	Untreated and infectious feeding adult
AD_{U}	Untreated and developing adults (both male and female)
AD_{T}	Treated and developing adults (both male and female)
AO_{U}	Untreated egg-laying adult females
AO_{T}	Treated egg-laying adult females
H_{US}	Untreated and susceptible cattle host
H_{UI}	Untreated and infectious cattle host
H_{UC}	Untreated and carrier cattle host
H_{TS}	Treated and susceptible cattle host
H_{TI}	Treated and infectious cattle host
H_{TC}	Treated and carrier cattle host

Appendix A.2: Model equations

$$\frac{dE_{US}}{dt} = AO_U(t)\left(\frac{E_{total}}{t_o}\right)\left(1-\frac{N_T(t)}{k_T}\right) + AO_T(t)\left(\frac{E_{total}(1-TFR)}{t_o+\tau_o}\right)\left(1-\frac{N_T(t)}{K_T}\right) - E_{US}(t)k_E - E_{US}(t)\mu_E$$

$$\frac{dLQ_{US}}{dt} = E_{US}(t)k_E - LQ_{US}(t)\mu_{LQ} - LQ_{US}(t)\alpha_L$$

$$\begin{aligned}\frac{dLF_{US}}{dt} = {} & LQ_{US}(t)\alpha_L - LF_{US}(t)\mu_{LF} \\ & - LF_{US}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}}\times(1-p_{LT})\right)\left(\frac{H_S}{H_{Total}} + \frac{H_I}{H_{Total}}\times(1-p_{LI}) + \frac{H_C}{H_{Total}}\times(1-p_{LC})\right){d_L}^{-1} \\ & - LF_{US}(t)\left(\frac{H_T}{H_{Total}}\times(p_{LT})\right)\left(\frac{H_S}{H_{Total}} + \frac{H_I}{H_{Total}}\times(1-p_{LI}) + \frac{H_C}{H_{Total}}\times(1-p_{LC})\right)(d_L+\tau_L)^{-1} \\ & - LF_{US}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}}\times(1-p_{LT})\right)\left(\frac{H_I}{H_{Total}}\times(p_{LI}) + \frac{H_C}{H_{Total}}\times(p_{LC})\right){d_L}^{-1} \\ & - LF_{US}(t)\left(\frac{H_T}{H_{Total}}\times(p_{LT})\right)\left(\frac{H_I}{H_{Total}}\times(p_{LI}) + \frac{H_C}{H_{Total}}\times(p_{LC})\right)(d_L+\tau_L)^{-1}\end{aligned}$$

$$\begin{aligned}\frac{dLD_{US}}{dt} = {} & LF_{US}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}}\times(1-p_{LT})\right)\left(\frac{H_S}{H_{Total}} + \frac{H_I}{H_{Total}}\times(1-p_{LI}) + \frac{H_C}{H_{Total}}\times(1-p_{LC})\right){d_L}^{-1} - LD_{US}(t)\mu_{LD} \\ & - LD_{US}(t)k_L\end{aligned}$$

$$\begin{aligned}\frac{dLD_{TS}}{dt} = {} & LF_{US}(t)\left(\frac{H_T}{H_{Total}}\times(p_{LT})\right)\left(\frac{H_S}{H_{Total}} + \frac{H_I}{H_{Total}}\times(1-p_{LI}) + \frac{H_C}{H_{Total}}\times(1-p_{LC})\right)(d_L+\tau_L)^{-1} - LD_{TS}(t)(\mu_{LD} \\ & + \mu_{LDF}) - LD_{TS}(t)k_L\end{aligned}$$

$$\frac{dLD_{UI}}{dt} = LF_{US}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}}\times(1-p_{LT})\right)\left(\frac{H_I}{H_{Total}}\times(p_{LI}) + \frac{H_C}{H_{Total}}\times(p_{LC})\right){d_L}^{-1} - LD_{UI}(t)\mu_{LD} - LD_{UI}(t)k_L$$

$$\frac{dLD_{TI}}{dt} = LF_{US}(t)\left(\frac{H_T}{H_{Total}}\times(p_{LT})\right)\left(\frac{H_I}{H_{Total}}\times(p_{LI}) + \frac{H_C}{H_{Total}}\times(p_{LC})\right)(d_L+\tau_L)^{-1} - LD_{TI}(t)(\mu_{LD}+\mu_{LDF}) - LD_{TI}(t)k_L$$

$$\frac{dNQ_{US}}{dt} = LD_{US}(t)k_L + LD_{TS}(t)k_L - NQ_{US}(t)\mu_{NQ} - NQ_{US}(t)\alpha_N$$

$$\frac{dNQ_{UI}}{dt} = LD_{UI}(t)k_L + LD_{TI}(t)k_L - NQ_{UI}(t)\mu_{NQ} - NQ_{UI}(t)\alpha_N$$

$$\begin{aligned}\frac{dNF_{US}}{dt} = {} & NQ_{US}(t)\alpha_N - NF_{US}(t)\mu_{NF} \\ & - NF_{US}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}}\times(1-p_{NT})\right)\left(\frac{H_S}{H_{Total}} + \frac{H_I}{H_{Total}}\times(1-p_{NI}) + \frac{H_C}{H_{Total}}\times(1-p_{NC})\right){d_N}^{-1} \\ & - NF_{US}(t)\left(\frac{H_T}{H_{Total}}\times(p_{NT})\right)\left(\frac{H_S}{H_{Total}} + \frac{H_I}{H_{Total}}\times(1-p_{NI}) + \frac{H_C}{H_{Total}}\times(1-p_{NC})\right)(d_N+\tau_N)^{-1} \\ & - NF_{US}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}}\times(1-p_{NT})\right)\left(\frac{H_I}{H_{Total}}\times(p_{NI}) + \frac{H_C}{H_{Total}}\times(p_{NC})\right){d_N}^{-1} \\ & - NF_{US}(t)\left(\frac{H_T}{H_{Total}}\times(p_{NT})\right)\left(\frac{H_I}{H_{Total}}\times(p_{NI}) + \frac{H_C}{H_{Total}}\times(p_{NC})\right)(d_N+\tau_N)^{-1}\end{aligned}$$

$$\begin{aligned}\frac{dNF_{UI}}{dt} = {} & NQ_{UI}(t)\alpha_N - NF_{UI}(t)\mu_{NF} - NF_{UI}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}}\times(1-p_{NT})\right){d_N}^{-1} \\ & - NF_{UI}(t)\left(\frac{H_T}{H_{Total}}\times(p_{NT})\right)(d_N+\tau_N)^{-1}\end{aligned}$$

