How relevant are *in vitro* culture models for study of tick-pathogen interactions?

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Abstract

Although tick-borne infectious diseases threaten human and animal health worldwide, with constantly increasing incidence, little knowledge is available regarding vector-pathogen interactions and pathogen transmission. *In vivo* laboratory study of these subjects using live, intact ticks is expensive, labour-intensive and challenging from the points of view of biosafety and ethics. Several *in vitro* models have been developed, including over 70 continuous cell lines derived from multiple tick species and a variety of tick organ culture systems, facilitating many research activities. However, some limitations have to be considered in the translation of the results from the *in vitro* environment to the *in vivo* situation of live, intact ticks and vertebrate hosts. In this review, we describe the available *in vitro* models and selected results from their application to the study of tick-borne viruses, bacteria and protozoa, where possible comparing these results to studies in live, intact ticks. Finally, we highlight the strengths and weaknesses of *in vitro* tick culture models and their essential role in tick-borne pathogen research.

Keywords: ticks, tick cell lines, organ culture, tick-borne, *in vitro* model, virus, bacteria, protozoa

1. Introduction

During the last two decades, the rate of emergence of vector-borne diseases has increased worldwide presenting a significant global economic burden. Ticks are second only to insects as vectors of human diseases, and are the most important vectors of livestock diseases especially in tropical countries [1]. Ticks, haematophagous ectoparasitic arthropods belonging to the order *Ixodida* of the class *Arachnida*, comprise two main families: the *Argasidae* (soft ticks) with 218 species [2] and the *Ixodidae* (hard ticks) with 742 species [3]. Ticks are widely distributed around the world, in particular in countries with warm, humid climates. As obligate bloodfeeders, ticks can acquire a variety of pathogenic bacteria, viruses, protozoa and helminths from their hosts during feeding [4], and subsequently transmit the pathogens during their next blood meal. Once infected, ticks may remain infective for a single stage or for life, depending on the pathogen species, and can transmit to vertebrate hosts and/or other ticks. Other ticks may be infected by the venereal route or by co-feeding in which the pathogen transmission occurs when a naïve tick acquires an infection after feeding in close proximity to an infected tick while the vertebrate host may remain uninfected [5]. In addition, numerous tick-borne pathogens can be passed vertically from adult females to their offspring, which can then transmit during their first or subsequent blood meal. Although the vertical transmission efficiency appears to be low for some microorganisms, it is essential for the persistence of pathogens for which ticks also represent the natural reservoir [6].

To understand tick-borne disease transmission, it is necessary to characterize the ability of the tick to serve as a vector and not only as an occasional host for a pathogen acquired during a blood meal on an infected vertebrate. In most cases, the pathogen must colonize tick tissues and replicate, allowing infection and subsequently transmission during the next blood meal. Study of vector-pathogen interactions is a key factor in unravelling the adaptation of pathogens to their hosts and developing new strategies for disease control.

Although a number of tools have been developed to study tick biology and tick-pathogen interactions, knowledge is still poor and limited by the difficulties of handling ticks in appropriate biocontainment facilities required for manipulating highly-pathogenic microorganisms [7]. To overcome this, several *in vitro* models have been developed to promote research on tick-borne pathogens. Here we review these *in vitro* models, highlighting their strengths and weaknesses.

2. Tick tissue culture-based models for study of pathogens *in vitro*

Over the past seven decades, the development of tick tissue, cell and organ culture systems has been driven predominantly by the need for *in vitro* models of tick-pathogen interactions to reduce the need for, or replace, live, intact ticks feeding on live vertebrate hosts. Maintaining a laboratory tick colony is labour-intensive, expensive and, in many countries, subject to restrictive legislation concerning host animal welfare and exotic pathogen carriers. *In vitro* model systems used to propagate and study tick-borne pathogens fall into three categories – relatively short-lived primary tick tissue and cell cultures, tick cell lines and tick organ cultures. Each system has advantages and disadvantages, and each has had a role to play historically in the development of *in vitro* models and in the understanding of tick-pathogen interactions.

2.1. Primary cultures

The first reported tick tissue cultures comprised tissue fragments or organs with some limited cell outgrowth or proliferation and a survival time of just a few days [8,9]. Techniques for longer-lived and repeatable primary ixodid tick tissue and cell cultures suitable for pathogen propagation were first developed in the 1960s [10-15]. These cultures, derived from developing adult ticks dissected out from the moulting nymphal integument, comprised viscera, adherent tissue fragments and cellular outgrowths, and survived for up to 25 weeks. The introduction of trypsin as a dispersing agent facilitated production of cell monolayers [16,17]. Some cell proliferation was observed, but continuous growth was not achieved. The main disadvantages of primary cultures as experimental models were the need for a constant supply of moulting nymphal ticks, the need for the ticks to be dissected aseptically and the lack of reproducibility. Attention was soon turned to tick eggs as a more easily-handled source of material for generation of reproducible primary cultures [18] (Figure 1), and this approach resulted in the first successful series of subcultures up to passage 14 [19]. The painstakingly-achieved improvements in techniques and culture media over the first two decades of tick tissue culture were about to yield dividends in the form of cell lines.

2.2 Cell lines

The first continuous ixodid tick cell lines were established in 1975 from developing adult *Rhipicephalus appendiculatus* tissues [20] and two of the original three lines are still in use today [21]. The first embryo-derived cell lines [22-24] were subsequently lost. However, these were soon followed by multiple cell lines derived from embryos of several *Dermacentor, Rhipicephalus* and *Hyalomma* spp. [25-32], most of which are currently extant [21].

Whereas all the aforementioned cell lines were derived from members of the Metastriata, the first, and subsequently most widely-used worldwide, cell lines from the prostriate tick species *Ixodes scapularis* were published in 1994 [33]. Establishment of the *I. scapularis* cell lines coincided with the explosion of interest in human pathogens transmitted by this and other tick species in North America [34-36], and subsequently the cells were distributed to research groups across the US and internationally, in particular the lines IDE8 [33] and ISE6 [37]. These were followed in the early 21st Century by cell lines from a second prostriate species, *Ixodes ricinus* [38,39], and several more metastriate species [21,40-49].

Despite their importance as vectors of human and livestock pathogens, cells from the argasid, or soft, ticks proved more challenging to propagate *in vitro* and were initially neglected. Apart from some early short-term primary cultures of soft tick haemocytes [50-52], sustained efforts to culture soft tick cells were made only within the past 15 years, resulting in embryo-derived cell lines from three argasid genera [21,53,54].

Cell lines (Figure 2) have now been established from most of the tick vectors of medical and veterinary importance in Europe, North and South America and Africa, and several of the species important in South and South-East Asia and Australia [21]. Notable exceptions include the genus *Haemaphysalis*, of which no cell lines currently exist, European and Asian *Dermacentor* spp., and significant Asian

prostriate vectors including *Ixodes persulcatus* and *Ixodes ovatus*. Techniques for their generation, maintenance and cryopreservation are well-established [21,32,33,39,53,54]; cell lines that are difficult or impossible to cryopreserve may instead be stored for several weeks or months at temperatures between 4 and 15 °C [39,53-55]. Details of the tick cell lines specifically mentioned in this review are presented in Table 1.

