Received: 16 March 2022 | Revised: 3 July 2022 | Accepted: 18 August 2022

DOI: 10.1111/tbed.14687

Transboundary and Emerging Diseases

WILEY

ORIGINAL ARTICLE

Sequence polymorphisms in a *Theileria annulata* surface protein (TaSP) known to augment the immunity induced by live attenuated cell line vaccine

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Funding information
Deutscher Akademischer Austauschdienst; DFG, Grant/Award Number: OB 490/2-1; Molecular epidemiology network for promotion and support of delivery of life vaccines against Theileria parva and Theileria annulata infection in Eastern and Northern Africa, Grant/Award Numbers: DFG-SE862/2-1, CL166/4-2

Abstract

Theileria annulata is a tick-borne protozoan causing tropical theileriosis in cattle. The use of attenuated cell line vaccines in combination with subunit vaccines has been relatively successful as a control method, as exemplified by a recent study in which immunization with a local cell line followed by booster vaccinations with recombinant *T. annulata* surface protein (TaSP) resulted in 100% protection upon field challenge in Sudan. However, these findings cannot be directly extrapolated to other countries as culture-attenuated live vaccines are generated using local strains and no systematic evaluation of genotype differences between countries has been undertaken. In this study, we sequenced the *TaSP* gene from *T. annulata* cell lines and field isolates from Tunisia (n = 28) and compared them to genotypes from Sudan (n = 25) and Morocco (n = 1; AJ316259.1). Our analyses revealed 20 unique *TaSP* genotypes in the Tunisian samples, which were all novel but similar to genotypes found in Asia. The impact of these polymorphisms on the ability of the *TaSP* antigen to boost the immunity engendered by live cell line vaccines, especially in Tunisia where studies with *TaSP* have not been conducted, remains to be examined. Interestingly, phylogenetic analyses of publicly available *TaSP* sequences resolved the sequences into two clusters with no correlation to the geographical origin of the isolates. The availability of candidate vaccines that were recently attenuated using local strains from Sudan, Tunisia, Egypt and Morocco should be exploited to generate a comprehensive catalogue of genetic variation across this regional collection of attenuated live vaccines.

KEYWORDS
subunit vaccine, TaSP, Theileria annulata

1 | INTRODUCTION

Theileria annulata infection, also known as bovine tropical theileriosis (TT), is one of the most important tick-borne diseases affecting taurine and zebu cattle breeds, as well as buffaloes in several African and Asian countries (Darghouth et al., 2011; Gharbi et al., 2020). The disease is a serious impediment to livestock production, affects animal wellbeing and causes large economic losses in terms of milk and meat production (Gharbi et al., 2006; Gharbi et al., 2015; Inci et al., 2007). *Theileria annulata* infections have a complex epidemiology involving interactions between the host, pathogen and tick vector (ticks of the genus *Hyalomma*).

Sporozoites of *T. annulata* mature in the salivary glands of infected ticks and are subsequently transmitted to cattle during tick feeding. In cattle, the sporozoites invade mononuclear cells where they develop into schizonts, which are the pathogenic stages responsible for lymphoproliferation. The microschizonts produce merozoites, which are subsequently released from the mononuclear cells and invade erythrocytes to develop into piroplasms. Ticks become infected with *T. annulata* when they feed on an infected animal. Sexual reproduction subsequently takes place in the tick midgut; kinetes penetrate the midgut and migrate to the salivary glands (Schein, 1975).

Transmission dynamics are influenced by various factors such as livestock production systems, agro-ecology and political and socio-economic environments, which makes the control of TT challenging. The control of TT is mainly based on the use of chemical acaricides for vector tick control, barn upgrading to eliminate shelter spaces for endophilic *Hyalomma* vector ticks, chemotherapy of clinical TT cases using buparvaquone and live attenuated vaccines (Darghouth, 2008; Gharbi et al., 2011; Gharbi et al., 2015). Some of these control options may however not be sustainable in the long term as exemplified by the emergence of resistant strains of *T. annulata* against buparvaquone due to a mutation in the *cytochrome b* gene (Mhadhbi et al., 2010, 2015) and resistance of *Hyalomma* ticks to acaricides (Gaur et al., 2017; Singh et al., 2014).

Vaccination represents a more sustainable approach to control. Immunization of cattle with live attenuated *Theileria annulata* schizont containing lymphoid cells results in the transfer of schizonts to lymphoid cells (mainly macrophages) of the recipient animal. Thus, vaccination results in establishment of infection in recipients. However, this infection is not lethal for the host, but it is protective against future pathogen challenge. It has been demonstrated that the immunity engendered by this live vaccination is mediated by class I MHC restricted cytotoxic CD8 T cell responses specific for parasitized cells. Some of these antigens that are the targets of this protective CD8 T cell response have been identified and are being evaluated as second-generation subunit vaccine candidates (Darghouth, 2008; Nene & Morrison, 2016).

