



Research paper

Infection dynamics of *Theileria annulata* over a disease season following cell line vaccination

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ABSTRACT

Tropical theileriosis is a tick-borne haemoparasitic disease of cattle caused by the protozoan parasite *Theileria annulata*. Globally, the economic impact of the disease is immense and enhanced control measures would improve livestock production in endemic regions. Immunisation with a live attenuated vaccine is an effective and widely used control method, however, the repeated use of live vaccines may have an impact on the field parasite population at a genetic level. Additionally, there has been an increasing number of reports of vaccine breakthrough cases in recent years. Thus, the present study was designed to evaluate the genetic composition of a parasite population over a disease season in a locality where live cell line vaccination is practised. A diverse range of parasite genotypes was identified and every *T. annulata* positive cattle blood sample harboured multiple parasite genotypes. An alteration in the major genotype and an increasing multiplicity of infection in individual animals was observed over the course of the disease season. Vaccination status was found not to effect within-host multiplicity of infection, while a significantly higher number of genotypes was detected in grazed cattle compared to non-grazed ones. A degree of genetic isolation was evident between parasite populations on a micro-geographic scale, which has not been reported previously for *T. annulata*. Analysis of parasite genotypes in vaccinated animals suggested only a transient effect of the vaccine genotype on the genetic diversity of the *T. annulata* population. The vaccine genotype was not detected among clones of two vaccine 'breakthrough' isolates and there is no suggestion that it was responsible for disease. The obtained data indicated that in the system studied there is no apparent risk of introducing the vaccine genotype into the population with only a transient effect on the genetic diversity of the parasite population during the disease season.

1. Introduction

Tropical theileriosis is caused by the protozoan parasite *Theileria annulata* and is transmitted by several species of Ixodid ticks of the genus *Hyalomma*. It is an economically important bovine disease, which is widespread between longitudes 30 °W–150 °E and latitudes 15 °N–60 °N. The parasite has a cattle-tick-cattle life cycle which, in the bovine

host, involves two major asexual replicative phases. The first of these takes place within leukocytes and the second within erythrocytes. After piroplasm-containing erythrocytes are ingested by a feeding tick, a sexual cycle occurs within the tick (Schein and Friedhoff, 1978). Male and female gametes are formed which fuse to form zygotes, which in turn differentiate into kinetes that migrate to the salivary glands, ultimately generating bovine-infective sporozoites (Gauer et al., 1995;

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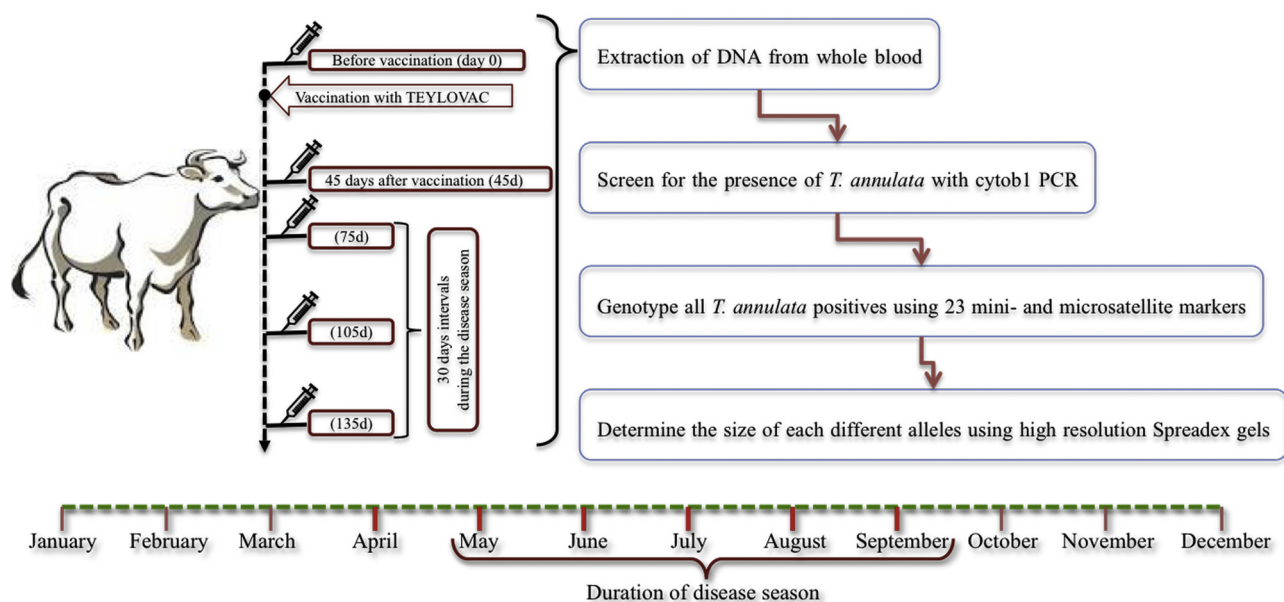


Fig. 1. A diagram showing the experimental work-flow of this study.

Schein and Friedhoff, 1978). Recent population genetic studies have provided further indirect evidence for the occurrence of a sexual phase in the parasite life cycle and these indicate that random mating is a feature of field populations of *T. annulata* (Al-Hamidhi et al., 2015; Gomes et al., 2016; Weir et al., 2007). Sexual recombination, together with a high transmission rate, is understood to play a significant role in generating *T. annulata* genetic diversity in different regions (Katzer et al., 2006; Pumpaibool et al., 2009).

Currently, prevention and control measures against tropical theileriosis comprise: (i) control of the tick vector, (ii) treatment of infected animals, (iii) use of disease-resistant breeds of cattle and (iv) vaccination with attenuated cell lines. Each of these methods, however, suffers from various drawbacks. Tick control using acaricides is unsustainable due to emerging resistance and food safety concerns (Graf et al., 2004; Khater et al., 2016). The drugs used for treatment, parvaquone and buparvaquone, have been in use since the 1980s and an increased rate of treatment failures has been observed in recent years, with buparvaquone-resistant parasites detected in Turkey (Hacilarlioglu, 2013), Tunisia (Mhadhbi et al., 2010) and Iran (Sharifiyazdi et al., 2012). A small number of indigenous cattle breeds from disease-endemic regions has been shown to possess innate disease-resistance, such as Sawihal and Kenana cattle in India (Glass et al., 2005). However, the ability of other breeds to resist or tolerate tropical theileriosis is largely unknown and a substantial amount of work is required to gauge the importance of breed resistance in combatting tropical theileriosis on a broad scale. Vaccinating cattle using attenuated *T. annulata* cell line vaccines has been shown to be an effective method for controlling disease (Darghouth et al., 1999; Seitzer and Ahmed, 2008) and this has been adopted in a number of countries, including Turkey. Attenuation of virulence of schizont-infected cell line cultures *via* long term *in vitro* passage has been associated with a reduction in the number of genotypes contained within the cell line (Darghouth et al., 1996; Pipano and Shkap, 2000). For example, the vaccine used in Turkey, based on the Pendik cell line, may comprise only a single haploid *T. annulata* genotype (Weir et al., 2011). The use of attenuated live cell line vaccines has been shown to provide solid immunity against homologous challenge and partial cross-protection against heterologous challenge (Darghouth et al., 1996; Gill et al., 1980; Hashemi-Fesharki, 1988). The protection provided by vaccination is not associated with the induction of sterile immunity and it may be hypothesised that vaccinating cattle exposed to field challenge with a single parasite genotype may perturb the parasite

population harboured by these animals. It may be further hypothesised that vaccination could result in the positive selection of genotypes which are poorly protected against, thereby altering the genetic composition of the local parasite population.