2.3 Organ cultures

These provide a different approach, focussing less on cell proliferation and more on maintaining the functions of cells, tissues and organs. Thus, tick organ cultures provide an environment well-suited to supporting host-dependent development of pathogens and studying their interactions with cells whose metabolic and physiological functions are maintained *in vitro*, rather than with isolated cells.

The earliest well-defined tick organ cultures comprised developing adult *R. appendiculatus* and *Dermacentor andersoni*, as mentioned above (section 2.1), maintained singly on coverslips in Leighton tubes or in groups in plastic flasks [10,14,56] (Figure 3A). These moulting nymph explants, when cultured between one-third and half way through the moulting period, can be maintained for several months, during which time digestion of the blood meal continues, midgut and Malpighian tubule peristalsis can be seen, chitinisation of parts of the external surface occurs (Figure 3B) and cellular outgrowth may occur [10]. Indeed, similar organ cultures initiated from developing nymphal (moulting larval) *A. variegatum* explants eventually gave rise to the continuous cell lines AVL/CTVM13 and AVL/CTVM17 [42,57].

Organ cultures initiated from unfed adult ticks fall into two categories – whole body explants and isolated organs. Whole body explants, with [56,58] or without [10] the adult integument, do not generally exhibit cell outgrowth or survive *in vitro* for as long as moulting nymphal explants, although 1/62 explants of adult *R. appendiculatus* whole body contents survived for at least 163 days with peristalsis and cell outgrowths [10]. Backless adult tick explants (Figure 3C) can survive for at least 32 days at 28 °C, during which tissue metabolism continues, indicated by accumulation of excretory products in the Malpighian tubules and rectal sac, and, if the mouthparts are not removed, some explants may imbibe the culture medium [58]. Legs and mouthparts may be removed from backless tick explants to minimise contamination in short-term organ cultures [59-62]. Whole body explants without the adult integument have been used successfully to isolate tick-borne bacteria when co-cultivated with tick cell lines [63-65].

Isolated organs from adult, and occasionally nymphal, ticks have been used in a variety of short- and longer-term studies focusing on tick physiology, pathogen propagation and/or development, and tick-pathogen interactions. The earliest studies, carried out on fed nymphal and adult female organs (ovaries, salivary glands, midguts and Malpighian tubules) from ticks of the genera *Hyalomma* and *Rhipicephalus*, demonstrated survival, determined by observation of peristalsis and examination of histological preparations, for 13-58 days *in vitro* [66]. Subsequent studies focussed on salivary glands; short-term organ cultures were used to unravel the mechanisms of salivation [67,68], and an elegant technique was developed for collection of saliva secreted by individual excised glands for up to 14 days *in vitro* [69]. Excised salivary glands, and occasionally other tissues such as midgut, synganglion, ovaries and Malpighian tubules, subsequently used in studies on pathogen metabolism, development and interaction with host tissues [58,70-73], were reported to survive for, at most, 9-12 days. However, when co-cultivated with tick cell lines, adult organs from some tick species may maintain viability as shown by midgut peristalsis for up to four months (Bell-Sakyi, unpublished observations of unfed adult *Dermacentor reticulatus* organs co-cultivated with BME/CTVM23 cells).

3. Studies on viruses

Several arbovirus families and genera have been studied using tick cell and organ cultures. Early reports delineated the wide range of tick- and insect-borne viruses that could be propagated in tick

primary cultures and cell lines [74]; more recently these models have been used in both fundamental and applied research to clarify many aspects of virus biology and virus-vector interactions. Selected studies are reviewed below.

3.1 Tick cell lines in arbovirus research

Arboviruses can infect tick cell lines, often persistently, with no inclusions or cytopathic effect visible in live cells. Interestingly, tick cell lines support the replication of tick-borne viruses (TBV) and some insect-borne viruses, while most non-vector-borne viruses failed to replicate [74,75]. Amongst TBV, viral replication may be sustained to higher titres and for longer in cell lines derived from known vectors, suggesting that tick cells may reflect some specificity of vector competence, as demonstrated for tick-borne encephalitis virus (TBEV) and Crimean-Congo hemorrhagic fever virus (CCHFV) [74,76]. Moreover, maintenance of TBV in tick cells may or may not modify the virus properties. After 10 serial passages in non-vector OME/CTVM21 cells, TBEV achieved virus titres similar to those in vector (IRE/CTVM19, IRE/CTVM20) cells and no change in plaque phenotype [54]. Similarly, *in vivo* studies on CCHFV and TBEV in ticks showed that persistent infection was not associated with significant changes in virus genome sequence over time [77,78]. In contrast, *in vitro* TBEV infections maintained by serial passage in either tick or mammalian cells were reported to promote the selection of variants that exhibited distinct plaque sizes and virulence in a mouse model [78]. The selection of virus variants seems to be linked to the co-existence of several sequences in the parental strain, suggesting that viruses such as TBEV exist as a heterogeneous population (quasispecies) that contains virus variants pre-adapted to reproduction in different environments, probably enabling virus survival in ticks and mammals [79].

Comparison of virus replication in mammalian and tick cells could allow identification of essential elements required for virus infection in tick cells. Although comparison of the morphogenesis of Dugbe virus, a close relative of the highly pathogenic CCHFV, in tick and mammalian cells showed a strong similarity in viral protein localization [80], the maturation process of TBEV exhibited different features in tick and mammalian cells [81,82]. Interestingly, Uukuniemi virus particles derived from vector tick cells were shown to have glycosylation and structural specificities that may influence the initial infection in mammalian hosts highlighting the importance of working with viruses originating from arthropod vector cells when investigating the biology of arbovirus transmission and entry into mammalian host cells [83]. In this regard, it has been reported that N-glycosylation of the TBEV envelope protein E affects protein trafficking and virus infectivity in mammalian cells but not in tick cells [84]. Moreover, a significant difference was observed in the mass spectrometric profiles of N-glycans linked to the E protein between TBEV grown in human neuronal and IRE/CTVM19 cells [85]. The nucleoprotein of CCHFV is characterized by a highly-conserved DEVD motif that is cleaved by caspase-3 during the induction of apoptosis in mammalian cells, and probably plays a role in apoptosis modulation [86]. However, tick cells can be persistently infected by CCHFV without any sign of cell death. A recombinant CCHFV with a mutated DEVD motif failed to replicate in HAE/CTVM9 cells while showing only slightly reduced replication in mammalian cells, suggesting an essential role for the DEVD motif only in vector cells [87].

Chimeric virus-like particles were developed to study TBEV viral genome packaging and cellular factors in ISE6 cells. Compared to mosquito-borne flaviviruses, they demonstrated the existence of specific cellular factors involved in vector specificity [88]. The role of protein C in both viral assembly and RNA replication of flaviviruses was demonstrated using chimaeric TBEV and West Nile virus in *I. ricinus* cells, probably by interaction with host cell factors required to set up the cell for RNA replication [89]. Proteins that could be associated with viral infection and replication were identified in the proteome of ISE6 cells following infection with Langat virus (LGTV) [90]. RNA interference (RNAi)-mediated transcript knockdown of ten tick genes in ISE6 cells resulted in decreased infectious LGTV replication for nine of the genes and reduced LGTV negative strand genome replication for two of the genes [91].