In Tunisia, the attenuated schizont infected cell line 'Beja' (previously also reported as 'CL1') was developed and used at passage 280 to immunize cattle under field conditions (Darghouth, 2008). This culture-attenuated vaccine, which was shown not to be tick-transmissible, showed a high level of effectiveness (up to 88%) and safety in endemic situations with low to moderate infection pressure. However, attenuated cell lines from Tunisia including the Beja cell line exhibited a low efficacy against heterologous challenge (Darghouth et al., 1996a; Darghouth, 2008). As an alternative to the use of live vaccines, several subunit vaccines have been evaluated. It was for instance previously shown that immunization with recombinant sporozoite surface antigen 1(SPAG-1) or the merozoite surface protein 1(Tams-1) provided partial protection against *T. annulata* challenge (Boulter et al., 1998), with a synergetic effect conferring better protection against heterologous challenges when SPAG-1 and Tams-1 were combined. However, the protection offered by the subunit vaccine cocktail was inferior to that of the live vaccine (Gharbi et al., 2011). A synergetic effect was also shown when SPAG-1 was combined with a Tunisian attenuated cell line (Darghouth et al., 2006) in experimentally infected calves exposed to a lethal sporozoites heterologous challenge. More recently, this synergetic effect has also been demonstrated in Sudan where boosting with an experimental subunit vaccine based on the *T. annulata* surface protein (TaSP) could augment the immunity conferred by a single immunization with the attenuated Atbara *T. annulata* cell line. In that study, the immunized animals were exposed to natural high tick challenge in a *T. annulata*-endemic area in Sudan. This combination allowed for the use of a lower dose of cell line vaccine (Saaid et al., 2020).

Polymorphisms within the *TaSP* gene are yet to be analysed in field isolates from endemic regions that are geographically widely separated. It therefore remains unclear what impact *TaSP* polymorphisms are likely to have on its ability to augment the immunity conferred by the live cell line vaccines. This issue is of practical relevance as the use of *TaSP* in combination with cell line vaccines may protect against lethal challenge and reduce the required live vaccine dose, which would ultimately reduce the cost of vaccination (Gharbi et al., 2020). Furthermore, part of the difficulty in the harmonization of vaccination strategies in North Africa relates to the perception that the introduction of 'foreign' parasite genotypes, because of persistent, tick-transmissible infections ('carrier state') induced by vaccination could result in enhanced disease problems (Darghouth et al., 1996a; Darghouth, 2008; Irvin et al., 2012). This has had the implication that each country resorted to independent generation of live attenuated culture from local parasite isolates.

The major objective of the present study was to discern genotype divergence among culture-attenuated vaccines and field isolates from Tunisia and Sudan based on polymorphisms in the *TaSP* antigen gene.

2 | MATERIALS AND METHODS

2.1 | Samples collection

We used 23 archived blood samples from the biobank of the École Nationale de Médecine Vétérinaire de Sidi Thabet, Tunisia. These samples were collected between 2014 and 2018 from Holstein cattle (*Bos taurus*) from five districts located in northern and central