The long-term effectiveness of current vaccines in endemic regions and the influence of vaccination on field parasite populations remain poorly understood. Clinical theileriosis has been observed in vaccinated cattle during the disease season in Turkey (Aysul et al., 2008). Recent field reports indicate an increasing number of ‘breakthrough’ cases in vaccinated animals (unpublished observation) and investigating the genetic basis of this phenomenon is now essential. Previous genetic analysis of *T. annulata* field populations using a panel of molecular markers has revealed a high level of genotypic diversity with large numbers of distinct parasite genotypes detected within limited geographical areas (Al-Hamidhi et al., 2015; Gomes et al., 2016; Weir et al., 2007; Yin et al., 2018). Furthermore, a previous study has indicated that the level of multiplicity of infection is influenced by vaccination status and that cell line vaccinated cattle tend to be infected with more genotypes than unvaccinated cattle (Weir et al., 2011). The influence of vaccination on field parasite population dynamics remains unknown. In the present study, we have investigated the dynamics of parasite infection over the course of a disease season following vaccination with a commercial cell line vaccine, measuring parasite genotypic diversity in the cattle population both pre- and post-immunisation together with investigating the genetic basis of ‘breakthrough’ cases in vaccinated animals.

2. Materials and methods

2.1. Parasite material

The study was conducted at seven farms, with a history of tropical theileriosis, located within four different villages (one farm from Seferler, three from Centrum, one from Sarikoy and two from Kabalar) in the Akçaova district of Aydın province in Western Turkey where tropical theileriosis is endemic. A map illustrating the geographical location of sampling sites is shown in **Supplementary Fig. S1**. A total of 143 cattle from Seferler ($n = 13$), Centrum ($n = 55$), Sarikoy ($n = 20$) and Kabalar ($n = 55$) were screened for *T. annulata* between April and September 2013. Both calves and adult cattle vaccinated with the attenuated schizont vaccine Teylovac™ (Vetal, Turkey) were

sampled pre- and post-immunisation. Blood samples were collected in EDTA tubes immediately prior to and 45 days after vaccination and subsequently at 30-day intervals during the disease season (Fig. 1). Cattle consisted of mainly dairy types (Holstein and Brown Swiss) and Simmental together with a small number of cross-bred cattle. Details of the animals sampled in this study are summarised in **Supplementary Table S1**, including location, sex, age, breed and grazing history.

In order to evaluate the genetic diversity of parasites in non-vaccinated co-grazed carrier cattle, samples from 44 non-vaccinated cattle in Sarikoy village were also collected at Day 105 following vaccination (**Supplementary Table S1**). In addition to blood samples taken for parasite detection and genotyping, peripheral blood mononuclear (PBM) cells isolated from cattle showing signs of clinical disease were collected in heparinised tubes during the disease season and macro-schizont-infected cell lines were established *in vitro* as previously described (Brown, 1987). *In vitro* cultivated isolates and the commercially available Turkish vaccine line TEYLOVAC™ (Vetal, Turkey), derived from the Pendik cell line (Boulter and Hall, 1999), were cloned by limiting dilution of cell lines using the method described by Shiels et al., (1986).

2.2. DNA preparation and screening for the presence of *T. annulata*

EDTA blood samples collected from animals were divided into aliquots and stored at -20°C . Frozen blood samples were thawed and 300 μl of whole blood was used to extract DNA using the Promega Wizard genomic DNA extraction kit (Madison, WI, USA) following the manufacturer's instructions. Extracted DNA was resuspended in 100 μl rehydration buffer and stored at -20°C until used. All DNA samples were initially screened for the presence of *T. annulata* using the Cytob1 PCR protocol as previously described (Bilgic et al., 2010) and parasite-positive samples were then subjected to genotyping. To allow analysis of 'breakthrough' isolates, DNA was prepared from cloned cell line cultures having 2×10^6 cells/ml using the same methodology.

2.3. Mini- and micro-satellite genotyping

A total of 23 polymorphic mini- and micro-satellite markers were used to genotype parasite isolates, as described previously (Bilgic et al., 2017; Weir et al., 2007). These included 14 microsatellites (Tmsc 1, 31, 33, 37, 45, 48, 68, 75, 77, 86 and TS 5, 9, 12, 16) and nine minisatellites (MSC 8, 19, 14 and TS 6, 8, 15, 20, 25, 31) distributed across each of the four chromosomes of the *T. annulata* genome. The reagents required and thermocycler conditions selected for PCR amplification have been previously described (Bilgic et al., 2017; Weir et al., 2007), except in the present study the PCR primers were not fluorescently tagged.

2.4. High-resolution separation of amplified alleles using 'Spreadex' gels

High-resolution Spreadex gels (Elchrom Scientific™) were used to determine the size of amplicons representing the alleles of each mini- and micro-satellite marker. Under optimal conditions, these gels provide a resolution of up to 3 bp. Following electrophoresis, gels were stained in 30 mM Tris-acetate-EDTA (TAE) buffer containing 0.4 $\mu\text{g}/\text{ml}$ of Gelred (Biotium, USA) for 40 min and then destained in distilled water for 30 min. Gels were then viewed under UV light (254 nm) and photographed. VisionWorksLS (Versiyon 6.8) software (UVP EC3 Bio-Imaging system, USA) was used to determine allele size by direct comparison with the M3 marker (Elchrom Scientific), which contains over 50 DNA fragments between the sizes of 75 bp and 622 bp. The M3 marker has been specifically designed for the accurate sizing of micro- and mini-satellite alleles that may differ in size by as little as three base pairs.