To characterize the cellular antiviral response in the vector using tick cells as a model, siRNAs were shown to play a role in suppressing Hazara virus replication in ISE6 cells following a mechanism similar to the one found in other eukaryotes [92,93]. Long dsRNAs were found to be more efficient than siRNAs to induce silencing of target genes in tick cells, expanding possibilities for studying the role of tick genes in modulation of virus infection [94]. Evaluation of the antiviral RNAi response of ISE6 cells against LGTV identified key Argonaute (Ago) proteins involved in RNAi, virus-derived small interfering RNAs longer (at 22 nucleotides) than those from other arbovirus vectors that mapped at highest frequency to the termini of the LGTV genome, and expression by tick-borne flaviviruses of subgenomic RNAs that interfere with tick RNAi [95].

In the context of virus-vector interactions, the role of the well-conserved *I. scapularis* organic anion transporting polypeptides (OATPs) was examined in ticks infected with LGTV [96]. While infection of unfed nymphal ticks with LGTV *in vivo* did not result in significant changes in *oatps* gene expression, specific genes were significantly downregulated upon LGTV infection of ISE6 cells *in vitro*. Treatment of tick cells with OATP inhibitor significantly reduced LGTV loads suggesting a role of arthropod OATPs in vector-virus interactions [96]. In the presence of LGTV, expression of arthropod IsSMase, a sphingomyelinase D that catalyzes the hydrolytic cleavage of substrates such as sphingomyelin (SM) lipids, was significantly reduced in both *I. scapularis* ticks *in vivo* and ISE6 cells *in vitro* [97]. The LGTV-mediated suppression of IsSMase allowed accumulation of SM lipid levels supporting membrane-associated viral replication and exosome biogenesis, suggesting a role for arthropod IsSMase in tick-LGTV interactions and its function in vector defence mechanism(s) against TBV infection and in anti-viral pathways [97]. *In vivo* studies in ticks are required to confirm the roles of other antiviral responses, identified in studies focusing on flaviviruses in *Ixodes* spp. cell lines [98-100].

Some arthropod genomes contain non-retroviral integrated RNA virus sequences (NIRVS) that are a substrate for the production of short RNAs involved in the response to viral infections. NIRVS originate by integration of DNA derived from the retrotranscription of small regions of viral RNA genomes and may modulate the outcome of infection. In particular, ISE6 cells and *I. scapularis* ticks both contain many bunya- and orthomyxo-like NIRVS sequences, suggesting that ticks are a dominant host for these virus groups [101]. Furthermore, the genomes of OME/CTVM21 and other *Ornithodoros moubata* cell lines, as well as some populations of *O. moubata* ticks, harbour African swine fever virus (ASFV)-like integrated elements that may interfere with ASFV infection [102].

3.2 Tick organ cultures in arbovirus research

In the first report describing the *in vitro* growth of an arbovirus in tick organ cultures, the kinetics of replication of Colorado tick fever virus (CTFV) were followed in vector (*D. andersoni*) developing adult explants [15]. A latent phase of ~10 days preceded detection of active viral replication, the virus persisted in the tissues until 166 days post infection, and the titre in the medium decreased with the senescence of the organ culture. Moreover, using nymphal ticks infected by feeding on viraemic animals, CTFV replicated at much higher levels *in vitro* in developing adult explants than *in vivo* in live, intact ticks, showing the efficiency of this system for virus propagation.

Hyalomma spp. developing adult explants were used to compare virus reproduction during single or mixed infections with TBEV and Powassan virus (POWV) [103]. Both viruses persisted for several months in tissue explants from *Hyalomma dromedarii* with reproduction peaking after 2-3 months and ceasing at the death of the cultured explants. Only POWV was able to persist in *Hyalomma anatolicum* explants while TBEV was apparently eliminated [103]. In a study of the variability of POWV after 11 serial passages in *H. anatolicum* ticks or prolonged maintenance (86 days) in developing adult explants, both *in vivo* and *in vitro* tick-derived viruses were less pathogenic in mice compared with the wild type strain [104].

Rhipicephalus evertsi developing adult explants and REE/CTVM28 cells were used to evaluate if tick midgut cells can be infected by alphaviruses, occasionally detected in, but not known to be transmitted by, ticks. Results obtained with an eGFP-expressing Semliki Forest virus (SFV), showed

that midgut cells were not infected, suggesting that alphaviruses can be ingested by ticks during feeding but they cannot infect midgut cells thereby establishing a systemic infection [105].

In a series of studies using short-term *I. scapularis* organ culture models to investigate flavivirus replication and dissemination, midgut and salivary glands remained metabolically active for 10 days and synganglion for 9 days [73]). The organ cultures were permissive to LGTV and POWV infections, determined using immunohistochemistry and an eGFP-expressing LGTV, and RNAi-mediated transcript knockdown of a viral 3'UTR genomic region was demonstrated in both midguts and salivary glands [73]. For both viruses, production of infective virus was quantified in salivary gland cultures from male and female ticks [106,107], and knockdown of the *I. scapularis* vanin gene confirmed its involvement in flavivirus replication [106], previously demonstrated in ISE6 cells [91].

4. Studies on bacteria

4.1. *Anaplasma marginale*

The obligate intraerythrocytic tick-borne bacterium *Anaplasma marginale* is the etiological agent of bovine anaplasmosis, a disease characterized by anaemia, fever, abortion, and death, leading to significant economic losses for dairy and beef producers worldwide [108].

The first successful *in vitro* cultivation of *A. marginale* was achieved in the mid-1990s, firstly in IDE8 cells [109] and later in ISE6 cells [110]. Since then, these non-vector cell lines have been successfully used to propagate and characterize different isolates of *A. marginale* throughout the world. However, the most important contribution from this system has been its suitability, usefulness and practicability for comparative *in vivo*/*in vitro* studies. As reviewed previously [111], cultivation of *A. marginale* in IDE8 or ISE6 cells allowed not only a considerable reduction in cattle for *in vivo* infections, but also a variety of *in vitro* studies, providing results comparable with *in vivo* models. The vector cell line BME26 has also been used for gene expression studies in response to *A. marginale* infection [112].

A. marginale cultures are initiated from infected bovine blood, collected during ascending bacteraemia. Culture flasks containing growing layers of IDE8 or ISE6 cells are inoculated with infected blood stabilates, sealed and incubated at 32-34 ^{o}C with weekly medium changes [109,110]. Initially, compact colonies are observed inside well-defined parasitophorous vacuoles; two or three weeks later, large colonies are formed, and their contents are released into the culture medium after disruption of the vacuole and cell membranes. *A. marginale*-infected cells can be propagated continuously by serial passage onto naïve tick cells, and can reach infection rates up to 80%, retaining their infectivity and antigenic properties after successive passages [109,113]. Cultured cells can be monitored by direct examination under an inverted microscope and/or by microscopic examination of Giemsa-stained cytocentrifuge smears (Figure 4A).

