Theileria: intracellular protozoan parasites of wild and domestic ruminants transmitted by ixodid ticks

R. BISHOP[1]*, A. MUSOKE[1], S. MORZARIA[1], M. GARDNER[2] *and* V. NENE[2]

[1] *The International Livestock Research Institute (ILRI), P.O. Box 30709, Nairobi, Kenya*
[2] *The Institute for Genomic Research (TIGR), 9712 Medical Center Drive, Rockville, MD 20850, USA*

SUMMARY

Theileria are economically important, intra-cellular protozoa, transmitted by ixodid ticks, which infect wild and domestic ruminants. In the mammalian host, parasites infect leukocytes and erythrocytes. In the arthropod vector they develop in gut epithelial cells and salivary glands. All four intra-cellular stages of *Theileria* survive free in the cytoplasm. The schizont stages of certain *Theileria* species induce a unique, cancer-like, phenotype in infected host leukocytes. *Theileria* undergoes an obligate sexual cycle, involving fusion of gametes in the tick gut, to produce a transiently diploid zygote. The existence of sexual recombination in *T. parva* has been confirmed in the laboratory, and is presumed to contribute to the extensive polymorphism observed in field isolates. Key parameters in *T. parva* population dynamics are the relative importance of asymptomatic carrier cattle and animals undergoing severe disease, in transmission of the parasite to ticks, and the extent of transmission by nymphs as compared to adult ticks. Tick populations differ in vector competence for specific *T. parva* stocks. Recombinant forms of *T. parva* and *T. annulata* sporozoite surface antigens induce protection against parasite challenge in cattle. In future, vaccines might be improved by inclusion of tick peptides in multivalent vaccines.

Key words: *Theileria*, intracellular protozoa, sporozoite, ixodid tick, transmission dynamics, recombinant vaccine.

INTRODUCTION

The genus *Theileria* comprises tick-transmitted sporozoan protozoa that are the causative agents of a variety of disease syndromes in domestic and wild ruminants, and are collectively responsible for economic losses amounting to hundreds of millions of dollars annually in Sub-Saharan Africa and Asia. *Theileria* are unique among protozoa in that certain species are capable of immortalizing either mammalian lymphocytes or cells of the monocyte/macrophage lineage that they infect. *Theileria* are currently classified in the class Sporozoa together with human pathogens, including *Plasmodium* and *Toxoplasma*. Along with other Sporozoa, *Theileria* has been included within a sub-phylum designated the Apicomplexa, based on the common possession of an apical complex containing secretory organelles involved in invasion, or establishment, in the cells of their mammalian and invertebrate hosts. However the evolutionary and functional equivalence of the apical complex between different genera and hence the taxonomic validity of the Apicomplexa remains unclear. Analysis of 18s ribosomal RNA gene sequences demonstrates that the genus *Theileria* is phylogenetically most closely related to *Babesia*, a genus of tick-borne protozoans infective to the red cells of mammals including domestic livestock, and more distantly to the genus *Plasmodium* which causes malaria in humans and other species of vertebrates (Allsopp *et al*. 1994). There are similarities, but also significant differences between features of life cycle, genome organization and mammalian host immune responses to infection when *Theileria* and *Plasmodium* are compared.

Economically important *Theileria* species that infect cattle and small ruminants are transmitted by ixodid ticks of the genera *Rhipicephalus*, *Amblyomma*, *Hyalomma* and *Haemaphysalis*. *Theileria* species infective to domestic ruminants are summarized in Table 1. Globally the most important species are *Theileria parva*, transmitted by *Rhipicephalus* ticks, that causes a rapidly fatal lympho-proliferative disease known as East Coast fever (ECF). The disease was estimated to have been responsible for 170 million US dollars worth of economic loss in 1989 alone (Mukhebi, Perry & Kruska, 1992) and limits introduction of more productive exotic (*Bos taurus*) cattle in much of eastern, central and southern Africa. The primary host of *T. parva* is the African cape buffalo (*Syncerus caffer*) in which the parasite typically does not cause any disease. *Theileria annulata*, originating from Asian water buffalo (*Bulbulus bubulis*), and transmitted by several *Hyalomma* tick species, is responsible for tropical theileriosis from Southern Europe to China, a vast region in which an estimated 250 million cattle are at risk. Both *T. parva* and *T. annulata* induce a transformation-like

* Corresponding author: Dr Richard Bishop, ILRI, P.O. Box 30709, Nairobi, Kenya. Tel: 254 2 630 743. Fax: 254 2 631 499. E-mail: R.Bishop@cgiar.org

Table 1. *Theileria* species infective to domestic ruminants, their ixodid tick vectors and geograpical distribution. * Indicates *Theileria* species infective to small ruminants. Other species are infective to cattle

Theileria species	Major ixodid vectors	Known distribution
T. parva	*Rhipicephalus appendiculatus* *R. zambesiensis* *R. duttoni*	Eastern, Central and Southern Africa
T. annulata	*Hyalomma anatolicum* and other *Hyalomma* species	Southern Europe Western, Southern and Eastern Asia Northern Africa
T. mutans	*Amblyomma variegatum* and four other *Amblyomma* species	Western, Eastern Central and Southern Africa Caribbean islands
T. velifera	*Amblyomma variegatum* and other *Amblyomma* species	Western, Eastern Central and Southern Africa
T. tarurotragi	*Rhipicephalus appendiculatus* *R. zambesiensis* *R. pulchellus*	Eastern, Central and Southern Africa
T. sergenti	*Haemaphysalis* species	Japan and Korea
T. buffeli	*Haemaphysalis* species	Europe, Asia, Australia, Eastern Africa
*T. lestoquardi**	*Hyalomma* species	Asia and Northern Africa
*T. ovis**	*Hyalomma* species	Asia
*T. separata**	*Hyalomma* species	Asia