2.5. Data analysis

Multi-locus genotypes (MLG) were defined for *T. annulata* positive samples collected from pre- and post-vaccinated animals, *in vitro* cultivated field isolates of *T. annulata* and clones derived from the Turkish vaccine line (Teylovac™) based on the sizes of the alleles detected at each locus. In samples where more than one allele was amplified at a particular locus, the predominant allele was identified on the basis of the intensity of Gelred staining. Alleles were binned using a model-based approach implemented in Tandem2 (Matschiner and Salzburger, 2009). For the majority of micro-satellite markers (13 of 14), the Tandem2-predicted period size corresponded to the identified period size in the reference *T. annulata* genome (Pain et al., 2005) (**Supplementary Table S2**). For seven of the nine mini-satellites markers (TS6, TS8, TS15, TS20, TS25, MSC8 and MSC14), for which allelic polymorphism may not be explained by a simple step-wise mutation model, a shorter period size was chosen (**Supplementary Table S2**). A genetic difference matrix was constructed and principle co-ordinate analysis (PCoA) was undertaken using GenAlEx v.6.5 (Peakall and Smouse, 2012). A second matrix was constructed which represented both the major and minor alleles present at each locus, thus providing a comprehensive genetic 'finger-print' representing the mixture of genotypes within each isolate. Multiplicity of infection (MOI) was estimated for each isolate by calculating the average number of alleles present per locus. To test for differences in vaccine-allele frequency between groups of animals, One-way ANOVA with Tukey HSD, Scheffé and Bonferroni and Holm *post hoc* tests of significance were utilised. To test for differences in proportions of infected/uninfected animals per group, Fisher's Exact test was used. To test for differences in the MOI between vaccinated and unvaccinated animals, Student's *t*-test was used. The probability of discovering the vaccine genotype among clones was calculated using a cumulative Binomial function.

2.6. Ethical statement

This study was approved by Adnan Menderes University Animal Experiment Ethics Committee dated 27/03/2015 in accordance with decision number B.30.2.ADÜ.0.00.00.00/050.03/2015/029.

3. Results

3.1. Screening for the presence of *T. annulata*

A sub-set of the cattle present at each of the premises was sampled before and after vaccination. The number of animals that could be sampled on each premises varied at different time-points during the disease season due to local management factors (Table 1), but where possible, the same animals were sampled on subsequent visits. A breakdown of the sampling schedule for individual animals over each time-point is provided in **Supplementary Table S3**. For comparative purposes, a number of non-vaccinated cattle, which were co-grazed with vaccinated cattle, were also sampled during the disease season. At the beginning of the disease season, immediately prior to vaccination (Day 0), only four of 143 animals (2.8%) were found to be infected with *T. annulata*. Twenty of the cattle in the study cohort had been vaccinated the previous year and except three animals (HO13, HO20 and ME05) that were parasite positive at the pre-vaccination time-point, all remaining cattle were negative. Over the course of the disease season, the parasite was detected in a large proportion of samples (Table 1) in both vaccinated and non-vaccinated animals. Sixty-five *T. annulata* positive samples were identified over the course of the study and these were collected from a total of 33 different animals. Of the four cattle that were positive at Day 0 (pre-vaccination) (Table 1), three of these (SC02, HO13 and ME05) remained positive throughout the disease season. Animal HO20 also remained positive throughout the disease season, except at Day 75. The remaining positives comprised the

Table 1
Detection of genotypes in *Theileria annulata* positive samples over time.

Group	Time-point	Total no. of sampled animals ^a	No. of <i>T. annulata</i> positives ^b	No. of samples genotyped ^c	Vaccination status
V0	Day 0	143	4 (grazed)	4	Unvaccinated (pre-vaccination sample)
V1	Day 45	124	27 (total) 15 (grazed) 12 (non-grazed)	27 15 12	Vaccinated
V2	Day 75	104	20 (total) 10 (grazed) 10 (non-grazed)	10 9 1	Vaccinated
V3	Day 105	88	7 (grazed)	7	Vaccinated
V4	Day 135	90	7 (grazed)	7	Vaccinated
NV	Day 105	44	29 (grazed)	29	Non-vaccinated

^a Total number of animals sampled at each time-point.

^b Number of samples found to be *T. annulata* positive after screening with the Cytob1 PCR.

^c Number of samples where a full or partial MLG could be determined.

samples collected from a total of 29 cattle over a number of time-points. *T. annulata* was also detected in 29 of 44 non-vaccinated cattle (Table 1) at Day 105, towards the end of the season. These cattle were located on a single farm (SC) and were co-grazed with vaccinated cattle.

3.2. Population diversity and geographical sub-structuring

Multi-locus genotypes were established for every *T. annulata* positive sample, and these are detailed in **Supplementary Table S4**. Principal co-ordinate analysis (PCoA) of multi-locus genotypes detected on six farms in Akçaova district demonstrated that a diverse range of parasite genotypes could be identified in the vaccinated cattle (Fig. 2). The two axes account for 32% of the variation in genetic diversity across the dataset. While the populations of six different farms are not entirely distinct, samples from the Sarikoy, Centrum and Kabalar study sites formed three distinguishable clusters, suggesting a degree of genetic isolation between *T. annulata* genotypes at a local level. The samples from Seferler are positioned within the Kabalar cluster. In order to investigate genetic diversity among isolates collected from individual cattle on a temporal basis, the genotypes from the three positive animals at all five time-points were identified and their centroids defined (Fig. 2). This clearly illustrates that there is a turnover of major genotype present in each individual animal. Compared to the MLGs detected in animals SC02 and ME05, those detected in HO13 showed lower diversity, illustrated by tighter clustering on the PCoA, with three of the MLGs being highly similar to one another.

3.3. Effect of grazing on genotyping

Across the study sites, the sampled animals were kept under similar management conditions (**Supplementary Table S1**). A small proportion of cattle were not grazed, and a number of these were sampled at Day 45 and Day 75 following vaccination for comparison with grazed animals. Over Day 45 and Day 75, a total of 47 *T. annulata* positive cattle were identified, 25 of which were grazed and 22 were not (Table 1). MLGs could be established from the majority of samples from grazed cattle but only a small number of non-grazed cattle. Strikingly, on Day 75, nine of ten infected grazed cattle could be genotyped, whereas only one of ten non-grazed infected animals generated a MLG. This is a statistically significant finding (Fisher's Exact Test, $P < 0.001$) and may be explained by a low-level parasite burden in non-grazed animals.

3.4. Multiplicity of infection

Multiple alleles at one or more loci were detected in every *T. annulata* positive field sample, indicating co-infection with two or more parasite genotypes. On average 1.7 alleles per locus were detected at the beginning of the disease season in grazing animals, immediately

prior to vaccination (Fig. 3A). This had dropped to 1.3 alleles per locus by Day 45, rising to 2.5 at Day 105. At Day 105, a cohort of non-vaccinated grazed cattle were sampled and found to have 2.7 alleles per locus, which was not significantly different from the vaccinated cohort (Student's *t*-test - two-tailed, $P = 0.30$). To investigate whether a rising level of MOI could be observed in individual animals at different farms, three cattle were longitudinally sampled. A rising MOI over the disease season was noted for each animal (Fig. 3B). Animal H013 maintained a higher number of parasite genotypes (> 2 alleles per locus) than the other two grazing cattle throughout the disease season.