$$\begin{aligned}\frac{dND_{US}}{dt} = {} & NF_{US}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}}\times(1-p_{NT})\right)\left(\frac{H_S}{H_{Total}} + \frac{H_I}{H_{Total}}\times(1-p_{NI}) + \frac{H_C}{H_{Total}}\times(1-p_{NC})\right){d_N}^{-1} \\ & - ND_{US}(t)\mu_{ND} - ND_{US}(t)k_N\end{aligned}$$

$$\frac{dND_{TS}}{dt} = NF_{US}(t)\left(\frac{H_T}{H_{Total}} \times (p_{NT})\right)\left(\frac{H_S}{H_{Total}} + \frac{H_I}{H_{Total}} \times (1 - p_{NI}) + \frac{H_C}{H_{Total}} \times (1 - p_{NC})\right)(d_N + \tau_N)^{-1} - ND_{TS}(t)(\mu_{ND} + \mu_{NDF}) - ND_{TS}(t)k_N$$

$$\frac{dND_{UI}}{dt} = NF_{US}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}} \times (1 - p_{NT})\right)\left(\frac{H_I}{H_{Total}} \times (p_{NI}) + \frac{H_C}{H_{Total}} \times (p_{NC})\right)d_N^{-1} + NF_{UI}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}} \times (1 - p_{NT})\right)d_N^{-1} - ND_{UI}(t)\mu_{ND} - ND_{UI}(t)k_N$$

$$\frac{dND_{TI}}{dt} = NF_{US}(t)\left(\frac{H_T}{H_{Total}} \times (p_{NT})\right)\left(\frac{H_I}{H_{Total}} \times (p_{NI}) + \frac{H_C}{H_{Total}} \times (p_{NC})\right)(d_N + \tau_N)^{-1} + NF_{UI}(t)\left(\frac{H_T}{H_{Total}} \times (p_{NT})\right)(d_N + \tau_N)^{-1} - ND_{TI}(t)(\mu_{ND} + \mu_{NDF}) - ND_{TI}(t)k_N$$

$$\frac{dAQ_{US}}{dt} = ND_{US}(t)k_N + ND_{TS}(t)k_N - AQ_{US}(t)\mu_{AQ} - AQ_{US}(t)\alpha_A$$

$$\frac{dAQ_{UI}}{dt} = ND_{UI}(t)k_N + ND_{TI}(t)k_N - AQ_{UI}(t)\mu_{AQ} - AQ_{UI}(t)\alpha_A$$

$$\frac{dAF_{US}}{dt} = AQ_{US}(t)\alpha_A - AF_{US}(t)\mu_{AF} - AF_{US}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}} \times (1 - p_{AT})\right)d_A^{-1} - AF_{US}(t)\left(\frac{H_T}{H_{Total}} \times (p_{AT})\right)(d_A + \tau_A)^{-1}$$

$$\frac{dAF_{UI}}{dt} = AQ_{UI}(t)\alpha_A - AF_{UI}(t)\mu_{AF} - AF_{UI}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}} \times (1 - p_{AT})\right)d_A^{-1} - AF_{UI}(t)\left(\frac{H_T}{H_{Total}} \times (p_{AT})\right)(d_A + \tau_A)^{-1}$$

$$\frac{dAD_U}{dt} = AF_{US}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}} \times (1 - p_{AT})\right)d_A^{-1} + AF_{UI}(t)\left(\frac{H_U}{H_{Total}} + \frac{H_T}{H_{Total}} \times (1 - p_{AT})\right)d_A^{-1} - AD_{U(t)}\mu_{AD} - AD_U(t)k_o^{-1}$$

$$\frac{dAD_T}{dt} = AF_{US}(t)\left(\frac{H_T}{H_{Total}} \times (p_{AT})\right)(d_A + \tau_A)^{-1} + AF_{UI}(t)\left(\frac{H_T}{H_{Total}} \times (p_{AT})\right)(d_A + \tau_A)^{-1} - AD_T(t)(\mu_{AD} + \mu_{ADF}) - AD_T(t)(k_o + k_\tau)^{-1}$$

$$\frac{dAO_U}{dt} = AD_U(t)\zeta k_o^{-1} - AO_U(t)\mu_{AO} - AO_U(t)\left(\frac{1}{t_o}\right)$$

$$\frac{dAO_T}{dt} = AD_T(t)\zeta(k_o + k_\tau)^{-1} - AO_T(t)\mu_{AO} - AO_T(t)\mu_{AOF} - AO_T(t)\left(\frac{1}{t_o + \tau_o}\right)$$

$$\frac{dH_{US}}{dt} = H_{Total}\mu_H + P_I H_I \mu_I + H_{TS}\delta - H_{US}\varphi - H_{US}\mu_H - H_{US}\alpha_N\left(\frac{NQ_{UI} + NQ_{US}}{H_{Total}}\right)\frac{NF_{UI}}{NF_{US} + NF_{UI}}P_{HN} - H_{US}\alpha_A\left(\frac{AQ_{UI} + AQ_{US}}{H_{Total}}\right)\frac{AF_{UI}}{AF_{US} + AF_{UI}}P_{HA}$$

$$\frac{dH_{TS}}{dt} = H_{US}\varphi - H_{TS}\delta - H_{TS}\mu_H - H_{TS}\alpha_N\left(\frac{NQ_{UI} + NQ_{US}}{H_{Total}}\right)\frac{NF_{UI}}{NF_{US} + NF_{UI}}P_{HN} - H_{TS}\alpha_A\left(\frac{AQ_{UI} + AQ_{US}}{H_{Total}}\right)\frac{AF_{UI}}{AF_{US} + AF_{UI}}P_{HA}$$

$$\frac{dH_{UI}}{dt} = H_{US}\alpha_N\left(\frac{NQ_{UI} + NQ_{US}}{H_{Total}}\right)\frac{NF_{UI}}{NF_{US} + NF_{UI}}P_{HN} + H_{US}\alpha_A\left(\frac{AQ_{UI} + AQ_{US}}{H_{Total}}\right)\frac{AF_{UI}}{AF_{US} + AF_{UI}}P_{HA} + H_{TI}\delta - H_{UI}\varphi - H_{UI}\sigma_H - H_{UI}\mu_H - P_I H_{UI}\mu_I$$

$$\frac{dH_{TI}}{dt} = H_{TS}\alpha_N\left(\frac{NQ_{UI} + NQ_{US}}{H_{Total}}\right)\frac{NF_{UI}}{NF_{US} + NF_{UI}}P_{HN} + H_{TS}\alpha_A\left(\frac{AQ_{UI} + AQ_{US}}{H_{Total}}\right)\frac{AF_{UI}}{AF_{US} + AF_{UI}}P_{HA} + H_{UI}\varphi - H_{TI}\delta$$
$$- H_{TI}\sigma_H - H_{TI}\mu_H - P_I H_{TI}\mu_I$$

$$\frac{dH_{UC}}{dt} = H_{UI}\sigma_H + H_{TC}\delta - H_{UC}\varphi - H_{UC}\mu_H$$

$$\frac{dH_{TC}}{dt} = H_{TI}\sigma_H + H_{UC}\varphi - H_{TC}\delta - H_{TC}\mu_H$$