phenotype in nucleated mammalian host cells, which is the major cause of pathology. Several other non-transforming *Theileria* are also responsible for disease in domestic ruminants as a result of anaemia induced by the intra-erythrocytic stage. *T. sergenti* and *T. buffeli* cause disease and economic loss in cattle in East Asia. *T. mutans* may be responsible for disease in cattle in sub-Saharan Africa particularly in mixed infections with other tick-borne pathogens, but the economic impact of this species has yet to be accurately assessed using sensitive and specific diagnostic tests. *Theileria lestoquardi* is the most pathogenic of several small ruminant *Theileria* species infective to sheep and goats in Northern Africa and widely across Asia. Most wild bovids in Africa are infected by one or more species of *Theileria* and the epidemiological situation can be complex with East African cattle potentially being infected with up to five *Theileria* species at one time (Norval, Perry & Young, 1992). Cattle can be infested with several species of tick and more than one species of *Theileria* can also be transmitted by the same tick. For example, the morphologically very similar sporozoites of *T. parva* and the normally non-pathogenic *T. taurotragi* can both occur together in *R. appendiculatus* salivary glands, although they can be distinguished by molecular methods based on hybridization to rRNA (Bishop *et al.* 1994). Additional *Theileria* species that are infective to livestock, and may possibly be pathogenic in specific circumstances, continue to be discovered. The range of *T. buffeli* has been extended to Africa (Ngumi *et al.* 1994), and a hitherto undescribed *Theileria* that is pathogenic to small ruminants has

been discovered in North Western China (Schnittger *et al.* 2000). A *Theileria* species originally described from schizont-infected lymphocyte culture isolates from Cape buffalo (Conrad *et al.* 1987; Allsopp *et al.* 1993) has recently been found in cattle subjected to challenge by ticks that are presumed to have fed on buffalo (Bishop & Musoke, unpublished data). *Theileria* infections have also been associated with bovine fatalities in the United States, and the presence of *Theileria* was confirmed in both *Amblyomma americanum* and *Dermacentor variabilis* by PCR amplification using primers derived from small subunit rRNA (SSU rRNA) genes sequences (Chae *et al.* 1999).

The *T. parva* genome, which is approximately 8·5 Mbp in size, is small for a eukaryote with a complex life cycle involving several distinct intracellular stages in both the arthropod vector and the mammalian host. The genome is divided into 4 chromosomes, containing 33 *Sfi*I fragments (Morzaria & Young, 1992). Both the *T. parva* (http://www.tigr.org/tdb/e2k1/tpa1/) and *T. annulata* (http://www.sanger.ac.uk/Projects/T_annulata/) genome sequences are currently nearing completion. The current status of knowledge relating to the *T. parva* genome has been reviewed recently (Nene, Morzaria & Bishop, 1998; Nene *et al.* 2000, 2002) and will not be discussed in detail in this article. Most research on *Theileria* to date has been devoted to the life-cycle stages occurring in the mammalian host, particularly the pathogenic, intra-cellular schizont stages of *T. parva* and *T. annulata*, which respectively infect cells of the lymphoid and myeloid lineages and activate
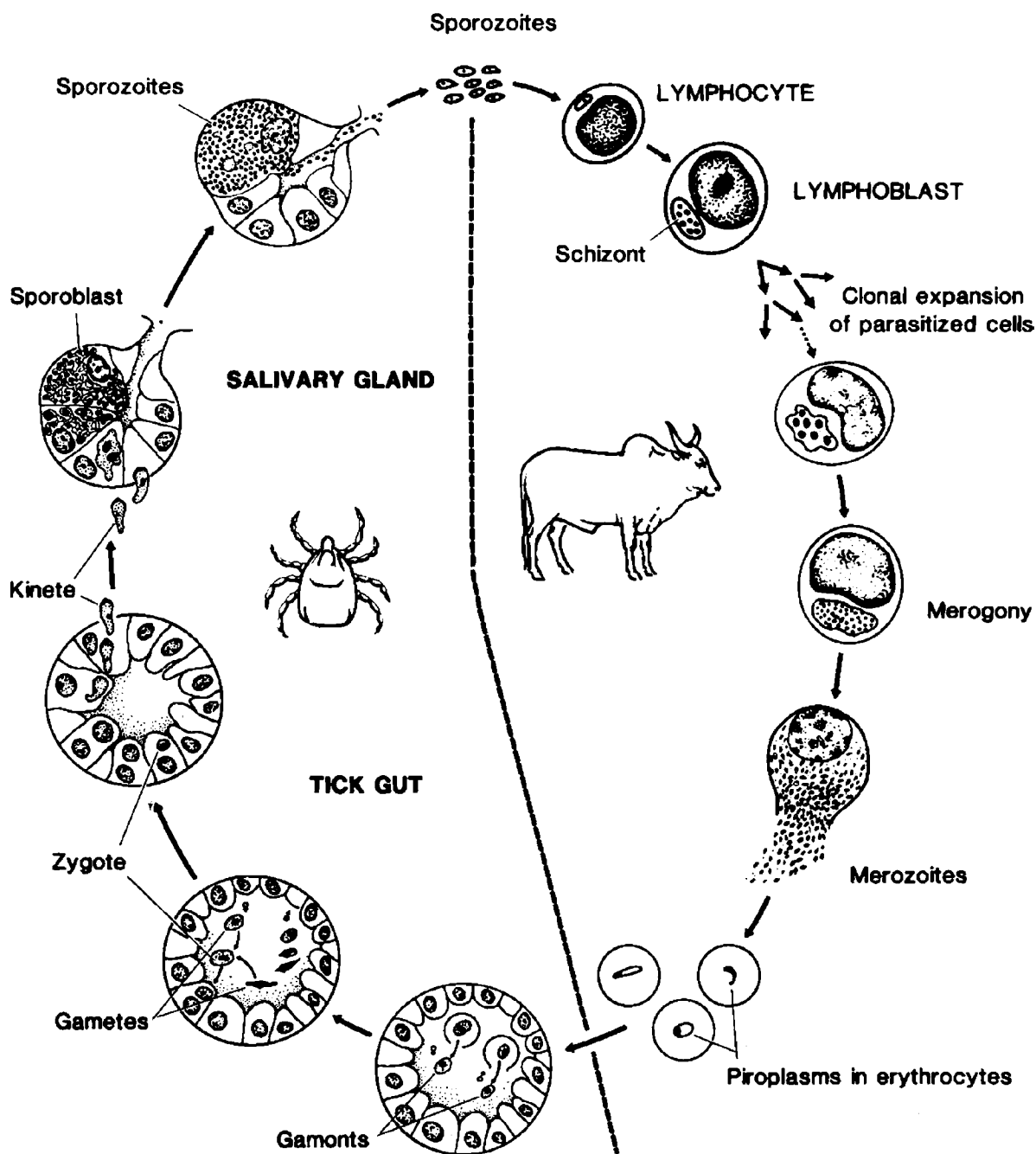
LIFE CYCLE OF *THEILERIA PARVA*