3.5. Cloning and genotyping of the vaccine strain

Cattle were vaccinated with a commercial cell line vaccine, Teylovac, early in the disease season (Day 0). To investigate genotypic diversity in the vaccine cell line, it was cloned by limiting dilution and 58 clones were genotyped at 21 loci; the results are presented in **Supplementary Table S5**. A very low proportion of markers failed to yield a product. Identical parasite genotypes were detected in each clone across 20 loci. Surprisingly, at MSC8, two alleles were detected; an allele of 426 bp was detected in 49 clones, while one of 421 bp was detected in the remaining nine. This corresponded to a heterozygosity of 0.262 for the marker MSC8 and an overall very low heterozygosity of 0.0114.

3.6. Comparison of field samples and vaccine strain

To determine if the Teylovac genotype could be detected in animals post-vaccination, the sets of predominant multi-locus genotypes for each time-point were screened for its presence, however the Teylovac genotype was not detected in any individual. In order to determine whether the Teylovac genotype represented a minor component within the complex mixture of genotypes in field isolates, the entire dataset was analysed to determine the frequency of Teylovac-type alleles on a marker-by-marker basis at each time-point post-vaccination. The results are illustrated in Fig. 4A. A relatively high frequency of vaccine-type alleles was detected in vaccinated animals at Day 45 post-vaccination, although this was not maintained throughout the rest of the season. A statistically significant difference in vaccine-allele frequency was detected among the various groups tested (One-way ANOVA, $P < 0.001$). *Post hoc* analysis using Tukey HSD, Scheffé and Bonferroni and Holm tests indicate that the Day 45 group has a higher vaccine-allele frequency than both the Day 75 group ($P < 0.01$) and the non-vaccinated cohort ($P < 0.01$). This suggests that the vaccine genotype has just a passing effect on the genetic diversity of the cattle-associated *T. annulata* population during the disease season. To investigate this further, the frequency of vaccine alleles at each marker at the Day 45 time-point was compared to that of the non-vaccinated animals (Fig. 4B). While

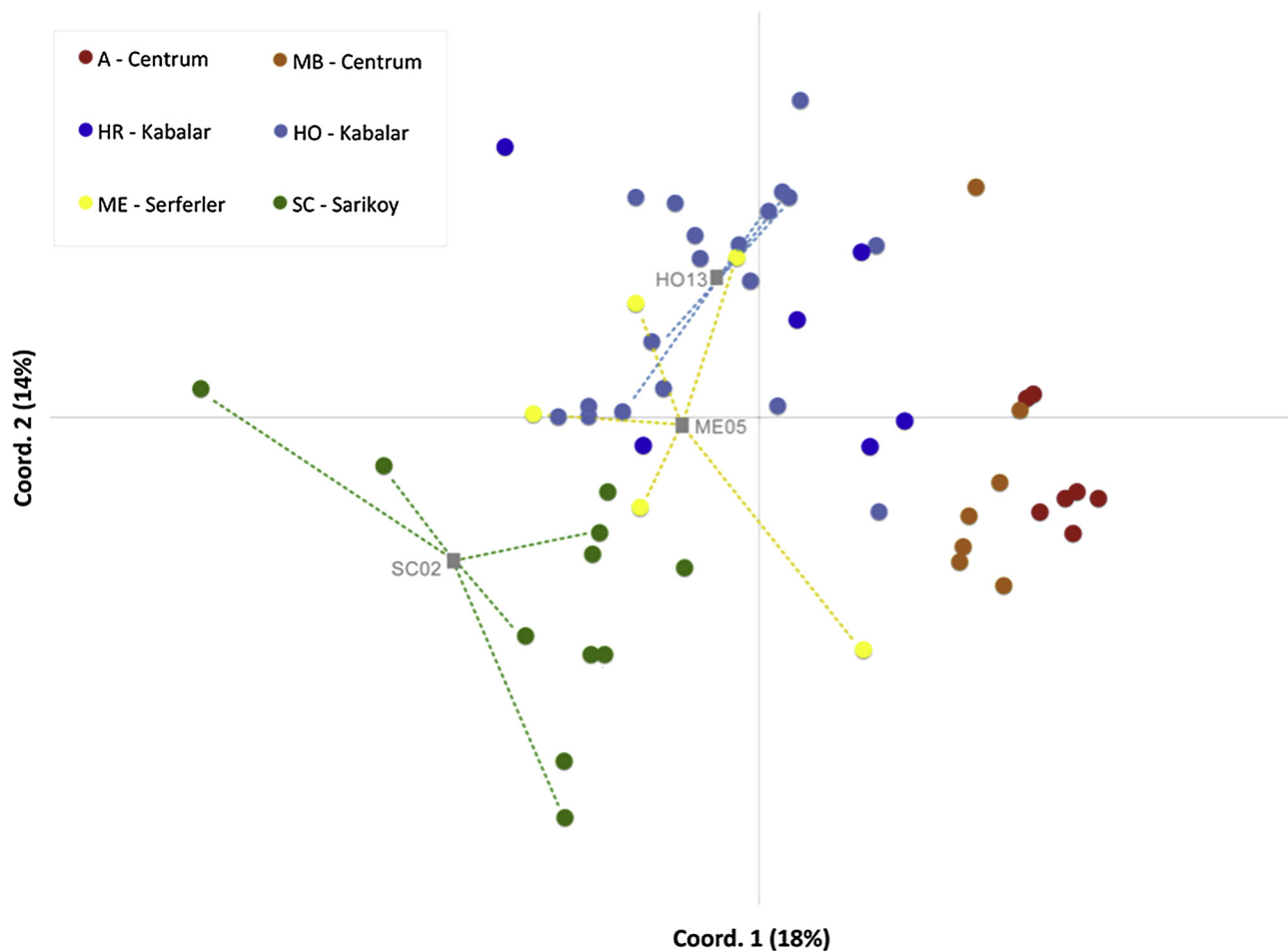


Fig. 2. Principle co-ordinate (PCoA) analysis of multi-locus genotypes detected on six farms in Akçaova district. The two principal axes generated by this analysis are presented, demonstrating a diverse range of parasite genotypes identified in the vaccinated cattle and a degree of genetic isolation between *T. annulata* genotypes at a local level. Data points representing different vaccinated animals are colour-coded to indicate their farms and the MLGs from the three positive animals at all five sampling time-points were identified and their centroids defined. The proportion of the variation in the dataset explained by each axis is indicated in parentheses.

the Day 45 group had a higher frequency at eight of the markers in the present study, at many loci the vaccine-type allele could not be detected in either group. This indicates that the enrichment for Teylovac alleles in the population is both subtle and transient.