Thus, apart from providing suitable material for diagnostic tests, immunization trials and ultrastructural characterization of distinct geographical isolates, these three cell lines, IDE8, ISE6 and BME26, have been widely used as *in vitro* models to evaluate a variety of interactions between *A. marginale* and tick cells, in studies on surface proteins [113], protein mutants [114], and functional studies using RNAi to discover genes/proteins that are differentially expressed in tick cells in response to infection with *A. marginale* [115].

The ISE6 cell model has been used in screening to identify *A. marginale* proteins upregulated during colonisation of the tick vector [116], while IDE8 cells have been used to evaluate activation of stress responses to *A. marginale* infections [117]. More recently, ISE6 cells provided the basis for *in vitro* experiments using transposon mutagenesis of *A. marginale*, coupled with *in vivo* assessment of altered phenotypes, to identify genes associated with virulence, leading to the possibility of inducing deliberately attenuated organisms with reduced infectivity for cattle [114].

4.2. *Anaplasma phagocytophilum*

Anaplasma phagocytophilum, a tick-transmitted granulocytotropic bacterium, is an emerging zoonotic infection [118], gaining increasing attention in veterinary medicine as the agent of tick-borne fever in ruminants and granulocytic anaplasmosis in companion animals, including dogs, cats, and horses [119]. Humans are accidental hosts [118], manifesting the so-called human granulocytic anaplasmosis. Cultures are initiated by adding granulocytes from blood of infected hosts, after hypotonic lysis of erythrocytes, into IDE8, ISE6, IRE/CTVM19 or IRE/CTVM20 cells, often under reduced O_2 [120-124]. Once established *in vitro*, culture conditions are basically the same as those for *A. marginale*.

ISE6 cells have allowed identification of an *A. phagocytophilum*-derived protein associated with the pathogen-occupied vacuolar membrane, expressed late during infection of tick salivary glands [125], and of genes involved in *A. phagocytophilum* infection/multiplication and the tick cell response to infection *in vivo*, with inhibition of apoptosis and promotion of cytoskeleton rearrangement for infection of tick cells [126]. IRE/CTVM19 cells have been used to confirm, through gene silencing, that *A. phagocytophilum* uses fucose to colonise ticks, revealing a novel mechanism of pathogen colonisation in arthropods [127], as well as to prove the induction of actin phosphorylation to selectively regulate gene transcription in *I. scapularis* ticks [128], and to identify a protein facilitating the migration of *A. phagocytophilum* from the tick midgut to the salivary glands [129]. Recently, IRE/CTVM20 cells allowed the identification of three *I. scapularis* genes potentially involved in the synthesis of α-Gal that is essential for tick feeding, suggesting that increased α-Gal levels in response to *A. phagocytophilum* infection occur to control bacterial infection [130].

Transcriptomic and proteomic studies to evaluate stress response proteins in ticks and ISE6 cells after *A. phagocytophilum* infection demonstrated activation of responses in both systems. However, these results did not reflect the natural vector-pathogen relationship, in which such responses were not strongly activated [117]. IDE8 and ISE6 cells were used to demonstrate that nuclease Tudor-SN is involved in tick dsRNA-mediated RNAi and tick feeding but not in response to *A. phagocytophilum* [131]. Through a quantitative proteomic approach, ISE6 cells were used to characterize *A. phagocytophilum* proteins involved in infection of the tick vector, allowing identification of differences in the proteome of *A. phagocytophilum* in infected ticks with higher impact on protein synthesis and processing than on bacterial replication in tick salivary glands [132]. In addition, ISE6 cells have been used to better understand the dynamics of *A. phagocytophilum*-tick interactions, such as the existence of plasticity in the immune deficiency pathway of arthropods, restricting *A. phagocytophilum* colonisation of *I. scapularis* [133] and the up-regulation by *A. phagocytophilum* of an *I. scapularis* organic anion transporting polypeptide for its survival in this tick species [134]. Further studies indicated that *A. phagocytophilum* uses a tick transcriptional activator protein-1 in regulation of an arthropod antifreeze gene, suggesting a novel mode of arthropod signalling for the survival of both pathogen and vector in the cold [135], and a tick kinase facilitating *A. phagocytophilum* colonisation and survival in the arthropod vector [136]. Regarding survival in the vector and transmission to the vertebrate host, another study provided evidence of down-regulation of tick microRNA-133, inducing organic anion transporting polypeptide expression, which appeared to be critical for *A. phagocytophilum* survival in the vector and its transmission to the vertebrate host [137]. Recently, a model combining IDE8 and ISE6 cells, *I. ricinus* ticks and sheep was established that mimics the entire transmission cycle of *A. phagocytophilum* in the laboratory; infection with an ovine strain was passed from tick cells into sheep, and from infected sheep to naïve sheep via either tick cells or feeding ticks [138].

Thus, for both *A. marginale* and *A. phagocytophilum*, these *in vitro* tick cell culture systems have opened a wide spectrum of possibilities to study a variety of tick-host-pathogen interactions in both vertebrate and invertebrate hosts under controlled conditions, allowing comparative *in vitro*/*in vivo* studies never possible before.

4.3 *Ehrlichia* and *Rickettsia*

Like the *Anaplasma* spp. covered in the previous section, most strictly tick-transmitted bacterial pathogens are obligately intracellular, and cannot replicate in the extracellular environment. These include other members of the order Rickettsiales – *Ehrlichia* spp. and *Neoehrlichia* spp. that form colonies of multiplying bacteria, or morulae, within cytoplasmic vacuoles, and *Rickettsia* spp. that grow directly in the host cell cytoplasm (Figure 4B). Some examples of the use of tick cell lines as models for study of *Ehrlichia* and *Rickettsia* spp. will be reviewed.

The immunodominant surface proteins of the human pathogen *Ehrlichia chaffeensis*, and the closely-related canine pathogen *Ehrlichia canis*, are encoded by multigene families. Protein expression studies of *E. chaffeensis* and *E. canis* grown in tick cell lines [44,139] confirmed previous observations on differential transcription of genes encoding their immunodominant outer membrane proteins in tick and mammalian hosts [140,141]. Two of the proteins encoded by members of the *E. chaffeensis* p28-Omp multigene family were predominantly expressed in infected canine macrophage (DH82) cultures, whereas a single, different p28-Omp protein was expressed in infected vector (AAE2) and non-vector (ISE6) tick cell lines [44,139]. Similarly, three of the proteins encoded by the *E. canis* p30-Omp multigene family were expressed in infected DH82 cultures, while the protein encoded by a single, different p30-Omp member was expressed in infected, non-vector (ISE6) tick cells.