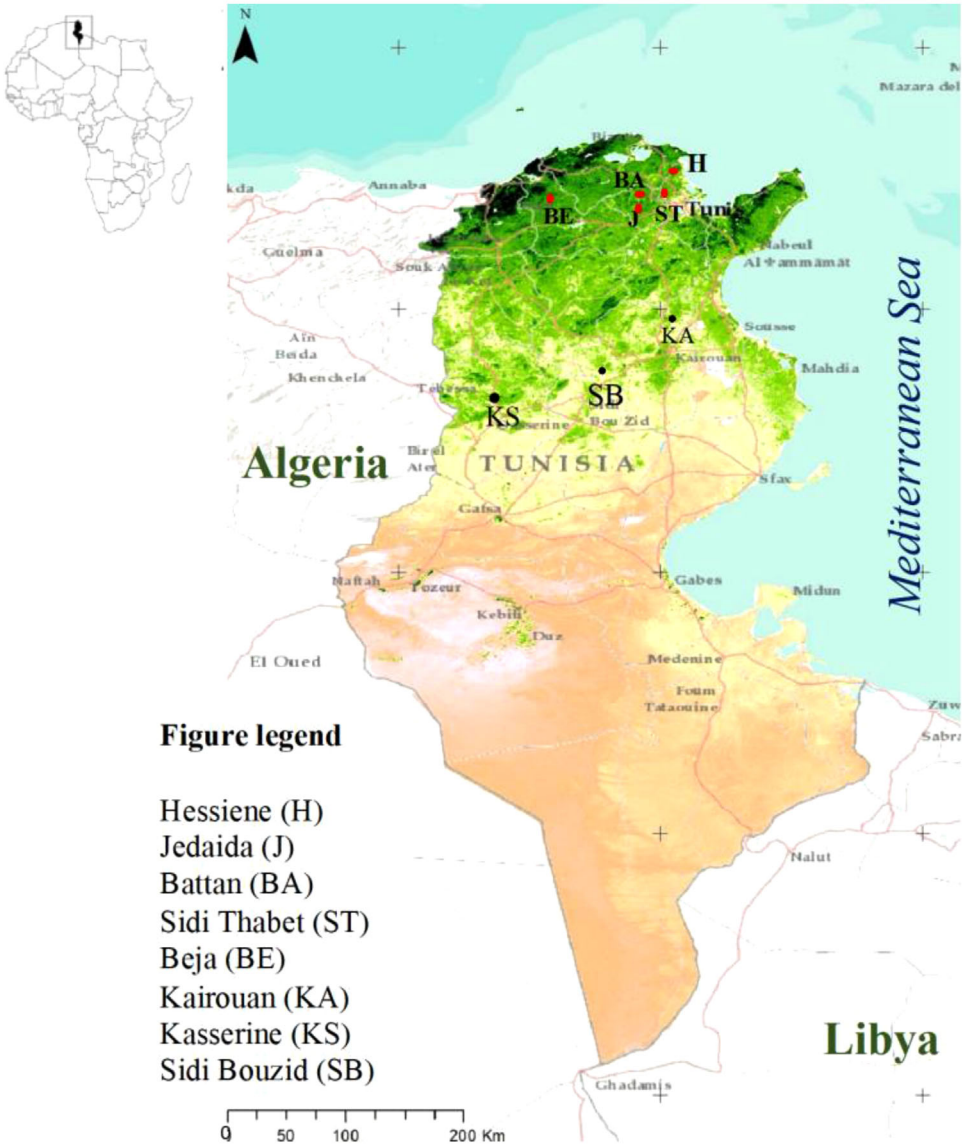

FIGURE 1 Geographic location of the sample collection sites in Tunisia

Tunisia, that have a semi-arid climate and arid climate, respectively (Figure 1).

We also analysed *TaSP* diversity in five cryopreserved *T. annulata* cell lines initially isolated from cattle and maintained by in vitro serial passage. These cell lines were Beja at passage 301(Beja P301), Battan P272, Jedaida P24, Hessiene P124 and Kairouan P9. It is important to note that although all cell lines were established for developing culture-attenuated cell line vaccines, only the Beja cell line was extensively evaluated. It showed significant efficacy in field trials and was also well-tolerated by lactating Holstein cows (Darghouth, 2008). For comparison, our analysis also included previously published *TaSP* sequences derived from the Sudanese cell lines Atbara (Nile state) ($n = 3$), Hantoub (El Gezira State) ($n = 5$) and Shambat (Khartoum state) ($n = 3$) (EU032567- EU032577) as well as 14 field isolates from Sudan (Ali et al., 2008).

2.2 | Genomic DNA /RNA extraction and amplification of the TaSP gene

The Nucleospin Genomic DNA extraction kit for tissue and cells (Macherey Nagel, Düren Germany) was used to extract genomic DNA from cell line samples, whereas the NucleoSpin Blood Mini (Macherey-Nagel) was used for DNA extraction from blood samples. RNA was extracted from three cell lines (Jedaida, Hesssiene and Beja) using the Direct-Zol RNA miniprep Plus kit (Zymo Research Europe GmbH, Freiburg, Germany). cDNA was synthesized using the iScript cDNA synthesis (Bio-Rad, California, USA) according to the manufacturer's instructions. For amplification of a fragment of 1100 bp of *TaSP* gene, forward primer *TaSP* F2-full (5′-ATGAAATTCTTCTACCTTTTTGTTCTATTTCC-3′) and reverse primer *TaSP* R2-full (5′-AATCTTCGTTAATGCGAGAAAAAGAGC-3′), based on

conserved regions of all *TaSP* sequences available in public repositories were used. PCRs were performed in a 20 μl reaction volumes and consisted of 5× Phusion HF Buffer, 0.2 mM dNTP, 0.5 μM of each primer, 0.02U/μl Phusion HSII Taq polymerase (ThermoFisher Scientific GmbH, Germany) and 2 μl of DNA. The cycling conditions were 98°C for 30 s, 35 amplification cycles of 98°C for 7 s, 62.5°C for 15 s and 72°C for 30 s, followed by a final elongation step at 72°C for 5 min. PCR products were analysed by electrophoresis in a 1.5% agarose gel. Successfully amplified products were purified using the DNA Clean & Concentrator-5 kit (Zymo Research) according to the manufacturer's instructions and bidirectionally sequenced using the Sanger sequencing method (LGC Genomics, Berlin).

2.3 | Cloning and Sanger sequencing

All amplicons were initially subjected to direct Sanger sequencing without cloning. However, based on the quality of the chromatograms, a subset of the amplicons was selected for cloning using the Strata Clone Blunt PCR Cloning Kit (Agilent Technologies, CA, USA) and recombinant plasmid vectors were transformed into StrataClone Solopack competent cells according the manufacturer's instructions. For each amplicon, six clones were selected for plasmid extraction and bidirectional Sanger sequencing using the M13 vector primers. All the contig assembly and variant calling steps were undertaken using the Geneious primesoftware (version 2021) (Kearse et al., 2012). All the unique sequences were submitted to GenBank under the accession numbers (OL675855-OL675859; OK256153-OK256164; OL985891-OL985893). The Ggplot R software was used to generate plots showing the distribution of pairwise per cent identities among the TaSP genotypes (R Core Team, 2020).