3.7. Genotyping of 'breakthrough' isolates

In order to analyse vaccine 'breakthroughs' for the presence of the Teylovac genotype, two exemplar vaccinated animals showing clinical signs were analysed in depth, one from farm HO and one from farm SC. The diagnosis of tropical theileriosis was confirmed in both these animals clinically, microscopically and by PCR. The HO breakthrough strain was isolated from a cow showing a variety of clinical signs including pyrexia (41 °C), lymphadenopathy and a drop in milk production at Day 75 post-vaccination. The SC breakthrough strain was isolated at Day 135 post-vaccination from a calf with more severe clinical signs. Forty clones were generated from the HO isolate and 16 from the SC isolate and in neither case was the vaccine genotype detected (Table 2). From the HO isolate, up to three alleles were identified at a single locus and five different genotypes were detected overall. In contrast, the SC clones were unique with up to six alleles detected at a single locus. Thus, the SC isolate showed much greater heterozygosity than the HO isolate. With this appreciable number of clones generated, the study was sufficiently powered to detect the Teylovac genotype if present as a 25% component in a mixed genotype infection. For these two clinical cases, the data indicates that the vaccine genotype does not

form a major component of the within-animal parasite population in a breakthrough infection and it is therefore unlikely to be associated with the clinical signs displayed.

4. Discussion

Tropical theileriosis hampers livestock production in endemic countries and has a particularly strong impact on the most productive cattle breeds. It is essential to investigate and quantify the dynamics of parasite infections to allow informed development and deployment of novel control strategies (Auburn et al., 2012; Weir et al., 2007). Among the control measures available to limit losses incurred by disease, live attenuated vaccination remains an important, effective and widely used method in endemic countries (Darghouth et al., 1996; Shkap et al., 2007). However, vaccination may potentially shape genetic diversity in *T. annulata* field populations (Weir et al., 2011) with repeated vaccination exerting a selective pressure on the parasite population (Darghouth et al., 1996). Thus, diversity within a local parasite population needs to be investigated over a disease season to help evaluate the sustainability of live vaccine-based control strategies.

Characterising the parasite genotypes co-infecting a single host is a useful investigative approach which can help to improve understanding of the dynamics of infections, the effects of transmission intensity and the genetic diversity of the local parasite population (Ross et al., 2012). In the present study, the dynamics of parasite infection over the course of a disease season following vaccination with a commercial cell line

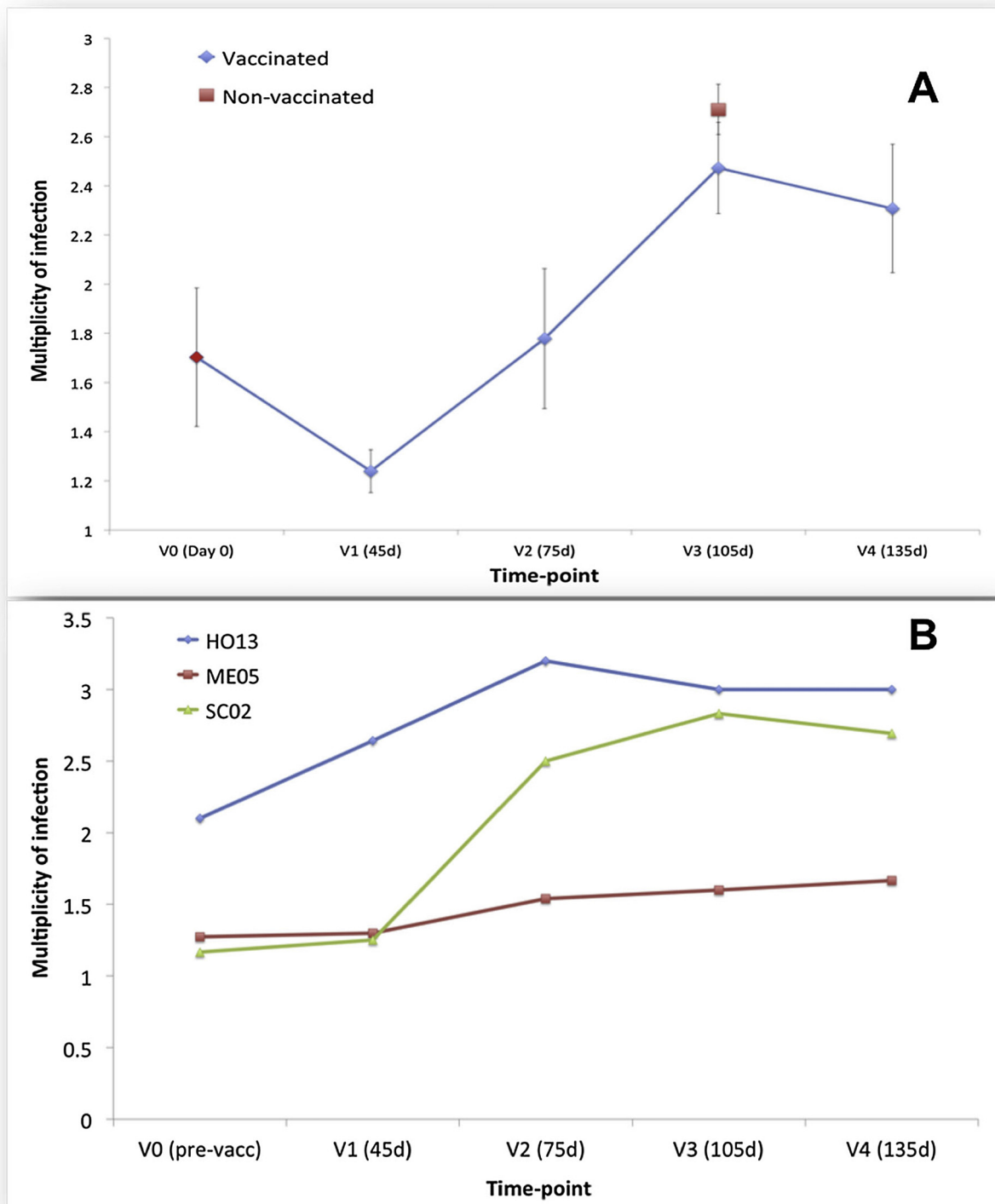


Fig. 3. Multiplicity of infection over the disease season in grazing animals (A) and in longitudinally sampled individual grazed cattle at different farms (B). These charts were generated for each isolate by calculating the average number of alleles present per locus.

vaccine, Teylovac, was investigated in samples collected during the pre- and post-immunisation periods. Each *T. annulata* positive field sample was found to harbour multiple alleles at one or more loci, indicating co-infection with two or more parasite genotypes. At the beginning of the disease season (Day 0), an average of 1.7 alleles per locus were detected, indicating the existence of co-infections in sampled animals just prior to vaccination. These animals had been vaccinated during

previous years and, as anticipated, this result provides clear evidence that vaccination does not induce sterile immunity. Following vaccination, the number of alleles detected at each locus rose to 2.4 at the end of the disease season (Day 135) and this clearly indicates an increase in the number of co-infecting parasites per animal over time (Fig. 3A). The necessity of discovering whether the MOI in vaccinated animals relates to pre- or post-vaccinal challenge has been indicated (Weir et al., 2011).