Confirmation that gene transcription and protein expression by *E. chaffeensis* grown in tick cell lines resembled that reported for immunologically important antigens *in vivo* led to a series of studies utilising tick cells as models. Genome-wide transcriptional analysis confirmed differential expression of over a third of *E. chaffeensis* genes, including the p28-Omp multigene family, in tick cells (ISE6 and AAE2) and human monocytes [142]. The immune response of mice inoculated with bacteria derived from ISE6 cells was found to be slower and initially less effective than that induced by canine cell-derived bacteria [143]. White-tailed deer, the natural host of *E. chaffeensis*, developed higher antibody levels and less frequently-detected persistent rickettsaemia following experimental infection with bacteria derived from tick (ISE6) cells than with mammalian (DH82) cells; the tick cell-derived bacteria induced an immune response similar to that induced by feeding infected adults of the natural vector *Amblyomma americanum* [144].

The major antigenic surface proteins of *Ehrlichia ruminantium*, causative agent of heartwater in ruminants, are also encoded by a multigene family, MAP-1 [145]. Comparison of transcription of the *map-1* genes in a panel of vector (AVL/CTVM13) and multiple non-vector tick cell lines with transcription in bovine endothelial cells revealed differences in expression patterns between genes in the various cell types, with only two of the 16 paralogs transcribed in all the cell lines. The *map1* gene predominated in bovine cells while the *map1-1* gene predominated in tick cells [41,146]. The importance of these two genes in the tick stages of *E. ruminantium* was confirmed when expression of *map1-1*, but not *map1-1*, was detected in midguts, but not salivary glands, of unfed, infected adult *A. variegatum* whereas both transcripts were detected in both tissues of 4-day fed ticks [147]. Proteomic analysis revealed expression of MAP1 in infected bovine endothelial cells and MAP1-1 in infected tick cells [148].

Interestingly, while other Anaplasmataceae grown in tick cell lines are infective and, in most cases, pathogenic when inoculated into susceptible mammalian hosts [109,120-123,143,149], *E. ruminantium* grown in either vector or non-vector tick cell lines failed to induce heartwater disease in almost all inoculated sheep [150]. However, infected vector (AVL/CTVM13) cells induced protection against homologous or heterologous needle challenge with bovine cell-derived *E. ruminantium* in 27/31 sheep, while infected IDE8 cells did not induce either a detectable antibody response or protection in 5/5 sheep [150]. Unfortunately, the ability of *E. ruminantium*-infected AVL/CTVM13 cells to protect against heartwater disease was subsequently lost, possibly as a result of disappearance from the cell line of a cell type essential for development of immunogenic bacteria [151], highlighting the inconsistent nature of some tick cell lines.

Most pathogenic *Rickettsia* spp. must be handled at BSL3, posing particular problems for studies on tick-bacterial interactions. Thus, as with highly pathogenic viruses such as CCHFV, tick cell cultures are a useful substitute for live, intact ticks enabling a range of studies at the cellular and molecular level. Growth of *Rickettsia rickettsii*, causative agent of Rocky Mountain spotted fever in humans, was compared in tick (DALBE3 and IDE2) and mammalian cell lines at temperatures between 28 and 34 °C; raising the incubation temperature induced expression of rickettsial proteins in infected tick cells possibly associated with pathogenicity for mammalian cells [152]. In the absence of a louse cell line, tick (ISE6) and insect (Sf9) cell lines were used as models to analyse the effect on the proteome of *Rickettsia prowazekii*, causative agent of louse-borne human epidemic typhus, of growth in arthropod and mammalian environments [153]. In that study, rickettsial stress response proteins were upregulated in both arthropod cell lines and in a murine cell line, compared to levels in bacteria grown in hen egg yolk sacs, indicating possible limitations of cell cultures to model the *in vivo* situation. Nevertheless, comparison of siRNA expression profiles and coding transcriptomes of *R. prowazekii* grown in tick (AAE2) and human cell lines revealed novel siRNAs unique to arthropod cells and evidence for alternative transcription start sites used by rickettsial genes depending on the host cell environment [154]. A review of tropism of a range of pathogenic *Rickettsia* spp. found that the arthropod host range *in vivo* was reflected in the susceptibility of tick and insect cell lines *in vitro*, with tick-borne spotted fever group *Rickettsia* generally growing better in tick cells and insect-borne typhus group *Rickettsia* growing better in insect cells [155]. A recent study using both tick cell lines and experimentally-infected vector ticks found that while two *Rickettsia parkeri* proteins, RickA and Sca2, played a role in actin polymerisation in tick cells *in vitro* and *in vivo*, their absence did not affect patterns of *R. parkeri* dissemination in live, intact ticks [156].

4.4 *Borrelia*

Unlike the aforementioned bacterial genera, *Borrelia* spp. spirochaetes, causative agents of Lyme borreliosis and relapsing fever, are predominantly extracellular, living within the tick midgut lumen and haemocoel. Tick cells are not essential for replication, but form a substrate for anchorage and can be used to study spirochaete-cell interactions *in vitro*. The *Borrelia burgdorferi sensu stricto* outer surface protein A (OspA) plays an important role in attachment of spirochaetes to tick midgut cells [157]; *B. burgdorferi* s.s. spirochaetes co-cultivated with vector tick (ISE6) cells at temperatures between 31 and 37 °C showed a greater reduction in OspA expression with increasing temperature than spirochaetes grown axenically [158]. In contrast, expression of the *B. burgdorferi* outer surface protein C (OspC), upregulated during transmission tick feeding [159], increased with temperature in spirochaetes co-cultivated with tick cells while remaining unchanged in axenic cultures [160]. The first study to demonstrate that *B. burgdorferi* s.s. has a functional stringent response, enabling it to respond to situations of nutrient depletion or starvation encountered in unfed, host-seeking ticks [160], was carried out in vector (IDE8 and ISE6) cell lines [161].

An interesting study compared the ability to phagocytose and destroy live *B. burgdorferi* s.s. spirochaetes of vector (IDE12 and ISE6) and non-vector (DAE15) tick cell lines [162]. IDE12 and DAE15 cells were highly phagocytic, with over 80% of cells containing spirochaetes after 24 h, while with ISE6 cells the spirochaetes remained extracellular and appeared viable. DAE15 cells phagocytosed spirochaetes faster and in higher numbers than IDE12 cells. The ability of the non-vector *D. andersoni* DAE15 cells to rapidly ingest and destroy *B. burgdorferi in vitro* [162] reflects the reported ability of non-vector *Dermacentor variabilis* ticks to destroy inoculated spirochaetes using both cellular and humoral responses [163]. More recently, siRNA-mediated RNAi transcript knockdown in ISE6 cells and in *I. scapularis* and *D. andersoni* ticks was used to examine the role of components of the tick IMD pathway in infection with *B. burgdorferi* s.s., *A. phagocytophilum* and *A. marginale* [133]. Good agreement was obtained between the *in vitro* ISE6 model and live, intact ticks inoculated with siRNAs for several IMD pathway genes identified as having positive or negative effects on replication of all three pathogens.

5. Studies on protozoa

Two genera of intracellular tick-borne protozoan parasites, *Babesia* and *Theileria*, transmitted exclusively by ticks during blood feeding, have been studied in tick culture systems; in addition, some protozoan parasites not known to be transmitted by ticks have also been propagated in association with tick cell lines.