6

Chapter 6

General discussion

Ticks and tick-borne diseases (TBDs) pose a considerable challenge to cattle health and productivity in sub-Saharan Africa (SSA). This is primarily due to morbidity and mortality in cattle, reduced draught power, poor weight gain (and hence poor meat production), reduced milk production, poor quality of hides, and the direct costs of treating and controlling TBDs (Gachohi et al., 2012; Kasaija et al., 2021; Kivaria, 2006). As is the case of most neglected tropical infectious diseases, one major weakness in the fight against ticks and TBDs is the lack of knowledge on the epidemiology of the diseases.

This thesis aimed to provide information on the epidemiology of tick-borne infections of cattle in coastal Kenya, and to unravel the potential of entomopathogenic fungi (EPF) for control of ticks and TBDs affecting cattle populations. This thesis presented the first report of the presence of *Ehrlichia minasensis* in ticks parasitizing cattle in Kenya (**Chapter 2**). Furthermore, this thesis reported the presence of etiological agents of anaplasmosis, babesiosis, heartwater and East Coast fever, which are important constraints to livestock production (**Chapter 3**). Malpractices in the control of ticks that may lead to acaricide failure and emergence of resistance were also identified (**Chapter 3**). In **Chapter 4**, I showed that Tickoff biopesticide (a formulation of the EPF *M. anisopliae* isolate ICIPE 7) can kill ticks yet is insufficient to result in a significant reduction in tick infestation and incidence of *A. marginale* and *T. parva* infections in cattle. However, the modeling approach showed that a meaningful epidemiological impact in cattle populations can be obtained by improving the persistence time of EPFs on treated cattle, provided that this is combined with appreciable coverage of cattle populations, treatment frequency, and an efficient spraying technique (**Chapter 5**). In this final **Chapter 6**, the findings of this thesis are discussed in a broader context of the current knowledge on epidemiology and control of ticks and TBDs in cattle. The practical implications, future research outlook, and main conclusions of the thesis are also highlighted.

Epidemiology of TBPs in cattle

The occurrence of TBPs of genera *Theileria*, *Ehrlichia*, *Babesia, Anaplasma, Rickettsia,* and *Coxiella*, has been reported in several countries in SSA, including Kenya (Gachohi et al., 2010; Haji et al., 2022; Kasaija et al., 2021; Lorusso et al., 2016; Simuunza et al., 2011). Nevertheless, the currently available information on the occurrence of TBPs remains fragmented and only available in specific geographical areas for the different countries. This thesis expanded the current knowledge of the geographical distribution of TBPs in Kenya by reporting the occurrence of diverse TBPs within the sampled ticks and cattle in coastal Kenya (**Table 1**;

Chapters 2 and 3). Of the detected pathogens, only *A. marginale, T. parva*, and *B. bigemina* are known to cause significant economic losses in cattle production systems in SSA through morbidity, mortality, and productivity losses (Gachohi et al., 2012; Kasaija et al., 2021; Kivaria, 2006). The occurrences of these detected TBPs in cattle have been associated with husbandry practices (grazing system and herd management practices), agro-ecological zone, and individual animal characteristics (breed and age) (Gachohi et al., 2012; Haji et al., 2022; Maloo et al., 2001a; Simuunza et al., 2011). However, this thesis did not find any significant association between the assessed risk factors and the occurrence of either *A. marginale* or *T. parva* in cattle (**Chapter 3**). This lack of significant risk factors for TBP presence in cattle may suggest that the study population was possibly too uniform in terms of herd management practices, and therefore it was difficult to detect clear differences in the classical risk factors; or there was insufficient transmission to detect a difference between groups.

Table 1. Summary of the epidemiology of tick-borne pathogens detected in coastal Kenya

Pathogen	Category	*Anaplasma marginale*	*Anaplasma platys*	*Theileria parva*	*Theileria velifera*	*Ehrlichia ruminantium*	*Ehrlichia minasensis*	*Babesia bigemina*	*Rickettsia africae*
Type	Bacterial	✓	✓			✓	✓		✓
	Protozoan			✓	✓			✓	
Disease	Name	Bovine anaplasmosis	Canine cyclic thrombocyto penia	East Coast fever	Non-pathogenic	Heartwater (cowdriosis)	?	Bovine babesiosis (redwater)	African tick bite fever
Importance	Veterinary	✓	✓	✓		✓	?	✓	
	Zoonotic								✓
Known tick vector(s)	Ticks distributed in Kenya	*Hyalomma rufipes, Rhipicephalus decoloratus, Rhipicephalus microplus, Rhipicephalus evertsi*	*Rhipicephalus sanguineus*	*Rhipicephalus appendiculatus*	*Amblyomma variegatum, Amblyomma lepidum*	*Amblyomma lepidum, Amblyomma variegatum*	*Rhipicephalus microplus*	*Rhipicephalus decoloratus, Rhipicephalus microplus*	*Amblyomma variegatum*
Tick life cycle	One-host	✓					✓	✓	
	Two-host	✓							
	Three-host		✓	✓	✓	✓			✓
Maintenance of pathogens in ticks	Transstadial	✓	✓	✓	✓	✓	✓	✓	✓
	Transovarial		✓					✓	✓
Transmission route (vector to host)	Biologically via tick bite	✓	✓	✓	✓	✓		✓	✓
	Mechanically by biting flies and by blood-contaminated fomites	✓							
Wildlife reservoir host in Africa				✓					

? Cattle are susceptible to experimental infection, but naturally occurring clinical disease has not been confirmed
A blank field indicates unknown.