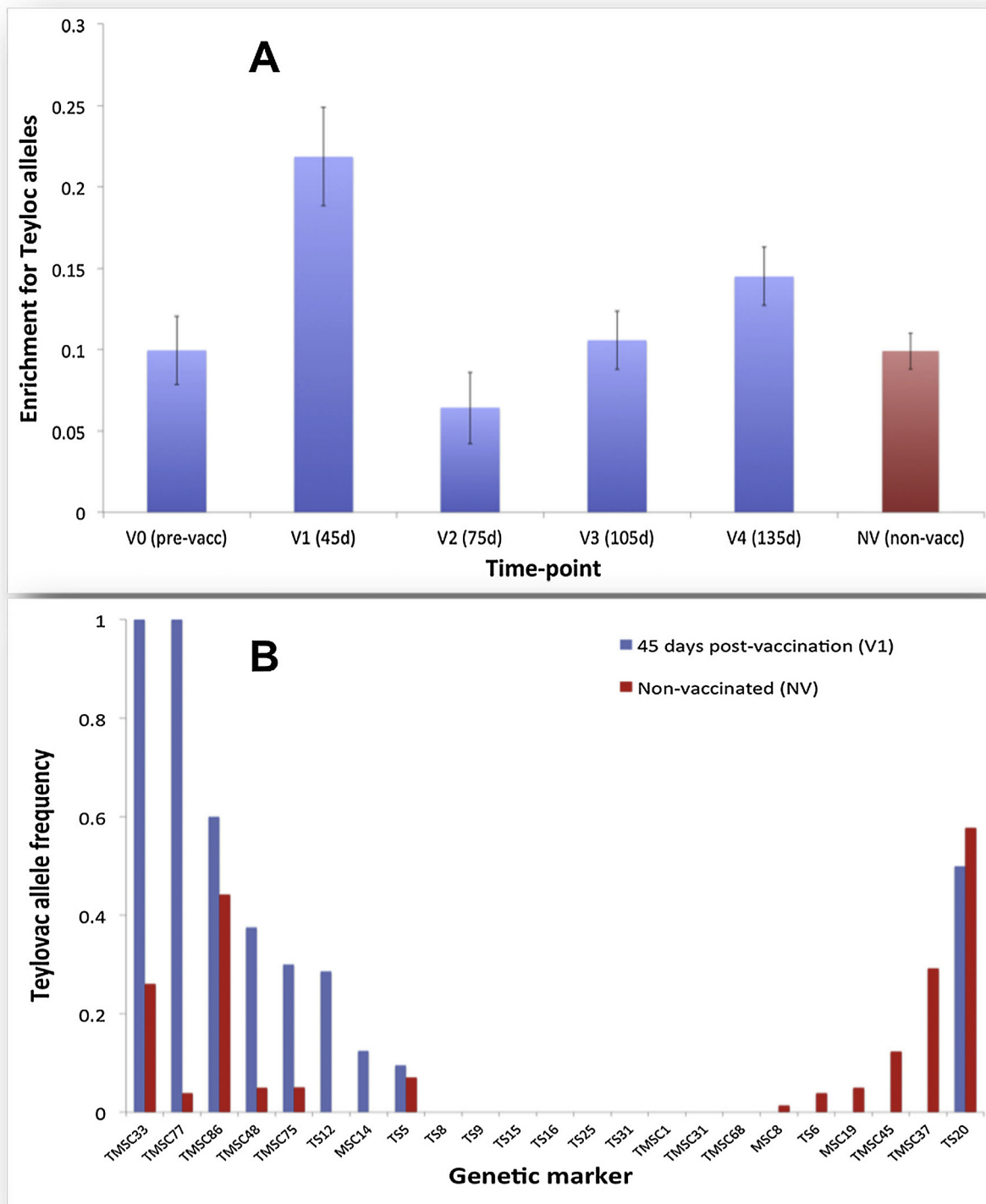


Fig. 4. Enrichment for Teylovac alleles to determine the presence of vaccine genotypes as a minor component within the complex mixture of field genotypes. (A) The frequency of Teylovac-type alleles on a marker-by-marker basis at each time-point post-vaccination. A difference in vaccine-allele frequency was detected between the groups (One-way ANOVA, $P < 0.001$). *Post hoc* analysis using Tukey HSD, Scheffé and Bonferroni and Holm tests indicate that the V1 group is higher than both the V2 ($P < 0.01$) and NV ($P < 0.01$) group. (B) Comparison of the frequency of vaccine alleles at each marker at the Day 45 time-point in vaccinated vs non-vaccinated cattle. These two histograms were directly generated from the multi-locus genotype data.

It is evident from the present study that the high MOI in vaccinated animals was primarily related to post-vaccinal challenge. Such a high level of multiple infection events is expected in areas where the disease is endemically present and multiple parasite genotypes are circulating

(Weir et al., 2011). The existence of multiple genotypes and the level of MOI in field samples may be associated with factors including pressure of tick infestations and the prevalence of *T. annulata* in the tick population (Elisa et al., 2015; Oura et al., 2005).

Table 2
Genotyping of vaccine breakthrough genotypes.

Sample	Number of clones genotyped	Number of distinct genotypes	Number of alleles per locus	Heterozygosity	Number of clones with the Teylovac genotype	Probability of vaccine genotype being present @ 25%
HO_INF	40	5	1-3	0.188	0	1.00×10^{-5}
SC_INF	16	16	1-6	0.938	0	1.00×10^{-2}

In the present study, the most abundant genotype detected in sampled animals changed over the course of the disease season and variation was observed in the level of MOI. The most parsimonious explanation for these results is that cattle are continuously challenged with a range of parasite genotypes from which cell line vaccination does not prevent from establishing a detectable infection. When analysed over the five time-points, some individuals harboured somewhat similar parasite genotypes, while in others a diverse range of genotypes was evident. For example, in animal HO13, despite the presence of a higher MOI (Fig. 3B), less genetic diversity was observed compared to MLGs detected in animals SC02 and ME05 (Fig. 2). Factors such as repeated vaccination using the same cell line may be predicted to result in an alteration in the *T. annulata* field population (Darghouth et al., 1996; Weir et al., 2011). For example, it may be hypothesised that positive selection of particular genotypes, either ‘escape’ genotypes or the vaccine strain itself, may occur and these may infect ticks. Such selection, followed by recombination events in ticks may generate multiple but closely-related genotypes. In *T. parva*, the sub-structuring of parasite populations at a micro-geographical scale has been interpreted as being the result of selection of closely-related genotypes (Asimwe et al., 2013) rather than a cross-fertilisation among diverse parasite genotypes. On a broad geographical scale, the variation in the MOI and genetic diversity among individual animals representing different sampling sites may be related to differential distribution of tick species or varying degrees of transmission intensities (Weir et al., 2011). However, the present study effectively controlled for these factors by sampling in a single locality.