2.4 | Nucleotide substitution model selection and phylogenetic inference

Phylogenetic inference for the TaSP antigen gene sequences was based on the maximum likelihood (ML) criterion. Following removal of the intron sequences as described by Schnittger et al. (2002), maximum-likelihood tree-search algorithms were implemented in PAUP 4.0 beta version using the parameter estimates for the best-fit nucleotide substitution model (Swofford, 2002). We also calculated branch support using 1000 bootstrap replicates. In addition to the Tunisian TaSP sequences, our data set for phylogenetic inference additionally comprised of sequences from Sudan (including the one used for the vaccine AJ345067.1 obtained from Ankara), Morocco, Turkey, China, India and Mongolia. The *Theileria lestoquardi* surface protein (SP) gene, SP-5 allele sequence was used as outgroup (GenBank accession number: AY274335.1). The best-fit nucleotide substitution model for the ML analysis was selected based on the Akaike Information Criterion corrected for small sample size (AICc). The model evaluation steps were implemented in jModelTest 2.1.10 (Posada, 2008). Nucleotide substitution rate parameters, as well as parameters based on base frequencies (+F), a proportion of invariable sites (+I), and rate variation among sites (+G) resulted in a candidate set of 88 models. Likelihood calculations for all the models were performed with PhyML 3.0 linux6 (Guindon & Gascuel, 2003).

3 | RESULTS

3.1 | The *TaSP* allele pool in Tunisian *T. annulata* isolates

We amplified a fragment of 1100 bp of *T. annulata TaSP* gene from five Tunisian *T. annulata* cell lines. These included the most widely used cell line for vaccination, Beja, as well as four other cell lines: Jedaida, Hessiene, Kairouan and Battan that have only been used on a limited scale (Darghouth et al., 1996a). Insertions and deletions unique to *TaSP* sequences derived from Tunisian isolates have been previously described (Schnittger et al., 2002).

To ascertain the level of similarity of the Tunisian *TaSP* alleles to previously published *TaSP* sequences, we conducted BLASTp searches against the NCBI non redundant protein collection. As shown in Table 1, this analysis revealed that all the sequences within the Tunisian *T. annulata TaSP* allele pool are novel. It is interesting to note that the Tunisian *TaSP* variants were more similar to alleles carried by *T. annulata* isolates from Asian countries (China, India and Turkey) (Table 1).

3.2 | Phylogenetic inference from *TaSP* sequences

To build the phylogenetic tree, the *TaSP* sequences generated during this study were aligned with representative *TaSP* sequences from Morocco (Gharb strain isolated in 1985 from Kenitra, Morocco) (Ouhelli et al., 1989) and Sudan but also with sequences from China, India, and Turkey to infer their phylogenetic relatedness. Since models of nucleotide substitution can bias the accuracy of the phylogenetic inference, we utilized both the likelihood scores and estimated model parameters for Akaike Information criterion selection of the best-fit model from a candidate set. The AICc ranking of the models resulted in most support for the GTR+I+G model (Akaike weight = 0.25814). The likelihood scores, model selection criteria and numerical values for model parameters are summarized in Table S2.

Phylogenetic analysis resolved the *T. annulata TaSP* gene sequences into two evolutionary lineages including previously described Tunisian sequences in the first and the Tunisian sequences described herein and all other previously described sequences from Sudan, Turkey, Moroco, India, China, and Mongolia in the second lineage. The two lineages were supported by bootstrap values of 100% and 97.9% in this analysis (Figure 2). The clustering of the *TaSP* sequences does not seem to correspond with the geographical origin of the isolates, as extensive intermingling of sequences from China, India, Tunisia, Sudan and Morocco is observed. Maximum likelihood analysis using the CIPRES

TABLE 1 TaSP genotype information for each sample

	Query			*Blast pSearch*		
Source	Isolate/location	Accession numbers	Length(bp)	Blast hit	% Identity	Country
Cell line	Hessiene	OL985892*	913	ACP74160.1	99.46	Inner Mongolia
	Beja	OL985893*	913	CAC87575.1	95.74	Turkey: Cokoren
	Kairouan	OK256159	550	ABK58721.1	96.17	India: Parbhani
	Battan	OL675857	550	CAC87575.1	95.63	Turkey: Cokoren
	Jedaida	OL985891*	904	CAC87576.1	98.39	Turkey: Ankara
Field isolate	Kasserine BL3	OK256164	520	ACP74160.1	99.42	China
	Kasserine BL1 (CL3)	OK256162	577	CAC87576.1	98.44	Turkey: Ankara
	Kasserine BL1 (CL5)	OK256161	577	CAC87576.1	98.44	Turkey: Ankara
	Hessiene BL4 (CL2)	OK256157	514	AAP36993.1	92.40	China: Lintan region
	Hessiene BL4 (CL1)	OK256160	577	ABK58721.1	96.35	India
	Sidi Bouzid BL1	OL675855	565	CAC87575.1	95.74	Turkey: Cokoren
	Beja BL4	OL675856	559	CAC87575.1	95.70	Turkey: Cokoren
	Kasserine BL2	OK256163	547	CAC87576.1	98.35	Turkey: Ankara
	Kasserine BL1 (CL4)	OL675859	581	AAP36993.1	93.26	China: Lintan region
	Hessiene BL1	OK256158	520	AAP36993.1	93.64	China: Lintan region
	Hessiene BL4 (CL6)	OK256155	547	AAP36993.1	91.76	China: Lintan region
	Hessiene BL5 (CL2)	OK256153	577	CAC87478.1	92.19	India: Hissar
	Sidi Thabet BL1	OK256156	520	CAC87576.1	91.91	Turkey: Ankara
	Hessiene BL3	OK256154	550	AAP36993.1	91.80	China: Lintan region
	Hessiene BL6	OL675858	547	CAC87574.1	95.05	Turkey: Ankara

Note: For each individual, the sample source, accession number, DNA sequence length and the BLASTp search results are given.
*cDNA used for amplification of the *TaSP* gene.
CL, clone; BL, blood.

portal RaxML tool provided a similar topology indicating the reliability of the tree.