Fig. 1. Life cycle of *Theileria parva* in cattle and the ixodid tick vector *Rhipicephalus appendiculatus*.

host signal transduction pathways thereby inducing cancer-like syndromes in infected mammalian lymphocytes, monocytes and macrophages. The only tick-expressed stage of *Theileria* for which any molecular information is currently available is the sporozoite, which is secreted from the tick salivary glands into the vertebrate host. This article will concentrate on tick vector-associated aspects of *Theileria* biology and will review the status of vaccine development based on an antigen isolated from the tick-derived sporozoite life cycle stage. The data presented primarily relates to *T. parva*, the species for which most information is currently available.

OVERVIEW OF THE *THEILERIA PARVA* LIFE CYCLE

Theileria parva life-cycle stages in the tick vector and bovine host have been comprehensively described previously and are summarized in Fig. 1.

The three-host brown ear tick *Rhiphicephalus appendiculatus* is the major vector of the parasite. *Theileria parva* exhibits a very narrow tick and mammalian host range, and there is no laboratory animal model susceptible to infection with the parasite. *Rhipicephalus appendiculatus* is classified as a three-host tick because the larvae, nymphs and adults feed on different hosts, which are not necessarily cattle. Transmission is trans-stadial, which means that larval or nymphal instars of the tick acquire infections from a blood-meal, which is then transmitted to a new host, after moulting by nymphs or adults, respectively. Transmission of *Theileria* has not been shown to be transovarian, unlike the related protozoan *Babesia* transmitted by the one host tick *Boophilus* (Norval, Perry & Young, 1992). The *Hyalomma* vectors of *T. annulata* are, by contrast, two host ticks, with larval and nymphal instars feeding on the same host. Sporogony takes place in the tick salivary glands in response to a combination of increased temperature and components present within the blood meal. Infective sporozoites are released from about day 3 to day 7 of tick feeding. One infected cell may contain 40–50 000 sporozoites, but the mechanics of tick feeding is believed to result in a trickle release of sporozoites from the salivary glands (Shaw, 2002; Shaw & Young, 1994). A tick with a single infected acinar cell can potentially cause severe disease and death. However, the severity of ECF is sporozoite dose dependent and individual animals exhibit different thresholds of susceptibility. Some animals undergo a mild disease reaction and recover due to development of protective cellular immune responses.

Once introduced into the host, *T. parva* sporozoites invade a restricted range of cattle and buffalo cells. *In vitro* they enter purified B cells or T cells which express receptors which are either CD4$^+$, CD8$^+$, or CD4$^-$/CD8$^-$ (Baldwin *et al.* 1988), and also afferent lymph veiled cells and monocytes (Shaw, Tilney & Mckeever, 1993), although the latter cannot subsequently be immortalised. The entry process is probably energy dependent and is likely to involve specific receptors located on the host cells (Shaw, 1997, 2002). *T. annulata* infects cells of the monocyte/macrophage lineage, although it can also enter B cells. Other species such as *T. taurotragi*, originally isolated from eland (*Taurotragus oryx*), appear to have a much wider host range, at least *in vitro* (Stagg *et al.* 1983). Like viruses transmitted by *R. appendiculatus* (see chapter by Nuttall & Labuda in this Supplement) components of tick saliva appear to promote *T. parva* pathogen transmission by facilitating sporozoite entry into host leukocytes (Shaw, Tilney & McKeever, 1993), although the mechanism may be an indirect one involving non-specific activation of host lymphocytes by saliva components. Unlike other sporozoans, *T. parva* sporozoites are not motile. They do not possess all of the components of a typical apical complex as described for other Apicomplexa, and also by contrast with *Plasmodium* entry into the host cells is not orientation specific, and the rhoptries and microspheres are involved in establishment in the host cell subsequent to entry rather than in the entry process itself (Shaw, Tilney & Musoke, 1991). Unlike other Apicomplexa, *T. parva* sporozoites also appear to lack morphologically distinguishable micronemes (Shaw, Tilney & Musoke, 1991). The newly internalized parasite is surrounded by a host cell membrane which is rapidly (< 15 min) removed by the parasite, thereby evading intracellular host cell defense mechanisms. In this respect, *Theileria* is similar to a phylogenetically diverse range of intracellular pathogens including *Listeria*, *Shigella* and *Trypanosoma cruzi* (reviewed by Andrews & Webster, 1991), presumably as a result of independent evolution of this solution to the problem of intracellular survival. Rapid dissolution of the host cell membrane by *T. parva* is closely correlated with the discharge of the contents of the rhoptries and microspheres. The major surface molecule of *T. parva* sporozoites, p67, and MHC class I molecules on the host cell are implicated in the entry process as monoclonal antibodies (mAbs) to these proteins inhibit sporozoite entry (Musoke *et al.* 1984; Shaw *et al.* 1991). Because of the restricted host cell specificity it seems unlikely that MHC class I molecules constitute the sole recognition site of sporozoites. Mobilization of calcium stores within sporozoites, parasite and host cell proteases, and signal transduction via host cell and parasite protein kinases and G proteins have all been demonstrated to play a role in the entry process (Shaw, 1997). Sporozoite organelles that discharge their contents after entry are likely to contain a microtubule nucleating factor as the free intracellular sporozoites are rapidly surrounded by a network of microtubules.