Repeated challenge by ticks carrying distinct parasite genotypes may be inferred from the PCoA of multi-locus genotypes detected in animals SC02 and ME05 (Fig. 2). The disease season in Turkey is between May and September, with a peak in clinical cases occurring in mid-summer which coincides with an increase of adult *Hyalomma* tick burdens (Sayin et al., 2003). Only a single round of mating per year is expected to occur in the *Hyalomma* population. So, one may expect to see a major peak of cases in the disease season due to completion of the life cycle in a limited time period. However, during this study we observed adult *Hyalomma* ticks feeding on cattle at each sampling time-point and farmers reported the existence of feeding ticks prior to the disease season. This indicated the existence of differences in activation times of infected ticks within the locality studied.

High genotypic diversity and co-infection with multiple genotypes is a common feature of bovine *T. annulata* infection (Al-Hamidhi et al., 2015; Ben Miled et al., 1994; Weir et al., 2007, 2011; Yin et al., 2018). In the present study, a diverse range of parasite genotypes was identified in the vaccinated cattle over a disease season (Fig. 2) and non-vaccinated animals at Day 105 of the sampling period (Fig. 3B). Geographical sub-structuring at a large geographical scale has been documented between *T. annulata* populations in Tunisia, Turkey, Portugal and Oman (Al-Hamidhi et al., 2015; Gomes et al., 2016; Weir et al., 2007), but this has not been clearly demonstrated at the within-country level. In this study, a diverse range of parasite genotypes was observed in vaccinated animals on six different farms located within the same district (Fig. 2). PCoA of multi-locus genotypes showed that parasite genotypes from some farms (Sarikoy, Centrum and Kabalar) separated into clusters, to an extent, suggesting a degree of genetic isolation. This may be partly explained by re-sampling the same cattle and thus identifying the same or closely related genotypes, resulting in a

perceived degree of genetic isolation at a local level. In *T. parva*, similar sub-structuring was observed in a single farm in Uganda with multiple divergent parasite genotypes observed in six animals sampled at different time-points over a nine-month period (Asimwe et al., 2013).

In the present study, a significantly higher number of genotypes was detected in grazed cattle compared to non-grazed ones (Fisher’s Exact Test, $P < 0.001$) which may indicate a lower level of exposure to challenge in cattle managed under non-grazing conditions with semi-closed and/or indoors management systems. Strikingly, in animals kept indoors, at farm AK, *T. annulata* was not detected (Supplementary Table S1). The influence of management systems on the prevalence of *T. annulata* has previously been highlighted (Calleja-Bueno et al., 2017) and variation in the prevalence of *T. annulata* infection among different management systems has been observed (Sayin et al., 2003) with a reduced rate of tick infestation and *T. annulata* infection reported in cattle being fed indoors compared to grazed cattle (Sayin et al., 2003).

Vaccination status of cattle has previously been shown to influence MOI and a significantly higher number of *T. annulata* genotypes has been demonstrated in vaccinated animals than in non-vaccinated animals over a wide area (Weir et al., 2011). This raised the question of whether vaccinated and non-vaccinated animals co-grazing in a locality or farm show different MOI or not. To evaluate this, samples were collected from a cohort of non-vaccinated cattle co-grazing with the vaccinated ones during the peak of the disease season (at Day 105 post-vaccination) in a single farm (SC). Interestingly, and in contrast with previous data (Weir et al., 2011), no significant difference in MOI was observed among vaccinated and non-vaccinated cattle. It may be speculated that the earlier cross-sectional study was not as well-controlled as the present one and that the finding of a higher MOI in vaccinated animals was in fact due to some unidentified confounding factor, such as time of year when samples were taken, a parameter which we have shown here results in MOI variation over the disease season.

The importance of understanding the genetic composition of vaccine strains has been previously highlighted (Combrink et al., 2014; Di Giulio et al., 2009). Thus, it was decided to characterise the genetic composition of one commercial vaccine against tropical theileriosis, Teylovac, by deriving parasites clones and applying a multi-locus genotyping approach. The development and use of live, attenuated vaccines against tropical theileriosis has been well documented (Boulter and Hall, 1999; Darghouth et al., 1996; Shkap et al., 2007). Attenuation of *T. annulata* cell lines is achieved by long-term *in vitro* passage. This process results in the selection of avirulent parasite sub-populations and has been shown to be associated with a consistent reduction in parasite diversity, which may reduce to a single haploid genotype (Darghouth et al., 1996; Hall et al., 1999). In a previous study twelve years ago, the Teylovac vaccine strain was genotyped and this cell line was shown to possess a single allele at each locus, providing evidence of a single clonal genotype (Weir, 2006). In contrast, the present study demonstrates a low but detectable level of genetic polymorphism in a recent preparation of the vaccine. All 58 parasite clones tested possessed identical alleles at 20 of the 21 loci tested, resulting in a very low overall heterozygosity of 0.0114. Two closely-sized alleles (421 bp and 426 bp) were detected by marker MSC08 and a heterozygosity of 0.262 was calculated for this marker alone (Supplementary Table S5). Shifts in dominant populations have been previously reported during the attenuation and mass production of vaccine cell lines (Baravalle et al.,

2012; Mazuz et al., 2012). However, in this case, the vaccine clones are identical at all other loci and the most parsimonious explanation is that this locus has experienced a relatively recent *de novo* mutation and that the original vaccine genotype was indeed a haploid clone. Microsatellite loci are known to be fast-evolving areas within eukaryotic genomes, which mutate by ‘strand slippage’ during the DNA replication process. The rate of such mutations is relatively high and has been estimated as between 10^{-6} and 10^{-2} per generation and instability is a common feature (Schlotterer, 2000). The vaccine strain was not cloned in the previous study and therefore a minor component beneath the detection threshold may have also been present.