3.3 | A comparison of *TaSP* sequence diversity in cell line and field isolates from Tunisia and Sudan

In the present study, the Tunisian *TaSP* variants derived from the cell lines (Beja, Jedaida, Hessiene, Batan, Kairouan) had a mean pairwise sequence similarity of 90.37% (± 4.5% SD; range = 96.9%–50.1%) at the deduced amino acid level after removal of intron sequences. Four of the five TaSP protein sequences obtained from the Tunisian cell lines were unique. Beja and Battan had identical *TaSP* sequences. This is in contrast to the eleven Sudanese cell line isolates (three from Atbara, five from Hantoub and three from Shambat) where only four were unique at the amino acid level. These were Atbara (EU032567), Hantoub (EU032572) and Shambat (EU032577 and EU032575). The distribution of pairwise sequence identity of the *TaSP* variants among the Tunisian cell lines, Sudanese cell lines and between Tunisian and Sudanese cell lines is shown in Figure 3.

A detailed pairwise comparison between the five Tunisian and three Sudanese cell lines is shown in Table S1 as per cent pairwise identities. The number of base pair differences for each comparison is shown in parenthesis and a single Moroccan cell line (CAC87477.1) (Schnittger et al., 2002) is also included in the analysis.

An alignment of all the *TaSP* sequences from all the Tunisian and Sudanese cell lines is shown in Figure 4.

Besides the cell lines, we also typed field isolates from five locations in northern and central Tunisia and assessed the degree of genetic divergence in the *TaSP* genes among these field isolates. Overall, this analysis identified 20 *TaSP* variants across the 28 samples sequenced. Among the unique *TaSP* variants from the field isolates, the mean amino acid pairwise sequence similarity was 93%. We found this level of genetic divergence of the *TaSP* gene in the field isolates of *T. annulata* in Tunisia to be more conserved relative to field isolates from Sudan from a previous study (Ali et al., 2008) where the mean pairwise sequence similarity at the deduced protein level was 85%. The distribution of pairwise identities among the field isolates is shown in Figure 5 and a comparison of the sequences from the Tunisian cell lines and field isolates to each other and to the attenuated subunit vaccine sequence is provided in Table S3. An alignment of the Tunisian and Sudanese *TaSP* sequences derived from the field isolates is provided as Figure S1.