The schizont is a multinucleated, syncytial body. Association of the schizont with the host cell nuclear spindle ensures that daughter host cells remain infected during cytokinesis (Carrington *et al.* 1995). Although the parasite and host cells divide in synchrony, schizont DNA synthesis occurs as the host cell enters mitosis and is immediately followed by division when the host cell is in metaphase (Irvin, Ocama & Spooner, 1982). The molecular basis of *T. parva* schizont-induced B and T cell transformation is not yet fully understood, but there is evidence for increased bovine casein kinase II activity (Ole-MoiYoi *et al.* 1993). Existing data indicate subversion of lymphocyte signal transduction pathways resulting in activation of the JNK mitogen-activated protein kinases, phosphatase PP2A and constitutive expression of nuclear factor kappa beta (NF-kB) and also IL2 and its receptor in the infected cells (reviewed by Heussler, 2002). Recently it has been demonstrated that constitutive activation of NF-kB

may be effected by the recruitment of the IKB regulatory kinase complex onto the surface of the schizont (Heussler *et al.* 2002). In testing a disease model for transformation induced by *T. parva*, it was demonstrated that dysregulation of casein kinase II mRNA expression, along with *myc* oncogene activation, results in the development of fulminant leukemia in transgenic mice (Ole-MoiYoi, 1995; Seldin & Leder, 1995). When injected sub-cutaneously into nude or SCID mice infected *T. parva* and *T. annulata* lymphoblastoid cells form a huge tumour mass (Irvin *et al.* 1975; Fell, Preston & Ansell, 1990). Despite these insights into parasite modulation of host cell phenotype, nothing is yet known about the parasite molecules involved in this process. The schizont stages of both *T. parva* and *T. annulata* are the major cause of pathology and disease. Merogony occurs within infected lymphocytes (Shaw & Tilney, 1992) and merozoites are released by host cell rupture. The merozoites invade red blood cells (RBCs) in a manner similar to that described for sporozoite entry into lymphocytes, where they develop into piroplasms, which, like the intra-lymphocytic schizont, lie free in the erythrocyte cytoplasm. There is very little multiplication of the piroplasm stage of *T. parva*, although intra-erythrocytic multiplication of the piroplasm does occur in *T. annulata* infections and this stage contributes to the pathology of tropical theileriosis. Other species of *Theileria*, especially the non-transforming ones, such as *T. mutans*, *T. buffeli* and *T. sergenti*, exhibit high levels of intra-erythrocytic merogony resulting in anaemia and pathology due to RBC lysis. Ticks become infected after feeding on blood containing piroplasm-infected RBCs which lyse in the tick gut. Morphologically distinct forms of the parasite are found in the tick gut which is the likely site of gametogenesis (Norval, Perry & Young, 1992). Gametes fuse to form a zygote which invades the cells of the gut epithelium. Kinetes are released into haemolymph and invade cells of the salivary gland, where they reside until suitable environmental conditions are presented. Full sporogony is initiated on stimuli received when the tick attaches to the mammalian host and appears to be closely linked with the moulting cycle of the tick. This completes the life cycle. All stages of the life cycle are haploid except for zygote in the tick gut.

LIFE CYCLE STAGES AND DEVELOPMENT OF *THEILERIA* IN THE IXODID TICK VECTOR

The life cycle stages of *Theileria* species in their ixodid vectors have been relatively little studied due to the difficulty of culturing them. The available information has been reviewed previously (Mehlhorn & Schein, 1984; Norval, Perry & Young, 1992; Shaw & Young, 1994; Shaw, 2002). This section draws heavily on these earlier accounts.