The transmission dynamics of the TBPs detected in this thesis study is complex, as the pathogens are maintained in natural cycles involving ticks, domestic ruminants, and/or wildlife reservoirs (Allsopp, 2010; Aubry & Geale, 2011; Bock et al., 2004; Nene et al., 2016). For each TBP, there exists one or several transmission routes and competent tick species, with each tick having its unique life cycle (**Table 1**). Ticks become infected with a pathogen by feeding

on infectious cattle and the pathogen is maintained within the ticks by transstadial (horizontal) and/or transovarial (vertical) transmission. Both transstadial and transovarial transmission may occur for some pathogens such as *B. bigemina* (Bock et al., 2004), *R. africae* (Socolovschi et al., 2009), and *A. platys* (Snellgrove et al., 2020). On the other hand, cattle may become infected with a pathogen either through an infectious tick bite or mechanically by blood-contaminated fomites and biting flies. All forms of pathogen transmission to cattle occur for *A. marginale* (Aubry & Geale, 2011; Kocan et al., 2010). Cattle that recover from anaplasmosis, heartwater, babesiosis and ECF become carriers of tick-transmissible infections (Allsopp, 2010; Bock et al., 2004; Kariuki et al., 1995; Kocan et al., 2010; Olds et al., 2018). This carrier state in cattle may last for a short period (e.g., 4 to 7 weeks for *B. bigemina*) (Bock et al., 2004), a long period (e.g., at least 361 days for *E. ruminantium*) (Allsopp, 2010), or a lifetime (e.g., for *T. parva*) (Nene et al., 2016). It is particularly this carrier state that was detected in this study (**Chapter 3**).

This complexity of the epidemiology of TBPs (as summarized in **Table 1**) presents a major challenge for TBD control in cattle. Firstly, the non-tick transmission routes cannot be reached by treatment programs and this may reduce the effectiveness of tick control products. Secondly, the presence of wild animals may reduce the effectiveness of treatment programs by reducing the effective coverage that can be reached. Lastly, the presence of carrier cattle and wildlife reservoir hosts within the general population may sustain the transmission of infection to the naïve cattle by acting as a source of tick-transmitted pathogens. The currently available methods for prevention and control of TBDs in cattle include immunization of cattle, treatment of clinical cases, and tick control using chemical acaricides. However, each of these approaches has its limitations. For example, while vaccination of cattle against ECF and anaplasmosis leads to the development of immunity to subsequent infections with homologous strains, this method may induce a tick-transmissible carrier state in vaccinated cattle (Aubry & Geale, 2011; Bishop et al., 2020; Magulu et al., 2019; Oura et al., 2007), thereby jeopardizing control efforts. Additionally, vaccination against anaplasmosis does not induce protection against all field strains found in other geographical locations (Aubry & Geale, 2011). On the other hand, treatment of clinical anaplasmosis by chemotherapeutic agents does not reliably eliminate carrier infections, and hence the cattle may become a source of infection for naïve population (Curtis et al., 2021). For babesiosis, treatment with a higher dose of imidocarb will be needed to eliminate the carrier status in recovered animals but this will interfere with the development of immunity following vaccination (Bock et al., 2004). The tick control approach using

chemical acaricides is threatened by emerging resistance in ticks (Githaka et al., 2022). Considering the critical role ticks play in maintaining and transmitting pathogens, tick control approach may offer a more suitable strategy for the prevention and control of TBDs in cattle as this has the potential to reduce contact between the tick vector and the cattle host.

Effectiveness of entomopathogenic fungi for control of tick infestation and TBDs in cattle

Entomopathogenic fungi (EPF) are one of many alternative vector control tools currently being evaluated for their potential to control tick infestation on cattle. The efficacy of EPFs on ticks has been demonstrated in several laboratory bioassays against a range of tick species (Hedimbi et al., 2011; Kaaya et al., 1996; Kaaya and Hedimbi, 2012), and recently there have been efforts to translate these successes to the field. However, research in this area has had mixed results as demonstrated by the inconsistencies in the level of control. For example, while some small-scale field studies have reported considerable reduction in tick infestations on cattle (Barbieri et al., 2023; Kaaya et al., 2011; Murigu et al., 2016), other studies have reported a lack of significant efficacy when compared to the respective controls (Correia et al., 1998; Leemon et al., 2008). All these studies were, however, limited by low statistical power and did not include data on epidemiological outcomes such as the incidence of tick-borne infections in cattle populations. This is despite the fact that the ultimate goal for tick control tools is to reduce the transmission of tick-borne infections. The large-scale randomized controlled trial design used in this study has several methodological advantages over the small-scale trials (**Chapter 4**). The stratification and randomization of eligible herds by village, herd size, and tick count prevented allocation bias within the village level. Randomization done at the herd level helped to capture the variations in tick count among the different herds. Treatment was also allocated at the herd level to ensure adequate protection of all cattle in a herd, with measurements of effectiveness conducted at the individual cattle level. The sample size calculation was powered to detect variations in tick count among the treatment groups. Contrary to the earlier studies, the robust statistical approach used in this thesis allowed for the simultaneous incorporation of covariate determinants of tick counts while handling robustly any clustering of tick counts within units of observation i.e., the individual cattle within the herd.

In the field trial, Tickoff® did not show a significant reduction in tick counts, and the incidence of *A. marginale* and *T. parva* infections in cattle when compared to the excipient (control group). However, in laboratory experiments, Tickoff® biopesticide induced significant mortality in field-collected *R. appendiculatus* ticks as compared to excipient control (**Chapter 4**). The median survival time of Tickoff®- treated ticks was estimated at 13 days. In **Chapter**

5, I developed a model to investigate how this delayed mortality may affect the epidemiological impact of this EPF formulation, using ECF as a case study. Results from the model showed that treatment with EPF will not be sufficient to reduce the tick infestation level and *T. parva* infection in cattle to an appreciable level within a year, and that failure to continue with the treatment program will inevitably lead to a rebound of tick infestation and infection in later years (**Chapter 5**). Insights from this model may help to explain the lack of significant effect in the Tickoff® treatment (**Chapter 4**). Firstly, in the field trial which was conducted for only seven months, it was expected that treatment with Tickoff® could reduce the tick count on the treated cattle with a subsequent reduction in tick-borne infections. However, the model results revealed that, due to delayed mortality, effects on tick counts are expected only through the reduction of the overall tick population by EPFs. Thus farmers will not see an immediate reduction in tick counts on their cattle, which, in turn, affects the perceived effectiveness of EPF products and the likelihood at which farmers may continue to use the product.