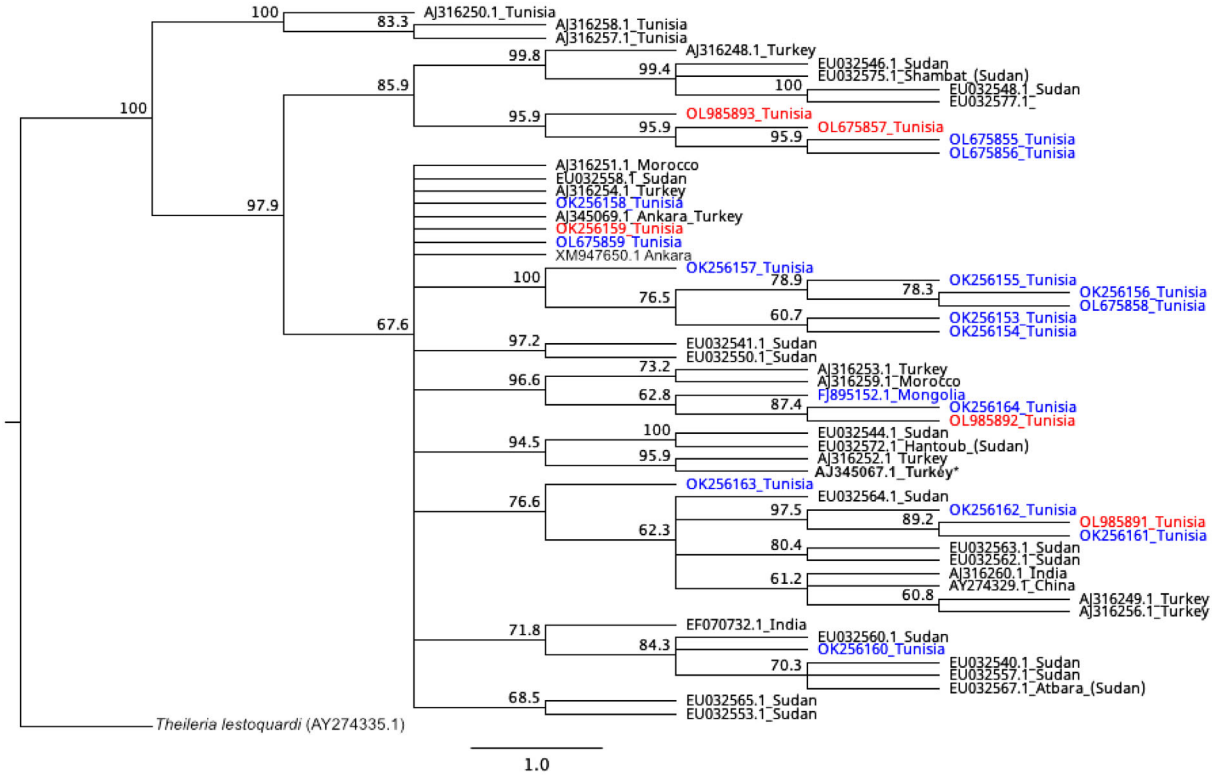

FIGURE 2 Maximum likelihood tree depicting the phylogenetic relationships of *T. annulata TaSP* sequences. The data set includes (i) 26 *TaSP* sequences from Sidi Bouzid, Sidi Thabet, Kairouan, Hessiene, Beja, Battan and Kasserine regions of Tunisia investigated in the present study (field isolates in blue colour, cell lines in red colour, sequence of the TaSP subunit vaccine used in Sudan in Bold and marked with star), (ii) sequences representing *T. annulata TaSP* sequences previously described in Sudan, Morocco, China, India and Turkey. Numbers on the branches refer to bootstrap values (percentages) based on 1000 replicates and the branch lengths are drawn to scale according to genetic difference

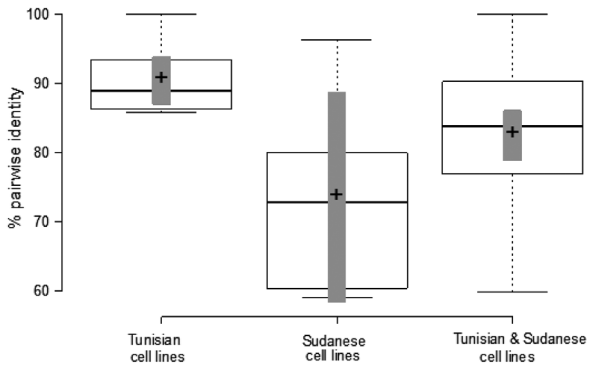

FIGURE 3 Comparison of pairwise identities of *TaSP* protein sequences both within and between Tunisian and Sudanese cell lines. Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; crosses represent sample means

4 | DISCUSSION

Tropical theileriosis constrains cattle health and production in Tunisia and other countries in the Mediterranean, Middle East and Asia, leading to substantial economic losses (Ayadi et al., 2016; Darghouth et al., 1996b; Gharbi et al., 2006, 2015; Latif, 1994). Numerous control measures have been implemented, targeting either the tick vector or the protozoan itself (Darghouth et al., 1999; Gharbi et al., 2011), but live attenuated vaccines remain the most sustainable control option across the different transmission patterns observed in Africa (Gharbi et al., 2011). It was demonstrated in earlier studies that the protective efficacy of the live vaccines can be enhanced by subunit vaccine candidates TamS-1, TamS-2 and SPAG-1 (Darghouth et al., 2006; d'Oliveira et al., 1997; Katzer et al., 1994, 1998). Subsequent studies aimed at building on these initial findings culminated in the recent demonstration in Sudan of a synergetic effect when *TaSP* was combined with the attenuated Atbara cell line, with 100% recovery from clinical disease of the immunized group despite the low efficacy of cell line alone against field challenge (Saaid et al., 2020).

The *T. annulata* surface protein, *TaSP*, is a single-copy gene expressed in both sporozoite and schizont stages of the parasite (Seitzer et al., 2007). *TaSP* is present as a phosphorylated protein in the infected leucocytes and interferes with the host signalling pathways as it is a target for the host cell kinase (Mackiewicz et al., 2020). It also interacts with host microtubulin during cell mitosis (Seitzer et al., 2010).

The use of recombinant proteins to augment the protective effects of live vaccines against theileriosis is of interest in light of the findings of the experimental trials in Sudan using the *TaSP* antigen (Saaid et al., 2020). However, the extent of polymorphisms in the *TaSP*