Tick gut stages

The life cycle of *Theileria* in the tick begins with the ingestion of piroplasm-infected erythrocytes within the blood meal, and at repletion millions of infected erythrocytes are ingested by adult ticks, even from an animal with a low piroplasm parasitaemia. The volumes of blood ingested by larvae and nymphs are much smaller and hence the numbers of parasites surviving and developing in the midgut will be correspondingly smaller. Due to the inability to culture the red blood cell stages of *T. parva* it is unknown whether gametocytes (stages that are predetermined to differentiate into gametes in the tick gut) occur in the circulating blood as they do in *Plasmodium* infections (reviewed by Sinden *et al.* 1996). Since there is currently no evidence for sexual differentiation within the mammalian erythrocytic stages, and there is also a marked drop in temperature after the tick completes repletion and falls to the ground, it is possible that the processes controlling sexual differentiation in *Theileria* and *Babesia* differ markedly from other sporozoan protozoa. It is believed that the vast majority of the ingested piroplasms are rapidly destroyed in the gut lumen possibly by acid phosphatases secreted by gut epithelial cells (Shaw & Young, 1994). Soon after the completion of feeding, structures designated 'Strahlenkorper', or ray bodies, can be observed in smear preparations by light microscopy. The significance of these as sexual structures, originally thought to represent microgametes, was initially recognized by Schein and colleagues (Mehlhorn & Schein, 1984). The process of gametogenesis appears similar in those species that have been studied, including *T. parva* and *T. annulata*, but there is no evidence regarding the factors required for triggering gamete development. Recent studies suggest that in *Babesia* (Gough, Jorgensen & Kemp, 1998) there is no clear indication of morphologically distinguishable micro- and macrogametes and the zygote is formed by the fusion of two similarly sized and structured ray bodies (gametes) that differ only in that one is more electron dense than the other. The recently reported formation of the zygote from two structurally similar gametes in *Theileria* and *Babesia* differs significantly from most other sporozoans, including *Plasmodium* (Shaw, 2002). Subsequently the spherical zygote invades a gut epithelial cell and differentiates into a motile kinete. It is currently not known what type of tick gut cell the zygotes enter, or what the mechanism of the entry process is. However it is possible that a stiletto-like apical arrowhead structure observed on the ray body is involved in this process, since a similar structure is present in the gut stages of *Babesia* and appears to be important in invasion of tick gut cells (Rudzinska *et al.* 1983). While the mechanism of entry of the zygote into gut epithelial cells is uncertain, the developing intracellular parasite is not

enclosed in a parasitophorous vacuole and lies free in the cytoplasm. Therefore, a common feature of all four intracellular stages of *Theileria*, the kinete in the tick gut, the sporozoite in the tick salivary gland and the intra-lymphoytic and erythrocytic stages in the mammalian host, is that they rapidly escape any enclosing host membrane and lie free in the cytoplasm. *Theileria* kinetes develop only in the tick gut cells and are typically uni-nucleate.

Salivary gland stages

The motile kinetes subsequently invade salivary glands and this process is very specific in that kinetes have not so far been recorded in other tick organs, including reproductive tissues. Development of kinetes and their appearance in the tick haemocoel is associated with the tick moulting cycle (Shaw, 2002). To reach the salivary gland, the kinete must cross several obstacles, the basal membrane and lamina to allow exit from the gut cells in which it is originally located, followed by other barriers, to allow entry into the salivary gland acinar cell in which sporogony occurs. Ixodid salivary glands contain four types of acini, I, II and III are present in both males and females, while type IV is in males only (see chapter by Bowman & Sauer, in this Supplement). Type I acini are non-granular and thought to be involved primarily in off-host osmoregulation, whereas types II, III and IV contain a variety of secretory cell types designated by lower case letters from a–h. It appears that the motile kinetes probably have the ability to recognize specifically the type III salivary gland acinar cells within the salivary glands and may also be able to invade selectively e cells within the acinus. It has been suggested that the carbohydrate composition of the type III acinus determines the susceptibility of this cell type to parasite infection (reviewed by Shaw, 2002). *Theileria* develop almost exclusively within the e cells of the type III acinus, although it has been demonstrated that in ticks very highly infected with *T. annulata* the d cells and type II acini can also be infected (Mehlhorn & Schein, 1984).

Sporogony, which is the only multiplicative stage within the tick, occurs within the salivary gland and is typically triggered by feeding, although it has been shown that sporogony can also be completed at high ambient temperatures in a proportion of acini (Young & Leitch, 1982). The number of sporozoites produced during sporogony has been estimated at between 30 000–50 000 per infected acinar cell for female *R. appendiculatus* and up to 140 000 for *T. taurotragi* (Norval, Perry & Young, 1992; Shaw & Young, 1994). There are indications that *T. parva* sporozoite numbers are lower in male *R. appendiculatus* than in females and it has also been demonstrated that numbers of sporozoites are lower in the e cells of nymphal compared to adult ticks, which has

potential consequences for the relative importance of different instars in *T. parva* transmission (Ochanda *et al.* 1996). *Theileria mutans* in the salivary glands of *Amblyomma variegatum* typically produces fewer (approximately 5000) but larger sporozoites. The ultrastructure of the mature sporozoites has been described in detail (Fawcett, Doxsey & Buscher, 1982). Unlike other genera of the Apicomplexa, there is no clearly defined apical complex. A spherical body enclosed by three or more membranes is located close to the nucleus and may correspond to the apicoplast described from *Plasmodium* and other genera of Sporozoa (reviewed in Wilson & Williamson, 1997). As in other life cycle stages of *Theileria* (Shaw & Tilney, 1992; Ebel *et al.* 1997) structures corresponding to the endoplasmic reticulum and Golgi apparatus of higher eukaryotes are not visible although proteins associated with the classical secretory pathway are present in *Theileria* (Janoo *et al.* 1999). There is a number of peripherally located membrane-bound bodies termed microspheres and also a group of up to six larger, membrane-bound rhoptries. Both these secretory organelles discharge after entry into the host cell in association with establishment of the parasite (Shaw *et al.* 1991). Specific proteins encoded by cloned genes have been localised to these organelles. These include the p104 antigen located in the rhoptries (Iams *et al.* 1990) and the p150 and PIM antigens in the microspheres (Skilton *et al.* 1998). There is some evidence to suggest that sporozoites are released gradually from an infected cell in a manner similar to the release of secretory granules by apocrine secretion (summarized in Shaw & Young, 1994). This will have implications for the nature of the parasite challenge to the mammalian host and hence for the viability of control strategies using novel recombinant vaccines as agents for control of *Theileria*.