5.1 *Babesia*

In early experiments with organs from nymphal and adult female *Rhipicephalus annulatus* infected with *Babesia bigemina*, infected tick tissues were kept alive in an artificial nutrient medium [70]. Further development of parasites was observed in intestinal and ovarian tissue and in haemocytes. An experimental infection with salivary gland forms of these parasites in the culture fluid was, however, negative in calves. Development of *Babesia merionis* (previously *Nuttalia danii*) was observed in cultured salivary glands removed from *Hyalomma anatolicum excavatum* nymphs fed as larvae on infected gerbils [71]. The parasite continued to develop in the cultivated glands; however, there was no evidence for development of mammalian-infective particles.

Several studies used merozoites derived from infected host erythrocytes in attempts to propagate *Babesia bovis* in tick cell cultures. In embryo-derived *Rhipicephalus microplus* cells inoculated with infected bovine erythrocytes, *B. bovis* merozoite numbers increased ~20-fold over two days, but resembled the blood forms more closely than those of the gut or salivary gland forms in ticks [164]. Using the cell line BmVIII, *B. bovis* merozoites derived from parasitised bovine erythrocytes were found in tick cells both as phagocytised free organisms and in phagocytised erythrocytes which were all lysed by 72 h [165]. No replication of *B. bovis* was observed. In contrast, in an electron microscopic study of BmVIII-SCC cells inoculated with *B. bovis*-infected erythrocytes, sexual stages of the parasite normally found only within tick intestine were observed [166]. However, it was not clear at what point the parasites transformed to the sexual stage.

Subsequent studies used kinetes derived from the haemolymph of ticks. *Babesia caballi* kinetes from haemolymph and organs of *Dermacentor nitens* (previously *Anocentor nitens*) were co-cultivated with vector and non-vector tick cell lines ANE58, RAE25 and RSE8 [31]. Cells infected with *B. caballi* degenerated and lysed. The parasites remained in the cultures for 3-5 days but did not develop further and disappeared after a week. Similarly, *B*. *bigemina* kinetes obtained from haemolymph of infected *R. microplus* ticks penetrated primary embryo-derived *R. microplus* cells [167]; after 10 days *in vitro*, the viability of the kinetes was 95%. A single round of *in vitro* multiplication of *B. bigemina* kinetes from haemolymph of engorged females of *R. microplus* was described in the IDE8 cell line in which further development of the parasite in tick cells was demonstrated by light microscopy [168]. In a different approach, haemocytes from engorged female *R. microplus* ticks infected with *B. bigemina* kinetes were cultured and immature and mature kinetes were recognized [169]. Cultured kinetes were cryopreserved in liquid nitrogen and were subsequently successfully resuscitated, demonstrating that the culture procedure had not appreciably interfered with pathogen viability.

5.2 *Theileria*

The theilerioses are tick-borne diseases of ruminants caused by obligate intracellular protozoa of the genus *Theileria,* which are responsible for immense losses in domestic livestock. Although these organisms are of great importance in the veterinary field, cultivation in tick cell lines has not been reported. Maturation *in vitro* of *Theileria parva* in backless tick explants was compared with that in cultured excised salivary glands derived from already-infected ticks [58]. Backless tick explants and excised salivary glands showed similar numbers of infected acini per infected tick when cultured at 36 °C, possibly due to the high temperature [170]. However, after 12 days at 28 °C, backless tick explants showed 20-30 times as many infected acini per infected tick as excised salivary glands.

Two organ culture techniques were applied to moulting nymphal and adult *H. anatolicum* ticks infected with *Theileria annulata* [56]. Moulting nymph explant cultures (Figure 3A), set up from

developing adult ticks before the time of kinete migration, released large numbers of *T. annulata* kinetes into the supernatant medium (Figure 4C) at the same time as they were seen in intact ticks. Some of the kinetes subsequently infected the salivary glands of the explants at levels comparable to those in intact ticks. Backless tick explant cultures set up from unfed, infected adult ticks supported the maturation of *T. annulata* from sporoblasts to sporozoites infective for bovine lymphocytes *in vitro*. Whole nymph explant cultures set up before the time of kinete migration supported development of *T. annulata* from zygotes to infective sporozoites, but at much lower levels than in intact ticks, indicating that the *in vitro* environment did not satisfactorily replicate conditions *in vivo*.

5.3 *Trypanosoma*

Members of the genus *Trypanosoma* are parasites of all vertebrate classes and numerous species are of medical and/or veterinary importance. The first observation of trypanosomes in ticks was made over 100 years ago [171] and in 1986 the first experimental biological transmission of *Trypanosoma theileri* to cattle by the tick *H. anatolicum* was described [172]. However, the role of ticks in natural transmission of trypanosomes is unclear. Several recent studies reporting isolation into tick cell culture of trypanosomes from field ticks confirmed the ability of these arthropods to harbour viable parasites, but left open the question of whether or not transmission could occur during tick feeding.

Successful isolation and propagation in IDE8 cultures of two novel species of the genus *Trypanosoma*, naturally infecting Brazilian ticks, was achieved with, respectively, haemolymph obtained from *R. microplus* removed from cattle and crushed nymphal and adult *Amblyomma brasiliense* from a white-lipped peccary *(Tayassu pecari)* [173,174]. After isolation, both species grew axenically in L-15B medium. Very recently, another trypanosome was isolated into ISE6 cells from questing *I. ricinus* and partially characterised [175]. Analysis of the resultant DNA sequences suggest that this trypanosome may be a new species closely related to several species or strains of trypanosomes isolated from, or detected in, ticks in South America and Asia [173,174,176], and to *Trypanosoma caninum* isolated from dogs in Brazil [177].

5.4 *Leishmania*

Leishmania spp. parasites cause leishmaniasis, an infectious disease that occurs worldwide in humans and domestic animals. In the sandfly vector, the parasites exist as extracellular flagellated promastigotes, whereas in the mammalian host, they are usually found within phagocytic cells as amastigotes.) *Leishmania donovani* and *Leishmania major* were propagated as intracytoplasmic amastigotes in unnamed tick cell lines derived from embryonic *R. appendiculatus* and *Rhipicephalus evertsi* [178] at 37 °C. When the temperature was lowered to 26 °C, motile promastigotes were observed. However, evaluation of the vectorial capacity of *Rhipicephalus sanguineus* for transmission of canine visceral leishmaniasis did not confirm that maintenance and multiplication of *Leishmania* occurs within the ticks [179].

5.5 *Besnoitia*

Besnoitia besnoiti is the causative agent of bovine besnoitiosis. It is an obligate intracellular cyst-forming coccidium and affects mainly young cattle. Cattle act as intermediate hosts and the final host is unknown. Experimentally, *B. besnoiti* can be transmitted between cattle by bloodsucking insects [180]; although there is no evidence that ticks are involved in transmission, it was suggested that this avenue should be explored [181]. In a series of studies, *B. besnoiti* was cultured in four different tick cell lines, RA243, BmVIII-SCC, RML-15 and RAE25, for up to 7 months [182-184]. The authors did not find appreciable differences in parasite proliferation in the various cell lines; however, the yield of parasites was lower in tick cells than in mammalian Vero cells.