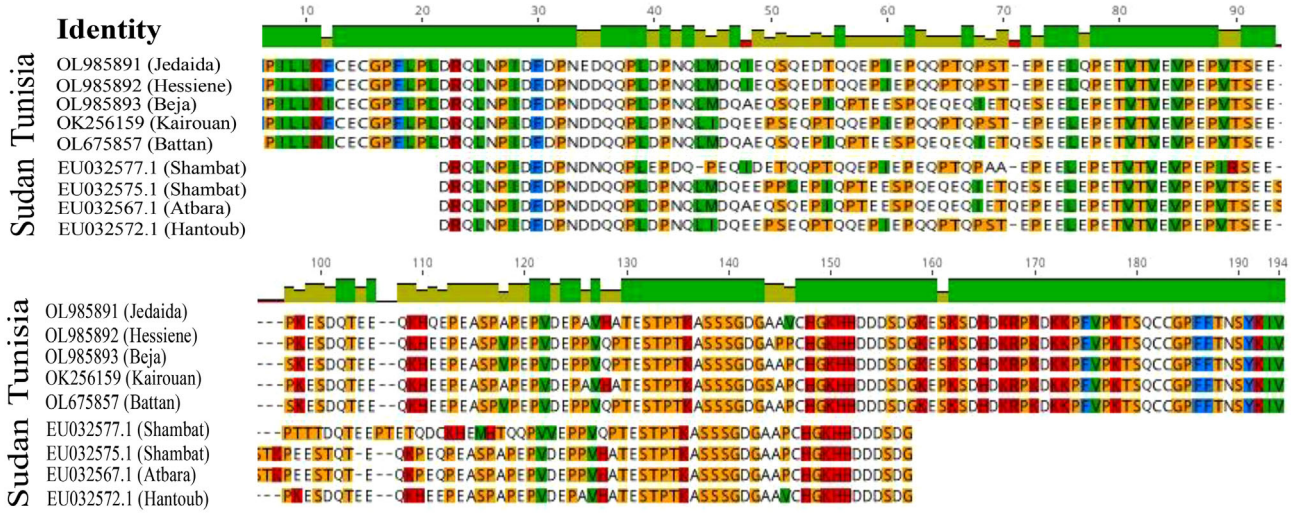

FIGURE 4 An alignment showing the *TaSP* genotypes present in the Tunisian and Sudanese cell lines

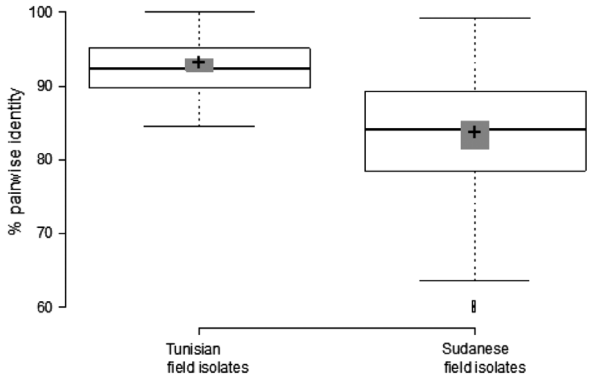

FIGURE 5 Comparison of pairwise identities of *TaSP* protein sequences both within and between Tunisian and Sudanese field isolates. Centre lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; crosses represent sample means

antigen gene and the possible impact of such polymorphisms on its ability to boost the immunity engendered by the live cell line vaccines, especially in Tunisia where no such TaSP boosting studies have been conducted, remains poorly understood.

In the present study, we provide evidence for limited variation in the *TaSP* antigen gene in Tunisia. All available cell lines from Tunisia were included in an analysis of *TaSP* antigen gene diversity. A comparison of the *TaSP* sequences amplified from cDNA and from genomic DNA templates revealed the presence of intronic sequences that were consistent with those described by Schnittger et al. (2002). The overall analysis revealed a mean per cent pairwise identity at deduced amino acid level of 90.3% (± 4.5% SD). Except Beja and Battan, which were 100% similar, the highest similarity among the four unique Tunisian cell lines was between Jedaida and Hessiene, which were over 95% identical. The most variation was between Kairouan and Battan (85.8%), but even these differed by 26 bp. This overall conservation in the *TaSP* gene present in the Tunisian cell lines is in contrast to Sudan where a high overall variation is observed between the Atbara (EU032567), Hantoub (EU032572) and Shambat (EU032577 and EU032575) cell lines with a lower mean per cent pairwise identity of 67.4 % (± 7.2 % SD). This analysis showed that Hantoub and Shambat are the least similar, differing by up to 52 bp. Similarly, Atbara and Hantoub differ by 50 bp in the region of the *TaSP* gene that was sequenced. The diversity in Sudanese sequences could be related to the abundance of the tick vector *Hyalomma anatolicum*, the time of sample collection, the transmission intensity and the infection pressure, which is higher in Sudan (Roy et al., 2021). In Sudan, there are more genetically diverse autochthonous cattle population compared to Tunisia where Holstein cattle are dominating. Also, transboundary movement of carrier cattle and its effect on the epidemiology of *T. annulata* in Sudan could be partly responsible (Salih et al., 2021).

The overall conservation in the *TaSP* antigen gene in Tunisia relative to Sudan is also apparent when field isolates are compared. In particular, among the unique *TaSP* variants from the field isolates in Tunisia, the mean amino acid pairwise sequence similarity was 93% compared to 85% in the field isolates from Sudan. When we compared Tunisian cell lines to the single Moroccan cell line (CAC87477.1), the overall mean pairwise per cent identity was 92.7% (± 4% SD). While it is not yet possible to be certain that the differences in the extent of *TaSP* polymorphisms between the Tunisian and Sudanese cell lines will impact the ability of *TaSP* to augment the protective effects of live vaccines, it seems likely that such variation would be less important in Tunisia relative to Sudan where *TaSP* boosting has been demonstrated and that the Sudanese *TaSP* antigen gene field isolates appear less conserved from our study.

It is important to emphasize that the *TaSP* polymorphisms data at best only offer a glimpse into the genotype divergence among culture-attenuated vaccine candidates from North Africa. A comprehensive catalogue of genetic variation across this regional collection of attenuated live vaccines and field isolates will have to await comparative molecular epidemiology and population genetics studies based on polymorphic variable number tandem repeat (VNTR) sequences,

nucleotide sequencing of genes that code for the targets of protective responses and whole genome sequencing.