Secondly, the field trial results suggest that EPFs do not have the potential to have epidemiological impact. However, the model results revealed that this delayed mortality effect of EPF may provide indirect protection by killing the ticks after feeding thereby preventing onward pathogen transmission to other cattle (both treated and untreated). This indirect (and thus community) effects of EPF could not be picked up effectively in the field trial as it was designed to measure only the direct effects (i.e. the effect on the treated cattle). Thus, future studies investigating the effectiveness of EPF formulations and other tick control tools could aim to design their studies in such a way that it will be possible to examine community-level effects. These studies should also ensure that high levels of population coverage and treatment frequency are reached if meaningful epidemiolocal impact is to be achieved (**Chapter 5**).

The model framework in this thesis also identified the property of EPF that could be best targeted for improvement. Notably, increasing the persistence time of EPF on treated cattle from 1 to 7 days could lead to a substantial epidemiological effect (**Chapter 5**). The low persistence of EPFs on treated surfaces is a factor that has long been an obstacle to their development for large-scale use in the field (Fernandes et al., 2012). Therefore, there is a need for increased financial support for further research and development of this novel tool before it can become a commercially viable alternative for tick control. The findings in **Chapter 5** provide information that could guide the optimal deployment upon improved performance of EPF.

Challenges of tick control studies and assessment of epidemiological impact

In this section, I describe common challenges and complexities with the design, conduct and interpretation of tick control studies, using the field trial described in **Chapter 4** as an example, and provide recommendations for improvements.

Power

There are several factors that are likely to have masked the epidemiological effect of Tickoff® in this study (**Chapter 4**), the most likely being the lack of power to discriminate a statistically significant difference in tick counts among treatment groups. The lack of power in the study was likely caused by two factors: First, the unexpected, prolonged drought in the study area might have led to a substantial decline in tick counts and thus low circulation of *A. marginale* and *T. parva* infections. As a result, this might have lowered the infection incidence in the study area needed to discriminate any reduction in either pathogen attributable to the use of Tickoff®. Secondly, the study was powered to detect differences in tick count among the treatment groups and not the epidemiological effects of the treatments on either pathogen. This likely undermined the study power required to observe a difference in the incidence of infections among the treatment groups. Future studies aiming to assess the epidemiological impact of tick control tools should ideally be carried out for more than one transmission season to avoid such problems.

Complex transmission dynamics

The epidemiological endpoint of the trial was the incidence of *A. marginale* and *T. parva* in cattle (**Chapter 4**). The level of protection against each pathogen is likely to depend on the biology and transmission dynamics of such pathogens. For example, it may be easier to control *T. parva* infection which can only be transmitted via a tick bite of one tick species than *A. marginale* which has alternative transmission routes other than by an infectious tick bite. The transmission of *A. marginale* can be effected both mechanically by biting flies or blood-contaminated fomites and biologically by approximately 20 different tick species (Kocan et al., 2010). These mechanical transmission routes are not affected by tick control treatments and therefore could potentially result in a smaller treatment effect. This may in part explain the lack of significant effect by both Tickoff® and chemical acaricides on the incidence of *A. marginale* infections in cattle (**Chapter 4**; Muraguri et al., 2003). Therefore, focusing on TBPs transmitted by a single tick species through a single transmission route could help to simplify the interpretation of trial results. When a pathogen is transmitted by various tick species and/or

through different transmission routes, then mathematical models may be helpful to clarify the results of the field trial. Models can also help to inform the implementation strategy that can potentially result in a maximum reduction in pathogen transmission. Field trials can in turn be used to verify model results.

Complex life cycle of ticks

Depending on their life cycle, ixodid ticks are classified as one-host, two-host, and three-host (Walker et al., 2003). In one-host ticks, all stages (larvae, nymphs, and adults) occur on the same individual host. Once each stage completes feeding, they remain on the host and molting occurs there. The ticks will only leave the host after complete engorgement as an adult. This type of life cycle occurs in all species of the *Boophilus* sub-genus of the *Rhipicephalus* genus. The two-host life cycle is similar but only the larvae and nymphs feed on the same individual host before nymphs drop to the ground to molt to the adult stage, which will then feed on a different host of the same or different species. This life cycle occurs in some species of the genera *Hyalomma* and *Rhipicephalus*. In three-host ticks, each stage feeds only once before dropping to the ground to molt to the next development stage. Each stage thus feeds on a different host. Ticks of the genera *Amblyomma* and some species of *Rhipicephalus* and *Hyalomma* have a three-host feeding pattern. Therefore, it may be easier to control one-host ticks which spend most of their life cycle on the host than the three-host ticks which spend less time on the host. Given the long-term life cycle of three-host ticks, and the relatively short duration they spend on host in their entire life cycle, it may take several years for the full impact of treatments to be adequately observed. Thus, targeting one-host ticks would be desirable to simplify the interpretation of trial results. If the effect of EPF and other tick control tools are to be assessed for all tick species, then the difference in the life cycle of the species present must be taken into account in the study design and in the interpretation of results. Unfortunately, in this thesis, it was logistically impossible to evaluate the effectiveness of Tickoff® per tick species (**Chapter 4**).

Environmental conditions

Abiotic factors such as temperature, saturation deficit, relative humidity, and rainfall patterns, directly influence the germination, viability, persistence, and consequently, the effectiveness of EPFs (Fernandes et al., 2012; Kaaya et al., 2011; Maranga et al., 2005). During the fieldwork (**Chapter 4**), there was an unexpected, prolonged drought that was marked by high temperatures, strong solar radiation, and low and erratic rainfall. Such adverse conditions are known to reduce the effectiveness of EPFs in field settings (Fernandes et al., 2012). It is

therefore possible that these unfavorable conditions reduced the treatment effect of Tickoff® biopesticide. Thus, this thesis study may need to be repeated in geographic areas that are predicted to be more suitable for the deployment of EPFs (Agbessenou et al., 2021; Guimapi et al., 2023).

Host biodiversity

The effect of EPFs is mostly through reducing the tick population rather than direct protection to the treated animal. Host biodiversity may complicate the analysis and interpretation of the treatment effects of EPFs. This is because immature tick stages can feed on small wild mammals such as hares, rabbits, rodents, and tortoises (Walker et al., 2003). These small mammals (acting as tick amplifiers) would not be reached by the treatment, and this may reduce the effectiveness of treatment programs. Additionally, proximity to wildlife reservoir hosts such as Cape buffalo (reservoir host of *T. parva*) can result in reduced effectiveness of the treatment programs (Walker et al., 2014). While the effect of host biodiversity was not accounted for, it cannot be ruled out that this may have obscured the treatment effect of Tickoff®. Therefore, future studies assessing the effectiveness of EPF formulations and other tick control tools should aim to account for the role of host biodiversity by estimating the tick infestation levels in wild mammals in the study area.