SEXUAL RECOMBINATION IN *THEILERIA* WITHIN THE IXODID VECTOR

As in *Plasmodium* species, *T. parva* undergoes an obligatory sexual cycle in the tick vector. While the mammalian life cycle stages are haploid, there is a transient diploid phase in the tick gut after fusion of gametes (Gauer *et al.* 1995). In *T. parva*, according to direct measurements of the DNA content of single cells by fluorescence microscopy, a post-zygotic meiosis appears to occur in the gut prior to differentiation of the kinete, rapidly restoring the haploid condition. In *T. annulata*, by contrast, the kinete appears to remain diploid and meiotic division may occur at a later stage during sporogony in the salivary glands (Gauer *et al.* 1995). Laboratory crosses have been performed between *T. parva* Muguga and either of two other parasite stocks, *T. parva* Uganda and *T. parva* Marikebuni by feeding ticks on cattle co-infected with these stocks (Morzaria *et al.* 1993).

Hybridisation of 'stock specific' oligonucleotides derived from *T. parva* Muguga and Uganda (Allsopp *et al.* 1989; Bishop *et al.* 1993) revealed that 38% of single-infected acini derived from progeny of the Muguga/Uganda cross appeared to be recombinant. Subsequent analysis of a cloned parasite derived from one of the putatively recombinant acini revealed mixed profiles using several probes in Southern blot assays. The data suggested that at least four independent, physical crossover events had occurred on the two smallest chromosomes. In the case of the *T. parva* Muguga/Marikebuni cross, three independent clonal progeny were isolated and in all three progeny isolated from this cross a *T. parva* Muguga-type TpR locus appeared to have been introgressed into a genome background in which the majority of other polymorphic loci were of Marikebuni type. These probably represented independent occurrences of similar recombination events, since the Muguga-type Tpr genotypes differed from one another and were different from that of the previously characterized *T. parva* Marikebuni TpR RFLP pattern (Bishop *et al.* 2002).

To what extent recombination occurs in *T. parva* in field populations is currently unknown, although as in *Plasmodium* (Paul & Day, 1998; Awadalla *et al.* 2001) there is considerable genetic heterogeneity between individuals and populations in the field, and it seems reasonable to assume that this is attributable at least partially to a consequence of the obligate sexual cycle. This issue of the extent of recombination between different parasite clones can only be resolved by direct examination of kinetes using PCR analysis of single copy allelic markers, as has been done for *Plasmodium* oocytes from mosquito populations in the field. In a recent study, a high percentage of apparently recombinant *P. falciparum* zygotes were found at a Tanzanian study site (Babiker *et al.* 1994). However, such studies will be technically more difficult for *Theileria* infections in ticks. According to research on *Plasmodium*, levels of outcrossing may vary according to the force of infection in different epidemiological situations, and even quite high levels of inbreeding may still result in linkage equilibrium between different alleles in *Plasmodium* populations (Paul *et al.* 1995; reviewed by Paul & Day, 1998). It is possible that factors such as host and vector movements may be more important in genetic substructuring of *T. parva* populations than the extent of meiotic recombination, and that this may differ substantially between cattle and the wildlife reservoir host buffalo (*S. caffer*). For example, the major determinant of genetic substructuring among nematode parasites of sheep, cattle and a wildlife reservoir host, white tailed deer, was host movement, with genetic differentiation of parasite populations being greater in the white tailed deer due to more extensive movement of the livestock species between different localities (Blouin *et al.*

1995). Another issue that has yet to be addressed for *Theileria* or other parasites, including *Plasmodium* (Day *et al.* 1992), is the extent to which additional processes of diversification, such as somatic mutation, genome rearrangements, gene deletions and gene conversion during the asexual multiplication phases, in addition to classical recombination during the obligate sexual cycle, are important in generation of diversity in *T. parva* parasite populations.

TRANSMISSION DYNAMICS OF *T. PARVA* AND FACTORS AFFECTING VECTOR COMPETENCE

The transmission dynamics and epidemiology of theilerosis vary in different areas of eastern, central and southern Africa according to a complex interplay of factors, including the level of tick control, cattle genotype and management regime, proximity of a wildlife reservoir of disease, and the interaction of tick and parasite populations with differing genetic composition. Initial transmission dynamic modelling of *T. parva* infections (Medley, Perry & Young, 1993) focused on a particular epidemiological situation described by the term 'endemic stability' (Norval, Perry & Young, 1992) in which African Zebu (*Bos indicus*) cattle, previously exposed to *T. parva* over several generations and subjected to little or no tick control, are continuously challenged by infected ticks, and either become immune or die by day 150 of exposure. In endemically stable situations the majority of ticks are believed to exhibit relatively low levels of infection in terms of both prevalence (percentage of ticks infected) and abundance (mean number of infected acini/tick), both of which are frequently low in these epidemiological circumstances (Young *et al.* 1986). Laboratory data on experimental tick infections (Buscher & Otim, 1986), and also field data (S. Morzaria, unpublished data) indicate that the abundance of *T. parva* infections can frequently be described by a negative binomial distribution. Therefore the abundance of mature *T. parva* infections in salivary glands is typically over-dispersed, with a small proportion of ticks having many infected acini, while a majority may contain only a single infected acinus. The key conclusion from preliminary transmission dynamic modelling work (Medley, Perry & Young, 1993) is that the degree to which animals that have recovered from theileriosis, but remain infected (known as 'the carrier state'), are able to transmit the infection to tick larvae or nymphs is a crucial determinant of the dynamics of infection in a herd. Currently there is no comparative experimental data on the relative tick transmissibility of *T. parva* parasites from carrier animals relative to those undergoing acute infections. It is known that the level of piroplasm parasitaemia is the most important parameter influencing levels of infection in *R. appendiculatus* ticks experimentally fed on cattle (Young *et al.* 1996). However,