Alteration in transmission dynamics and population structure of the parasite population due to sustained use of live vaccines in endemic regions has previously been shown for the closely-related parasite, *T. parva* (Di Giulio et al., 2009; Oura et al., 2004) and it has been proposed that such changes should be monitored for an extended time period at vaccination locations using a standardised protocol (Di Giulio et al., 2009). However, a low risk of spreading the vaccine strains into the field (Gubbels et al., 2000) was previously reported for *T. annulata* due to the complete attenuation in *T. annulata* schizont-infected cells. This contrasts with *T. parva*, for which attenuation of virulence is incomplete (Radley et al., 1975), and an infection and treatment method is used for immunisation. In *T. annulata*, following complete attenuation, the parasite loses its ability to differentiate into the piroplasm stage, and no piroplasms were reported in blood samples of cattle vaccinated with a *T. annulata* attenuated cell line in one trial (Boulter and Hall, 1999). However, other work has demonstrated a small number of piroplasms in calves inoculated with the Tunisian attenuated vaccine (Darghouth et al., 2006) and piroplasms have also reportedly been detected in animals at one month post-vaccination (Gubbels et al., 2000; Singh et al., 2001). Thus, the risk of introducing the vaccine strain into field populations remains a poorly quantified but undeniable risk and raises the question of whether repeated vaccination in an endemic region has a detectable impact on the field parasite population. In the present study, analysis of the entire dataset on marker-by-marker basis at each time-point post-vaccination showed a significantly higher vaccine-allele frequency in vaccinated animals 45 days post-immunisation (V1) than at the subsequent time-point (V2) and compared to the non-vaccinated group ($P < 0.01$) (Fig. 4A). The frequency of the vaccine-type alleles in vaccinated animals eventually fell in line with the frequency pre-vaccination and thus there was no evidence of an ongoing enrichment of the population. Additionally, the absence of the vaccine-type allele at the majority of loci in both V1 (Day 45) group and the non-vaccinated animals (Fig. 4B) suggested only a subtle and transient impact on the genetic diversity of *T. annulata* population during the disease season. One explanation for this finding is that the Pendik vaccine strain has been attenuated for differentiation to the piroplasm and this stage is poorly represented in the red-cell population within vaccinated animals. This would represent a significant fitness cost to the vaccine genotype in the short-term, particularly in the face of competition with other genotypes continually infecting immunised cattle. Importantly, with the vaccine genotype not persisting in vaccinated animals at a detectable level in the transmissible piroplasm stage, the opportunity for ticks to become infected and for recombination and ongoing transmission to occur must be limited if not absent.

Since a proportion of animals were vaccinated in previous years, we decided to test whether the vaccine genotype could be detected in any animal before this year's vaccination. During the pre-vaccination sampling period, multi-locus genotypes corresponding to the Teylovac strain were not detected in any of the cattle vaccinated during the previous year. This result provides further evidence that the vaccine genotype does not persist in the field population. It is encouraging that no direct evidence was found for the spread of the immunising genotype into the field population following repeated use of vaccination. However, it should be appreciated that while vaccination does not induce a carrier state characterised by detectable *T. annulata* in the

circulation, the possibility that the immunising genotype is maintained in a sub-set of non-circulating leukocytes cannot be excluded.

It is known that cell line induced immunity is not wholly protective against heterologous challenge (Pipano, 1981) and this has been linked to strain specificity of the cytotoxic T lymphocyte response (Machugh et al., 2008; Seitzer and Ahmed, 2008). This partial protection against heterologous genotypes has the potential to positively select for genotypes, which are not protected against, thereby altering the genetic composition of the parasite population. Analysis of parasite-population structure is therefore essential to investigate the nature of the protection engendered by attenuated cell culture vaccines against heterologous field challenge (Gubbels et al., 2001). As such, long-term effectiveness of vaccines in endemic regions and the influence of vaccination on field parasite population dynamics remain unknown. In the present study, the effectiveness of the vaccine, Teylovac, was assessed over the course of a disease season. Considering the high-level of diversity in the local *T. annulata* population, it may be expected that the strain-specific immune response (Seitzer and Ahmed, 2008) induced in animals vaccinated with an essentially clonal cell line may be incapable of protecting cattle against challenge with heterologous genotypes of *T. annulata*. In this study, vaccine ‘breakthroughs’ (HO and SC) isolated from two vaccinated animals experiencing clinical tropical theileriosis was compared. An important consideration is whether cell line vaccine strains themselves can revert to virulence (Timms et al., 1990). The Teylovac genotype was not detected in either of the ‘breakthrough’ isolates and clinical disease was attributed to field challenge. The clinical signs observed in the SC ‘breakthrough’ case were more severe than that of HO case and the isolate from the latter showed lower heterozygosity and fewer distinct genotypes compare to the SC isolate. However, both isolates were genetically diverse and there was, therefore, no evidence of a clonal ‘breakthrough’ strain. Failure to protect may be related to factors such as pressure of infection and the immune competence of the individual cow response rather than on parasite genotype. The protection induced by attenuated cell lines depends on both antigenicity and the severity of the challenged infection (Darghouth, 2008) and previously, parasite diversity has been suggested to have a limited effect on the protective immunity induced by vaccination (Pipano and Shkap, 2000). While it is very possible that the genetic composition of the challenge strain does have a bearing on whether breakthrough is achieved, the genetic determinants of strain-specific immunity can not be resolved using a sparse multi-locus approach to genotyping, such as the one used in this study.

5. Conclusion

This study describes, for the first time, the dynamics of *T. annulata* infection and parasite genotypic diversity in animals vaccinated with a commercial cell line vaccine over the course of a disease season. The field parasite population was found to be highly diverse and an alteration in the major genotype and MOI was observed over the course of the season. Interestingly, vaccination status was shown not to affect within-host diversity in the middle of the disease season. From the results of this study, it can be concluded that there is no appreciable risk of introducing this vaccine genotype into the environment studied and there was only a subtle and transient effect on the genetic diversity of the *T. annulata* population post vaccination, with vaccine genotypes showing no evidence of persistence. However, the epidemiological situation may change from area to area with different parasite and tick populations, levels of challenge (Di Giulio et al., 2009) and vaccines used. These factors may influence the protectiveness of vaccination and it is therefore recommended that transmission dynamics and parasite population structure should be monitored in other areas using a standardised protocol (Di Giulio et al., 2009). These results and those of others clearly indicate the necessity of evaluating parameters related to host, vector and parasite including genotypic diversity of the parasite within the cattle and tick, intensity of tick burden on cattle, infection

rates of the ticks and genotypic composition of the vaccine (Auburn et al., 2012; Elisa et al., 2015) before the deployment of live, vaccine based control strategies. Using genetically and/or immunologically characterised region-specific vaccines may be suitable in regions where presumed vaccine breakthroughs are likely to happen (Di Giulio et al., 2009). As highlighted previously (Darghouth et al., 2006), there is a need for development of new control strategies such as the identification of conserved 'hidden' parasite vaccine antigens that are able to block transmission of the parasite in the tick, circumventing problems associated with highly diverse field populations.

Competing interests

None of the authors of this study have any financial or personal relationships with other people or organisations that could have inappropriately influenced this work.

Authors contributions

HBB, SB and TK designed the study and interpreted the data. HBB, AA, AHU, OK, SH and SB carried out the experimental work. WW performed the data analysis. HBB, TK and WW wrote the manuscript. All authors read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetpar.2018.11.012>.

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