Findings from such studies will be relevant in addressing the practical issue of how parasite population structures are likely to be altered in case of cross-border vaccination strategies or anthropogenic movement of vaccinated cattle. This is because country-based, as opposed to transboundary, live vaccination strategies against *T. annulata* have been favoured due to the perception that the introduction of 'foreign' parasite genotypes could result in novel genotypes that may cause enhanced disease problems (Darghouth et al., 1996a; Darghouth, 2008). This concern has been addressed for the *T. parva* live vaccine where the original trivalent Muguga cocktail version of infection and treatment method (ITM) which comprises three stocks (Muguga, Serengeti-transformed and Kiambu) has been extensively deployed in Tanzania, Kenya and parts of Uganda (Bishop et al., 2020; Oura et al., 2007). A recent study monitored ITM vaccinated animals for induction and longevity of carrier state. Although evidence exists that unvaccinated cattle shared sequences with those found in the vaccine, no evidence was found that vaccination stock replaces local ones or severely impacts parasite population genetic structure after deployment (Gwakisa et al., 2020). Here it is important to note that the genetic make-up of parasites that comprise the ITM cocktail have been characterized (Hemmink et al., 2016; Norling et al., 2015) and no comparable data exists for *T. annulata* as studies aimed at discerning genotype divergence among attenuated strains used as local vaccines have not been performed.

Even if alteration of parasite population structure in case of a transboundary vaccine deployment were demonstrated, it would only become a serious concern if development of the tick-transmissible infections after vaccine deployment occurs. Unlike *T. parva*, where the carrier state has been demonstrated repeatedly (Nene & Morrison, 2016), a few studies have shown that the carrier state is not a typical outcome following *T. annulata* vaccine use (Darghouth, 2008; Gubbels et al., 2000; Gharbi et al., 2006; Singh et al., 2001). Besides, given the documented extensive polymorphisms in *T. annulata* field isolates, and known recombination process that occur in the tick vector, it is possible that the parasite genotypes are continuously changing in field situations even in the absence of transboundary vaccinations. This point can be further illustrated by reference to the *T. parva* polymorphic immunodominant molecule (PIM) that is known to be extensively polymorphic even within two of the three strains that comprise the Muguga ITM live vaccine cocktail (Norling et al., 2015). Schnittger et al. (2002) have previously suggested that *TaSP* may actually be the *T. annulata* homologue of PIM. Despite widespread use of the cocktail in the Tanzania, Kenya and Uganda, recent studies have failed to demonstrate that local parasite population structures are altered as a result of vaccination or that the vaccine strains replace the local isolates.

5 | CONCLUSION

The observed *TaSP* polymorphism data suggest that Tunisia harbours a more homogeneous *T. annulata* parasite population relative to Sudan. It however remains to be evaluated whether the differences in the extent of *TaSP* variation between the Tunisian and Sudanese cell lines will impact the ability of *TaSP* to augment the protective effects of live vaccines. If such an impact would be demonstrated, it seems likely that such variation would not be a major problem in Tunisia relative to Sudan. A more comprehensive catalogue of genotype divergence among culture-attenuated vaccine candidates from North Africa will be useful in addressing the practical issue of the possible impact on parasite population genetic structure in the case of a transboundary cell line vaccine deployment or movement of vaccinated cattle. As previous studies showed that a carrier state is not a typical outcome following *T. annulata* vaccine use, there would be less concerns about alterations of parasite populations in the field following vaccine use. It is also important to emphasize that identification of the protective epitopes on the TaSP protein remains a priority for future research.

AUTHOR CONTRIBUTIONS

Conception and design of the study: Khawla Elati, Isaiah Obara, Mohamed Aziz Darghouth; Acquisition of data: Khawla Elati, Micky M. Mwamuye, Vahel Ameen, Moez Mhadhbi; Analysis and/or interpretation of data: Khawla Elati, Isaiah Obara, Ard Menzo Nijhof; Drafting the manuscript: Khawla Elati, Isaiah Obara; Revising and Editing of the manuscript: Ard Menzo Nijhof, Mohamed Aziz Darghouth, Moez Mhadhbi, Micky M. Mwamuye; Approval of the version of the manuscript to be published: Khawla Elati, Ard Menzo Nijhof, Micky M. Mwamuye, Vahel Ameen, Moez Mhadhbi, Mohamed Aziz Darghouth & Isaiah Obara.

ACKNOWLEDGMENTS

Part of the work was supported by DFG project 'Molecular epidemiology network for promotion and support of delivery of life vaccines against *Theileria parva* and *Theileria annulata* infection in Eastern and Northern Africa' (DFG-SE862/2-1 and CL166/4-2). Khawla Elati was supported by a doctoral scholarship from the German Academic Exchange Service (DAAD). Isaiah Obara was supported by DFG grant OB 490/2-1.

Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in [GenBank] at [https://www.ncbi.nlm.nih.gov/genbank/].

ETHICAL STATEMENT

The study adhered to local animal welfare regulations and practices and conformed to ethical guidelines for animal usage in research of the National School of Veterinary Medicine of Sidi Thabet (Tunisia) and the Association Tunisienne des Sciences des Animaux de Laboratoire (ATSAL, Tunisia).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Elati, K., Nijhof, A. M., Mwamuye, M. M., Ameen, V., Mhadhbi, M., Darghouth, M. A., & Obara, I. (2022). Sequence polymorphisms in a *Theileria annulata* surface protein (TaSP) known to augment the immunity induced by live attenuated cell line vaccine. *Transboundary and Emerging Diseases*, *69*, 3350–3359. https://doi.org/10.1111/tbed.14687