6. Tick cell cultures as models for studying tick-pathogen interactions: advantages and limitations

6.1 Tick cell lines

Tick cell lines, all derived as a result of spontaneous growth initiated after prolonged *in vitro* maintenance, are phenotypically heterogeneous, usually comprising a mixture of cells that may be relatively undifferentiated or cells such as haemocytes that are clearly differentiated but still undergo multiplication and exhibit functions such as phagocytosis [21,162,185]. Tick cell line genomes may be modified through gain or loss of chromosomes [186], and it is therefore important to account for the differences in encoded genetic information and downstream cellular processes when interpreting particular sets of data comparing studies in live ticks and tick cell lines.

In contrast to tumour-derived mammalian cell lines, in which cellular pathways linked to oncogenes are deregulated thereby influencing outcomes of pathogen interaction with the cells [187], arthropod cell lines are spontaneously immortalised and thus more likely reflect the cell biology of the vector from which they are derived. Yet some arthropod cell lines may not fully reflect the genetics of the arthropod from which they are derived. For instance, the *Aedes albopictus* cell line C6/36 [188] has a defective RNAi pathway and its genome contains integrated endogenous viral elements (EVEs) in the form of DNA copies of a flavivirus genome [189]. These changes in the cell line may explain why it is highly permissive to a wide range of arboviruses [189]. As EVEs in arthropods/arthropod cells are suspected to act as templates for generating PIWI-interacting RNAs (piRNAs), they may represent a source of immunological memory in these cells [190]. EVEs have been identified in genomes of both insects and ticks including sequences of viruses classified within several genera [191-195]. For example, the ASFV-related EVEs within the genomes of *O. moubata* ticks and cell lines are likely the origin of siRNA and piRNA interference responses against ASFV in ticks [102]. While infection of *O. moubata* and *O. porcinus* ticks resulted in detection of low levels of viral RNA transcripts, the cell lines OME/CTVM21, OME/CTVM22, OME/CTVM24 and OME/CTVM27 were resistant to infection with ASFV, with no detectable viral transcription. These results should be interpreted with caution, since the resistance of cell lines may not necessarily be linked to an innate immune response triggered by EVEs, rather it could be a question of susceptibility to infection linked to cell attachment and entry. It is thus difficult in this case to consider the cell line as reflecting virus-vector interactions in live ticks.

Tick cell lines also harbour endogenous viruses [48,196], including St Croix River virus, the first endogenous tick virus identified over a decade ago in IDE2 cells [197]. Some argasid cell lines examined by electron microscopy showed structures suggestive of bunyaviruses further confirmed by sequencing [48,196]. Viral sequences related to iflaviruses, bunyaviruses, Drosophila A virus and *I. scapularis*-associated virus-1 (which was also identified in *I. scapularis ticks)* were identified in ISE6 cells [198] and a novel rhabdovirus was detected in IRE/CTVM19 cells [199].

Replication of endogenous viruses in insect cell lines may significantly reduce or upregulate replication of other viruses [196,200]. Similarly, endogenous viruses in tick cells may modulate pathogen growth or be themselves modulated by the infecting pathogen. Such viruses must have originated from the parent arthropods; however, they may or may not modulate pathogen growth *in vivo*. Arthropod-specific viruses have been proposed as control strategies to modulate arbovirus replication in hematophagous arthropods [201].

TBV are usually associated with specific tick genera or species. For instance, *D. andersoni* cells support the growth of CTFV [15], while *I. scapularis* cells are non-permissive to the virus. CTFV produced in BHK-21 cells and inoculated into IDE2 or IDE8 cells, at a multiplicity of infection of 1 plaque-forming unit (pfu)/cell, failed to replicate in either of the two tick cell lines. Real-time RT-PCR targeting genome segment 9, carried out on RNA extracted 7 and 14 days post-inoculation, showed no evidence of CTFV genome replication (Attoui and Mohd Jaafar, unpublished observation). The choice of the species from which cells are derived is crucial in terms of relevance, as certain TBV can infect cell lines derived from multiple different tick genera/species [74]. For instance, Alkhumra haemorrhagic fever virus, which has been found in *Ornithodoros savignyi*, replicates in the tick cell lines HAE/CTVM9, RAE/CTVM1 and

OME/CTVM24 [202]. Yet detecting viral RNA by RT-PCR, or viral antigens by immunohistochemistry, does not reflect full replication functionality in a particular cell line and/or virus assembly. Indeed, while progeny infectious viruses were detected in both RAE/CTVM1 and OME/CTVM24, none could be detected in HAE/CTVM9, reflecting a probable abortive replication. Recent studies in *I. ricinus* ticks and cell lines of Kemerovo virus (KEMV), transmitted by *I. persulcatus* and, rarely, *I. ricinus*, suggest that the virus replicates in IRE/CTVM20 but not IRE11 or IRE/CTVM19 cells (Migné et al, manuscript in preparation). Despite initial virus titers of $>10^6$ pfu/ml produced in IRE/CTVM20 cells, KEMV replication was undetectable after three months. Replication in an arthropod cell line does not imply that the virus can be transmitted by the particular arthropod from which the cells were derived. While insect-borne viruses such as the mosquito-borne alphavirus SFV replicate well in multiple tick cell lines [74,105], the biological significance of this is far from reflecting vector capacities of the parent ticks or inferring relevant data regarding virus-vector interactions *in vivo*.

As with TBV, the pairing of live tick and tick cell line model is essential to allow a relevant comparison. In the study of *R. parkeri* in non-vector ISE6 cells, mobility was shown to be driven by two rickettsial proteins, which when mutated no longer permit actin polymerisation, thus inhibiting actin-based mobility [156]. However, despite mutating these two proteins in vector *Amblyomma maculatum* ticks, the bacterial dissemination pattern was unaffected. In the absence of any *A. maculatum* cell lines, this study would have been more informative if the *in vitro* work had used another *Amblyomma* sp. cell line. Thus, in the study of the role of innate immune response in ISE6 tick cells and *I. scapularis* ticks, silencing of genes such as Bendless, uev1a and relish made both ISE6 cells and live ticks more susceptible to infection by *A. phagocytophilum* [133]. However, the heightened susceptibility to the bacteria of ISE6 cells upon silencing other genes such as caspar was not observed in live ticks, in which infection was rather reduced. Therefore, while care should be taken when extrapolating *in vitro* results to the *in vivo* situation, sometimes an *in vitro* result, however unlikely, may be subsequently validated *in vivo*, as seen with cultivation of *E. ruminantium* in *R. microplus* cells [42] and the recent demonstration of transovarial transmission of the bacterium in *R. microplus* field ticks in West Africa [203].