no correlation has been found between the number of zygotes and kinetes present in the tick gut and haemolymph, respectively, and the number of sporoblasts in the salivary glands (Buscher & Otim, 1986). This implies that there is some degree of parasite destruction occurring either with the gut cells or during kinete migration through the haemolymph. Although the mechanisms are not yet fully understood there may be an element of *Theileria* specificity, since *R. appendiculatus* haemolymph exhibited no apparent effect on bacteria *in vitro* (Watt *et al.* 2001). The issue of arthropod vector immune responses to parasites is an emerging area of research that has been most extensively researched in *Drosophila* and *Anopheles* mosquitoes (reviewed by Dimopoulos, 2003). Recent experimental work suggests that certain stocks may not induce a long-term carrier state, and that even when experimentally infected or vaccinated cattle are persistently infected with *T. parva*, as judged by positivity using a PCR-based assay with *T. parva*-specific primers, the infection may not always be experimentally tick-transmissible (Skilton *et al.* 2002). Data generated using PCR assays also indicate that animals from endemic areas that are often close to 100% serologically positive for *Theileria* infection, according to application of immunofluorescence or ELISA tests (Young *et al.* 1986), may not necessarily be carriers of *T. parva* parasites based on the failure of PCR assays to detect parasite DNA in these animals (R. Bishop, unpublished data).

Two important parameters in tick-borne disease transmission dynamics for which most currently available data relates to transmission of *T. parva* by *R. appendiculatus* have potential relevance to transmission of other livestock and human pathogens by ixodid tick species: (1) differences in vector competence for pathogens between different tick stocks: (2) the importance of nymphal/larval transmission of pathogens relative to larval/nymphal transmission. Distinct *R. appendiculatus* tick stocks have varying effectiveness in their ability to transmit two different *T. parva* stocks (from Kenya and Zimbabwe) to cattle in an experimental context in the laboratory (Ochanda *et al.* 1998). These authors also demonstrated interaction between parasite and tick in terms of transmission efficiency. Specific tick stocks transmitted certain parasites efficiently and other parasite stocks less well. This effect was dependent on the particular combination of tick and parasite genotpye (Ochanda *et al.* 1998). Furthermore, the offspring of individual ticks within families derived from two different *R. appendiculatus* stocks (Kiambu and Muguga) have been demonstrated to have heritabilities between approximately 0·4 and 0·6 for susceptibility to infection with *T. parva* (Young *et al.* 1995), indicating that these differences are, at least in part, genetically determined. The basis of such differences has not yet been investigated for ticks

but, by analogy with insects, may be related to factors such as levels of expression of nitric oxide in tick gut tissues or antimicrobial peptides, such as cecropins and serpins, in a variety of tissues. This suggests that selection of tick lines that are refractory or susceptible to *T. parva* infection may be possible. The first study to attempt to make an objective comparison of transmission of *T. parva* between different instars is that of Ochanda *et al.* (1998). The conclusion of this research was that larval/nymphal transmission is less efficient than nympal/adult transmission. One factor probably underlying this result is that that mean numbers of type III acini are 1736 in females, 1346 in males and only 87 in nymphae (Ochanda *et al.* 1996). However, since numbers of nymphal and larval ticks infecting livestock are higher than that of adults, Norval *et al.* (1992) suggested a ratio of 1:100:1000 for adult/ nymphal and larval instars and, in addition, the numbers of the nymphal and larval immature stages are technically much more difficult to quantify than adults, the issue of the relative quantitative importance of nymphal versus larval transmission of *T. parva* is not yet fully resolved (Ochanda *et al.* 1996). It is interesting to note that, due to being infected by markedly lower numbers of parasites, nymphal transmission may typically result in milder *T. parva* infections and hence be important in induction of immunity in cattle that maintain the carrier state.

Vector biology and population dynamics have obvious relevance to *Theileria* transmission. This topic has been reviewed previously (Norval, Perry & Young, 1992; Shaw & Young, 1994) and is only covered briefly in this article. The ixodid ticks that are most important for transmission of economically important *Theileria* species, namely, *R. appendiculatus*, *H. anatolicum* and *A. variegatum*, have a wide variety of hosts but all have a preference for feeding on large mammals, including wild as well as domestic species as adults. However, the larvae and nymphs also frequently infest small mammals, mainly carnivores and lagomorphs in the case of *Rhipicephalus*, but additional vertebrate taxa including reptiles and birds for *Hyalomma* and *Ambylomma* (see Norval, Perry & Young, 1992 for further details). There are typically several tick species capable of transmitting the same *Theileria* species. For example in southern Africa, *R. appendiculatus* and *A. variegatum* are replaced by *R. zambeziensis* and *A. hebraeum* as major agents in the transmission of *T. parva* and *T. mutans*, respectively. Another issue is geographical variation in the behaviour of tick populations which can affect transmission. For example in Southern Africa, *R. appendiculatus* undergoes diapause during the cold dry season resulting in a distinct seasonality of transmission which is different from the situation in Eastern Africa (Norval, Perry & Young, 1992; Shaw & Young, 1994). The existence of mildly pathogenic,

or non-pathogenic, *Theileria* that can also be transmitted to domestic livestock and co-infect the same ticks complicates the epidemiological picture. Another issue in relation to vector population dynamics is the extent to which infection with *Theileria* may impact on the survival of the vector. Although, as mentioned previously, most *T. parva* infections in the field are at low levels and probably do not impact significantly on vector survival, *Hyalomma* ticks have been found in the Sudan with sufficiently high infections of *T. annulata* to reduce their feeding efficiency and survival (Walker *et al*. 1983).