Future perspectives

Role of cattle movement in the spread of ticks and tick-borne pathogens in Africa

In recent decades, the geographical range of *R. microplus* tick populations in Africa has expanded dramatically (Adakal et al., 2013; Madder et al., 2011; Makenov et al., 2021; Nyabongo et al., 2021; Silatsa et al., 2019). Additionally, the spread of TBPs to new geographical areas in Africa continues to be reported, including *A. centrale, T. parva, T. velifera,* and *T. annulata* (De Deken et al., 2007; Mamman et al., 2021; Marcellino et al., 2017; Ouedraogo et al., 2021a; Ouedraogo et al., 2021ba; Silatsa et al., 2020). While these trends have been blamed on the uncontrolled movements of cattle due to trade and transhumance, no study has actually attempted to quantify the role of these cattle movements in the spread of ticks and TBPs in Africa. In Canada, a quantitative study implicated migratory birds in the range expansion of *Ixodes scapularis* ticks (Ogden et al., 2008). Elsewhere, results from mechanistic movement models have suggested that the stopover behavior of migrating birds is a key determinant of the spread of ticks, with longer stopovers more likely to reduce the total dispersal distance of ticks (Tardy et al., 2021, 2023). Therefore, future studies could aim to quantify the extent to which cattle movement impacts the epidemiology of TBDs in Africa.

This may include estimating the movement distance by migrating herds, herd movement rate (i.e., movement speed) in space and time, herd movement behavior (i.e., direction of movement, and frequency and duration of stopovers), the proportion of moving cattle that are infested with ticks, mean infestation intensity, the prevalence of infections in those ticks and cattle, and the prevalence and genetic diversity of questing ticks along the migration routes and stopover points. The movement patterns of herds can be measured by a combination of GPS (Global Positioning System) and retrospective movement surveys, and the diffusion kernels could be used to analyze these movement patterns. Insights from such analyses, combined with knowledge of spatial distribution and genetic diversity of ticks and TBPs, will be crucial for quantifying the possible impact of cattle as spreaders of ticks and TBPs.

Trial design for measuring indirect effects

To date, few trials have attempted to estimate the impact of tick control tools on the transmission of TBPs in cattle populations (**Chapter 4**; Muhanguzi et al., 2014; Muraguri et al., 2003). However, those studies were designed to measure the direct effects on the treated cattle and may not be suitable for investigating the impact of slow-acting EPFs, which only offers indirect protection at the community level and limited direct protection at the individual cattle level. Therefore, future trials assessing the epidemiological impact of EPFs and other tick control tools should consider using cluster-randomized controlled trials (CRTs) instead of the conventional individually randomized trials. The CRTs would be the ideal study design when considering measuring both direct and indirect effects of an intervention (Wilson et al., 2015; World Health Organization, 2017). Treatments should be randomly allocated to spatial clusters rather than at the herd or individual cattle level. This cluster allocation may help to reduce contamination between treatment groups that may occur if herds within the same cluster receive different treatments. This CRT study will only be useful if the treatments are administered to a large proportion of the cattle population in a cluster and under strict adherence to treatment schedule (**Chapter 5**). Without this, it would be impossible to know whether an observed lack of effect is due to low coverage, low compliance or lack of efficacy of the tick control tool. The unit of measurement will also need to be at the herd level rather than the individual cattle. The indirect effect of the interventions can then be estimated by comparing the incidence of tick-borne infection among the herds in the treatment cluster that did not receive the treatment with that in the control clusters (World Health Organization, 2017). To measure the direct and indirect effects of the tick control tool in CRT designs, consider one block under treatment and one under control (**Figure 1**).

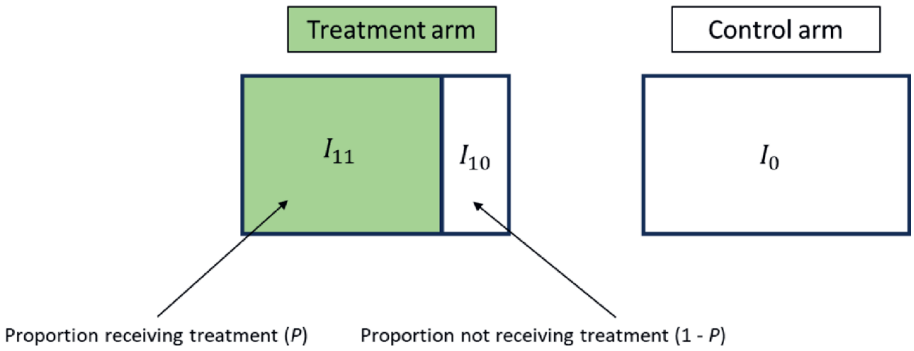

Figure 1. Diagram showing incidence rates in treatment and control arms used to determine measures of direct, indirect, total and overall effects (adopted from World Health Organization, 2017).

The direct effect of the treatment i.e., the protective efficacy (PE_{direct}) is expressed as a percentage and can be estimated by comparing the incidence of tick-borne infection in herds receiving the treatment (I_{11}) and those not receiving it (I_{10}) within the treatment clusters:

$$PE_{direct} = 1 - \frac{I_{11}}{I_{10}}$$

The indirect effect of the treatment can be estimated by comparing the incidence of tick-borne infection in herds in the treatment cluster but which did not receive the treatment (I_{10}) with the incidence in herds in the control clusters (I_0):

$$PE_{indirect} = 1 - \frac{I_{10}}{I_0}$$

The total effect of the treatment can be calculated by comparing the incidence among herds receiving the treatment in the treated clusters (I_{11}) with the incidence in the control clusters (I_0):

$$PE_{total} = 1 - \frac{I_{11}}{I_0}$$

Finally, the overall effect of the treatment, which encompasses both the direct and indirect effects of treatment, can be obtained by comparing the overall incidence in the treatment and control arms:

$$PE_{overall} = 1 - \frac{I_1}{I_0} = 1 - \frac{PI_{11} + (1-P)I_{10}}{I_0}$$

Conclusions

This thesis has shown that diverse tick species parasitize cattle in coastal Kenya. It further reports the presence and possible circulation of TBPs which are etiological agents of anaplasmosis, babesiosis, heartwater, East Coast fever, and rickettsiosis in coastal Kenya. There was no significant risk factor that could be associated with the occurrence of these tick-borne infections in the sampled cattle. The majority of cattle owners seemed to have a good understanding of tick control and treatment of TBDs, yet malpractices were still found in this study area. In the field trial, I demonstrated that Tickoff® biopesticide can kill ticks in laboratory conditions yet it was insufficient to result in a significant reduction in tick infestation and incidence of tick-borne infections in cattle in field settings. Interestingly, the model developed in this thesis revealed that EPF formulations such as Tickoff® can achieve a meaningful epidemiological impact provided that the population coverage levels, treatment frequency, and persistence time of conidia on treated cattle are sufficiently high. CRTs will be required to verify these results. Nonetheless, it will take much effort to achieve this ambition, and product advancements will be needed for this to become feasible. Overall, this thesis has provided insight into the epidemiology and control of TBDs in cattle that will be useful in the design of evidence-based control strategies.