SPOROZOITE-BASED VACCINES AGAINST *THEILERIA PARVA*

Currently management of ECF is primarily effected through control of the tick vector using acaricides although this is unsustainable in the medium term (Tatchell, 1987). An 'infection and treatment' live vaccine based on the injection of a potentially lethal dose of sporozoites together with a long-acting dose of tetracycline (Radley *et al*. 1975) has been developed. However, the live vaccine has not been widely deployed due to problems related to use of potentially lethal live parasites, the parasite strain-specificity of the immunity induced in a proportion of vaccinated cattle and, most importantly, the requirement for a cold chain and skilled veterinarians for effective delivery.

Research has shown that humoral immune responses directed against surface epitopes on the sporozoite stage can also be protective by neutralising infectivity of sporozoites for bovine lymphocytes (Dobbelaere *et al*. 1984; Musoke *et al*. 1984). The gene encoding p67, the sporozoite surface protein that is a major target of this neutralizing activity, has been cloned (Nene *et al*. 1992) and demonstrated to induce protection in cattle against experimental needle challenge with a lethal dose of sporozoites (Musoke *et al*. 1992). Both a bacterially-expressed recombinant (Musoke *et al*. 1992) and a form expressed in insect cells (Nene *et al*. 1995) have consistently been shown to induce approximately 70% protection against lethal needle challenge with sporozoite stabilates using susceptible taurine cattle in the laboratory. Furthermore, nucleotide sequence analysis of the p67 gene reveals absolute conservation among cattle-derived parasites (Nene *et al*. 1996) indicating that the vaccine should be robust against heterologous parasite challenge, a hypothesis which has since been experimentally confirmed. In order to promote further development and optimisation of the p67 recombinant vaccine, neutralising epitopes with the protein have recently been identified using synthetic peptides (Nene *et al*. 1999). In addition, an 80 amino acid section located near the C-terminus of the protein, which is easier to produce in recombinant form, has recently been demonstrated to provide equivalent levels of protection to the full-length molecule (Bishop *et al*. 2003). Several p67 vaccine field trials have been performed in Kenya in order to determine protective efficacy against tick challenge within different production systems and epidemiological situations. These indicated reduction in severe disease of 47–50% relative to un-immunised cattle (Musoke, unpublished data). Since there is no demonstrable heterogeneity in the p67 gene among cattle-derived parasites (Nene *et al*. 1996), factors that contribute to the lower levels of protection against *T. parva* sporozoite challenge observed under field conditions may well include differences in the immune responses to vaccination, differences in the individual threshold of infectivity and immuno-modulatory molecules secreted by ticks during feeding. These p67 field trial results suggest that the *T. parva* p67 experimental vaccine would be a useful model system to evaluate whether incorporation of tick salivary gland antigen components into a multivalent vaccine might improve the efficacy of a recombinant anti-pathogen vaccine when subjected to a field tick challenge.

Recombinant sporozoite vaccines against other Theileria *species*

The major surface antigen of *T. annulata*, known as SPAG1, exhibits significant sequence identity (40%) with p67. SPAG1 shares cross-reacting neutralizing determinants that inhibit invasion of leukocytes in an *in vitro* assay and it has been demonstrated that recombinant forms of these proteins exhibit a level of mutual cross-protection on heterologous challenge (Knight *et al*. 1996; reviewed by Boulter & Hall, 1999). By contrast with p67, several *T. annulata* SPAG1 isolates from cattle are quite polymorphic in deduced amino acid sequence (Katzer, Carrington & Knight, 1994). An orthologous gene has also been cloned from sporozoite RNA of the pathogenic small ruminant parasite *T. lestoquardi* (Skilton *et al*. 2000). This molecule has been expressed in a recombinant form but not yet evaluated against sporozoite challenge in sheep or goats. The molecule (designated SLAG1) is closer to the SPAG1 (58% identity) than to p67 (40% identity) in amino acid sequence.

CONCLUSIONS AND OPPORTUNITIES FOR FUTURE RESEARCH

Although significant information has been accumulated regarding the tick-infective stages of *Theileria* over the last 25 years, much of this currently relates to the sporozoite stage. Knowledge of the tick gut and haemolymph stages, including molecular processes involved in infection of ticks by the intra-erythrocytic stages of *Theileria*, factors triggering differentiation of gametes, and the invasion of the gut epithelia and subsequent development of the motile

kinete, is still rudimentary. This may remain true in the near future due to the difficulty of isolating and culturing these life cycle stages. The completion of two *Theileria* genome sequences, combined with comprehensive genome-wide micro array studies using RNA from different life cycle stages, and generation of extensive Expressed Sequence Tag (EST) data from *T. parva*-infected *R. appendiculatus* salivary glands (V. Nene, M. Gardner & R. Bishop, unpublished data), will generate new information relating to *T. parva* genes that are specifically expressed in sporozoites, and the tick-infective intra-erythrocytic piroplasm stages. As EST and proteomics data become available from proteins expressed in tick salivary glands, understanding of host–parasite interaction at the molecular level will increase. This should include mechanisms of action of tick molecules involved in modulation of mammalian immune and inflammatory systems and insight into how tick-secreted salivary gland molecules promote *Theileria* transmission. Another potential research area is on the interaction of *Theileria* spp. and their ixodid vectors in relation to differences in vector competence. Molecular markers for genotyping the parasite have recently been developed and are now available to for application in such studies. Field work on *Theileria* population genetics using new molecular markers and immunological assays will allow data be collected on how the immune response of the host impacts on parasite transmission as a result of re-infection by *Theileria* parasites with differing genoptypes. This information, combined with data on the relative infectivity to ticks resulting from different types of host infection, should result in the generation of more realistic models of transmission dynamics. This will improve understanding of parasite transmission and prediction of the impact of control measures. The possibility of combining the p67 sporozoite recombinant vaccine with one or more tick saliva components in order to enhance protection against tick challenge is an attractive future option for the control of East Coast fever.

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