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Summary

Ticks and tick-borne pathogens (TBPs) undermine cattle health and productivity, resulting in large economic losses in the livestock sector in sub-Saharan Africa (SSA) including Kenya. This loss is primarily due to morbidity and mortality in cattle, reduced draught power, poor weight gain, reduced milk production, and the direct costs of control. Although ticks and TBPs ticks have long been recognized for their negative effects on livestock production, little knowledge is available on their occurrence, diversity, predisposing factors, and control strategies practiced in coastal Kenya. Additionally, there has been a surge in interest in biological control of ticks on cattle using biopesticides containing entomopathogenic fungi (EPF). However, the effectiveness of this biopesticide in the context of an extensive grazing system has not been established. The main aim of this study was, therefore, to generate information on tick-borne diseases (TBDs) epidemiology and the effectiveness of EPF formulation as a control means. This information then can be used to formulate and optimize a control strategy in coastal Kenya and elsewhere.

The work presented herein firstly provides a review of the existing knowledge on the epidemiology of ticks and TBPs in SSA including Kenya, and control strategies that have been explored. It then identifies gaps present in the existing knowledge, leading to the rationale of this study (**Chapter 1**).

A first step was to provide comprehensive information on tick species infesting cattle and their associated pathogens in coastal Kenya (**Chapter 2**). A total of 3,213 adult ticks were sampled from extensively grazed zebu cattle, identified based on morphology and molecular methods and tested for the presence of bacterial and protozoan TBPs using PCR with high-resolution melting analysis and gene sequencing. The results from this study revealed that eight tick species are parasitizing cattle, including *Rhipicephalus appendiculatus*, *R. evertsi*, *R. microplus*, *R. pulchellus*, *Amblyomma gemma*, *A. variegatum*, *Hyalomma rufipes*, and *H. albiparmatum*. Ticks were infected with several pathogens of zoonotic and veterinary importance, including *Rickettsia africae*, *Ehrlichia ruminantium*, and *Theileria parva.* Additionally, *E. minasensis* and *T. velifera* were detected in ticks but whose importance is not clear. *Coxiella* sp. endosymbionts were also detected in the *Rhipicephalus* and *Amblyomma* ticks.

Chapter 3 describes a cross-sectional study that was undertaken to estimate the prevalence, identify the associated risk factors of TBPs, and document control strategies practiced in extensively managed zebu cattle. Blood samples from 1,486 cattle from 160 herds were

screened for the presence of tick-borne bacterial and protozoan pathogens using PCR with high-resolution melting analysis and sequencing. Standardized questionnaires were used to collect data on herd structure and herd management practices. Multivariable mixed-effect logistic regression was used to identify risk factors for the occurrence of *A. marginale* and *T. parva*. Chemical control was the mainstay approach for tick control on cattle, with the amidine group (mainly Triatix®) being the most frequently used acaricides. Malpractices regarding the dilution of acaricides were noted in some farms. Anaplasmosis (caused by *A. marginale*) and East coast fever (caused by *T. parva*) were perceived as the most important TBDs of cattle by cattle owners. Treatment of cases was mainly informed by clinical signs rather than laboratory diagnosis. The overall animal- and herd-level prevalence for TBPs were 24.2% (95% confidence interval (CI): 22.0-26.4%) and 75.6% (95% CI: 68.2-82.1%), respectively. Cattle were infected with *A. marginale, Babesia bigemina,* and *T. parva,* which are economically important in livestock production. Additionally, the study reported *Anaplasma* sp., *A. platys,* and *T. velifera,* whose epidemiology and association with clinical disease in cattle are still unclear. None of the assessed potential risk factors for the occurrence of either *A. marginale* or *T. parva* in cattle were statistically significant.

In Chapter 4, a randomized controlled field trial was undertaken to evaluate the effectiveness of Tickoff biopesticide (a formulation of the entomopathogenic fungus *M. anisopliae* ICPE 7) for control of tick infestations and transmission of *A. marginale* and *T. parva* infections in extensively grazed zebu cattle. The study combined laboratory experiments with an intervention study in the field. A total of 217 eligible herds comprising 1,459 zebu cattle were randomized in a 1:1:1 ratio to Tickoff®, Triatix®, or Tickoff® excipients. Tick counts and treatment administrations were performed every two weeks for seven months. A sample of ticks were collected from treated cattle and maintained in laboratory conditions while monitoring their mortality. Infections with *A. marginale* and *T. parva* were monitored every two months. Tickoff® demonstrated no significant effect on tick infestation ($p=0.869$) or infection incidence ($p>0.05$) when compared to the control group receiving excipients. In contrast, Triatix® significantly decreased tick infestation ($p<0.001$) and the incidence of *T. parva* ($p=0.042$), though not *A. marginale* ($p=0.509$), in comparison to the reference Tickoff®. Interestingly, Tickoff® demonstrated significant pathogenicity to ticks relative to excipients (hazard ratio: 8.50, 95% CI: 4.67 – 15.47) in laboratory experiments. The findings from this trial signified the need to improve the quality of Tickoff® and other EPFs before recommending their use in extensive grazing systems. Nevertheless, the unexpected prolonged

drought that occurred towards the end of the study period reduced the power of the study and hence the need for new trials. The results obtained in this thesis may therefore differ in other trials, especially those conducted under more favorable conditions. Furthermore, this work recognizes the importance of conducting diverse trials, particularly those emphasizing community effects.

In **Chapter 5**, a modeling approach was used to estimate the impact of EPFs on the transmission of ECF when deployed at population level. The model framework was further used to explore the implementation strategies and product properties needed to achieve a meaningful epidemiological impact. A deterministic model of tick–host–pathogen interactions was developed and parameterized using experimental data on Tickoff® biopesticide and data reported in the literature. The model results indicated that the greatest impact on ECF transmission is expected to result from the delayed mortality effect of EPF. This EPF-induced mortality would not only reduce the onward *T. parva* transmission to cattle (both treated and untreated) but will also cause a reduction in the tick to cattle ratio and cattle exposure to ticks, hence reducing the probability of cattle populations encountering an infectious tick. For EPF to achieve a meaningful epidemiological impact, it would require high levels of population coverage and treatment frequency in the cattle population. Substantial improvements can be obtained by improving the persistence time of EPF on treated cattle from 1 to 7 days. These model results offer support for further research and development of this biological tick control tool and provide insights into the planning of future deployment strategies upon their further development.

In the final **Chapter 6**, the main results in Chapters 2 to 5 are discussed and integrated with the current knowledge on epidemiology and control of ticks and TBPs. The relevance of knowledge generated in this thesis as well as future directions for research on the epidemiology and control of TBDs are also highlighted. This includes investigating the impact of cattle movement on TBD epidemiology in Africa by quantifying migration patterns, infection prevalence and incidence, tick infestation rates, and genetic diversity. Additionally, future trials assessing the effectiveness of EPF formulations and other tick control products should be designed to measure both direct and indirect effects. Overall, this thesis has provided insight into the epidemiology and control of TBDs in cattle that will be useful in the design of evidence-based control strategies.

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Curriculum vitae

Joseph Wang'ang'a Oundo was born on 3rd July 1991 in Kakamega County, Kenya. He completed his bachelor's degree in Biomedical science and Technology at Egerton University, Kenya, in 2015. In 2019, he obtained his MSc degree in Applied Parasitology from the University of Nairobi, Kenya. His MSc thesis research focused on the pathogens and blood-feeding patterns of questing ticks in the Maasai Mara wildlife ecosystem in Kenya. The same year he started his PhD fellowship at the International Centre of Insect Physiology and Ecology (ICIPE), a PhD registered at the Quantitative Veterinary Epidemiology group of Wageningen University and Research. His research focused on the epidemiology and control of bovine tickborne diseases which resulted in this thesis. As his Ph.D. has now come to an end, he intends to pursue a postdoctoral position focusing on the epidemiology and control of vector-borne diseases.

Publications

Peer-reviewed publications

- **Oundo, J. W.,** Kalayou, S., ten Bosch, Q., Villinger, J., Koenraadt, C. J. M., & Masiga, D. (2024). Ticks (Acari: Ixodidae) infesting cattle in coastal Kenya harbor a diverse array of tick-borne pathogens. *Ticks and Tick-Borne Diseases*, *15*(1), 102266. https://doi.org/10.1016/J.TTBDIS.2023.102266
- **Oundo, J. W.,** Masiga, D., Okal, M. N., Bron, G. M., Akutse, K. S., Subramanian, S., ten Bosch, Q., Koenraadt, C. J. M., & Kalayou, S. (2022). Evaluating the efficacy of Mazao Tickoff (*Metarhizium anisopliae* ICIPE 7) in controlling natural tick infestations on cattle in coastal Kenya: Study protocol for a randomized controlled trial. *PloS One*, *17*(8), e0272865. https://doi.org/10.1371/journal.pone.0272865
- **Oundo, J. W.,** Masiga, D., ten Bosch, Q., Villinger, J., Koenraadt, C. J. M., & Kalayou, S. (2022). Epidemiology of tick-borne pathogens of cattle and tick control practices in coastal Kenya. *Preventive Veterinary Medicine*, *209*, 105777. https://doi.org/https://doi.org/10.1016/j.prevetmed.2022.105777
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Submitted

- **Oundo, J. W.,** Kalayou, S., Gort, G., Bron, G. M., Koenraadt, C. J. M., ten Bosch, Q., & Masiga, D. A randomized controlled trial of Tickoff® (*Metarhizium anisopliae* ICIPE 7) for control of tick infestations and transmission of tick-borne infections in extensively grazed zebu cattle in coastal Kenya (*Chapter 4 in this thesis*)
- **Oundo, J. W.,** Hartemink, N., de Jong, M. C. M., Koenraadt, C. J. M., Kalayou, S., Masiga, D., & ten Bosch, Q. Biological tick control: modeling the potential impact of entomopathogenic fungi on the transmission of East Coast fever in cattle (*Chapter 5 in this thesis*)

Training and education statement

Training and Supervision Plan (TSP)

Graduate School WIAS

A. The Basic Package (1.7 ECTS)	**year**
WIAS Introduction Day	2023
Scientific Integrity	2022
Ethics and Animal Sciences	2022
B. Disciplinary Competences (12.1 ECTS)	
Advanced Statistics course Design of Experiments	2023
Decision WIAS PhD Proposal	2021
Mathematical Models of Infectious Diseases	2022
Stochastic Epidemic Models with Inference	2022
MCMC I for Infectious Diseases	2022
MCMC II for Infectious Diseases	2022
Clinic on Dynamical Approaches to Infectious Disease Data	2022
Infectious Disease Transmission Models for Decision-Makers	2022
Statistical Methods and Data Analysis with R	2022
C. Professional Competences (13.4 ECTS)	
The Essentials of Scientific Writing & Presenting	2023
Research Management & Leadership Workshop	2023
Presenting with Impact	2023
Searching and Organising Literature for PhD	2023
Efficient Writing Strategies	2023
WIAS Course The Final Touch: Writing the General Introduction & Discussion	2023
Effective behaviour in your professional surroundings	2023
Research and Data Management part 1-3	2023
The Choice: Un-box your PhD process & take charge of your performance	2023
Writing Grant Proposals	2022
Scientific Writing	2023
Project & Time Management online	2022
D. Societal Relevance (1.5 ECTS)	
WIAS course Societal impact of your research	2023
E. Presentation Skills (2.0 ECTS)	
Insights into the diversity of tick-borne pathogens in ticks and zebu cattle from coastal Kenya, World Association for the Advancement of Veterinary Parasitology (WAAVP), Chennai, India, August 20-24, 2023 Poster	2023
Unravelling the epidemiology of bovine tick-borne diseases and potential impact of a novel biopesticide, WIAS Lunch lecture, Wageningen University, 5th December 2023, Oral	2023
Education and Training Total	**30.7 ECTS**

**One ECTS credit equals a studyload of approximately 28 hours*

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