6.2 Tick organ cultures

As they more closely replicate the functions of tick tissues, organ cultures are attractive models for studying vector-pathogen interactions, as detailed in the preceding sections. However, even here caution is required, as in organ culture a pathogen may perform better, as in the case of increased replication of CTFV in *D. andersoni* nymphal explants [15], or worse, as in the case of reduced development of *T. annulata* in *H. anatolicum* nymphal explants [56], than in the live, intact tick. The studies of *I. scapularis* organ cultures infected with tick-borne flaviviruses [73,106,107] demonstrated the usefulness of these cultures in assessing replication in different organs, particularly the midgut which represents an important barrier to tick infection following blood feeding, and salivary glands which can be a barrier to transmission if not permissive to virus replication. Susceptibility and permissiveness of organs can therefore be examined *in vitro* and likely reflect the biological infection in live ticks. Organ cultures are also useful for RNAi studies and thus are relevant in studies of virus-vector interactions.

7. Conclusions and future prospects

Considering the expansion of tick populations and the increasing incidence of tick-borne diseases, it is essential to promote tick-borne pathogen research to prevent the risk of outbreaks from pathogens affecting human and animal health. We have shown that tick cell and organ culture systems have an important and relevant role to play in such research; however, caution is required when extrapolating from the *in vitro* model to the *in vivo* situation, particularly in the areas of vector competence and pathogen transmission. An *in vitro* model can never fully replicate the physiological and immunological complexities of a living tick; in the absence of sufficient physiological triggers, protozoan development

may be diminished [56,58] while the absence of some components of the immune response may allow multiplication of viruses to levels much higher than those seen *in vivo* [15,78]. However, using *in vitro* models characterized by increasing complexity, from a cell line to an organ culture, it is possible to characterize many biological aspects of pathogen evolution, development and interaction with the vector. This is most applicable to viruses, in which we can easily detect point mutations, recombination or reassortment of genomes that give rise to new biological properties [78,79], while tick organ cultures offer as-yet unexplored possibilities to investigate tick-protozoan interactions at the cellular and molecular levels [56,58]. Of course, *in vivo* validation is required but *in vitro* models can speed up the progress of research and increase the number of laboratories working on tick-borne pathogens without the need for facilities and expertise to work with live infected ticks and host animals. While there is a need to characterize the multiple phenotypes present in tick cell lines [21] and tick organ culture methods could be refined [62], financial, legislative and practical constraints on tick-borne disease research will ensure that tick *in vitro* culture models maintain and expand their central position, especially when applied to highly-pathogenic microorganisms.

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Author contributions

CS drafted section 1; LBS drafted section 2; SM and CS drafted section 3; LD and LBS drafted section 4; EZ drafted section 5; HA drafted section 6; CS and LBS drafted section 7; LBS edited all sections and all authors revised and agreed to the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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Table 1. Tick cell lines used as model systems for arthropod-borne pathogens, currently available from the Tick Cell Biobank (https://www.liverpool.ac.uk/infection-and-global-health/research/tick-cell-biobank/) and mentioned in this review.

Cell lines	Species of origin	Stage of origin	Reference
AAE2	*Amblyomma americanum*	Embryo	[43]
ANE58	*Dermacentor nitens*	Embryo	[31]
AVL/CTVM13	*Amblyomma variegatum*	Moulting larva	[57]
AVL/CTVM17	*Amblyomma variegatum*	Moulting larva	[42]
BmVIII, BmVIII-SCC	*Rhipicephalus microplus*	Embryo	[25,26]
BME26	*Rhipicephalus microplus*	Embryo	[204]
BME/CTVM6	*Rhipicephalus microplus*	Embryo	[42]
BME/CTVM23	*Rhipicephalus microplus*	Embryo	[47]
DAE15	*Dermacentor andersoni*	Embryo	[43]
DALBE3	*Dermacentor albipictus*	Embryo	[152]
HAE/CTVM9	*Hyalomma anatolicum*	Embryo	[32]
IDE2, IDE8, IDE12	*Ixodes scapularis*	Embryo	[33]
IRE11	*Ixodes ricinus*	Embryo	[38]
IRE/CTVM19, IRE/CTVM20	*Ixodes ricinus*	Embryo	[39]
ISE6	*Ixodes scapularis*	Embryo	[37]
OME/CTVM21, OME/CTVM22, OME/CTVM24, OME/CTVM27	*Ornithodoros moubata*	Embryo	[54]
RA243	*Rhipicephalus appendiculatus*	Moulting nymph	[20]
RAE25	*Rhipicephalus appendiculatus*	Embryo	[29]
RAE/CTVM1	*Rhipicephalus appendiculatus*	Embryo	[42]
REE/CTVM28	*Rhipicephalus evertsi*	Embryo	[48]
RML-15*	*Rhipicephalus sanguineus**	Embryo	[27]
RSE8	*Rhipicephalus sanguineus*	Embryo	[30]

* Originally published as derived from *D. variabilis* [28]; subsequently found to be derived from *R. sanguineus* [205]

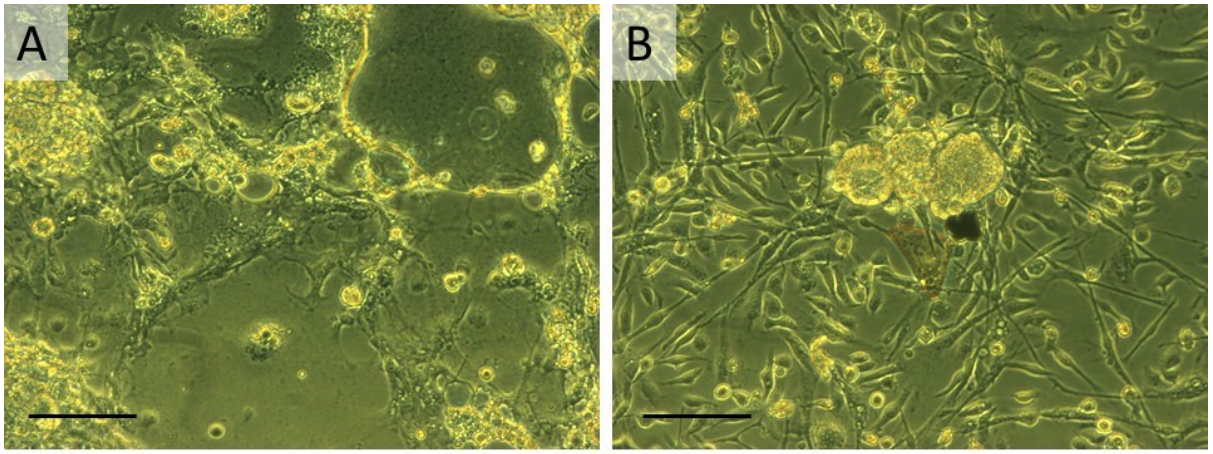

Figure 1. Light micrographs of primary embryo-derived tick cell cultures derived from (A) *Hyalomma dromedarii* and (B) *Dermacentor reticulatus* using published methods [32,47], illustrating the diversity of cell phenotypes and presence of tissue clumps typical of primary cultures. Live, phase-contrast inverted microscope (Zeiss Axiovert); scale bars = 100 µm.

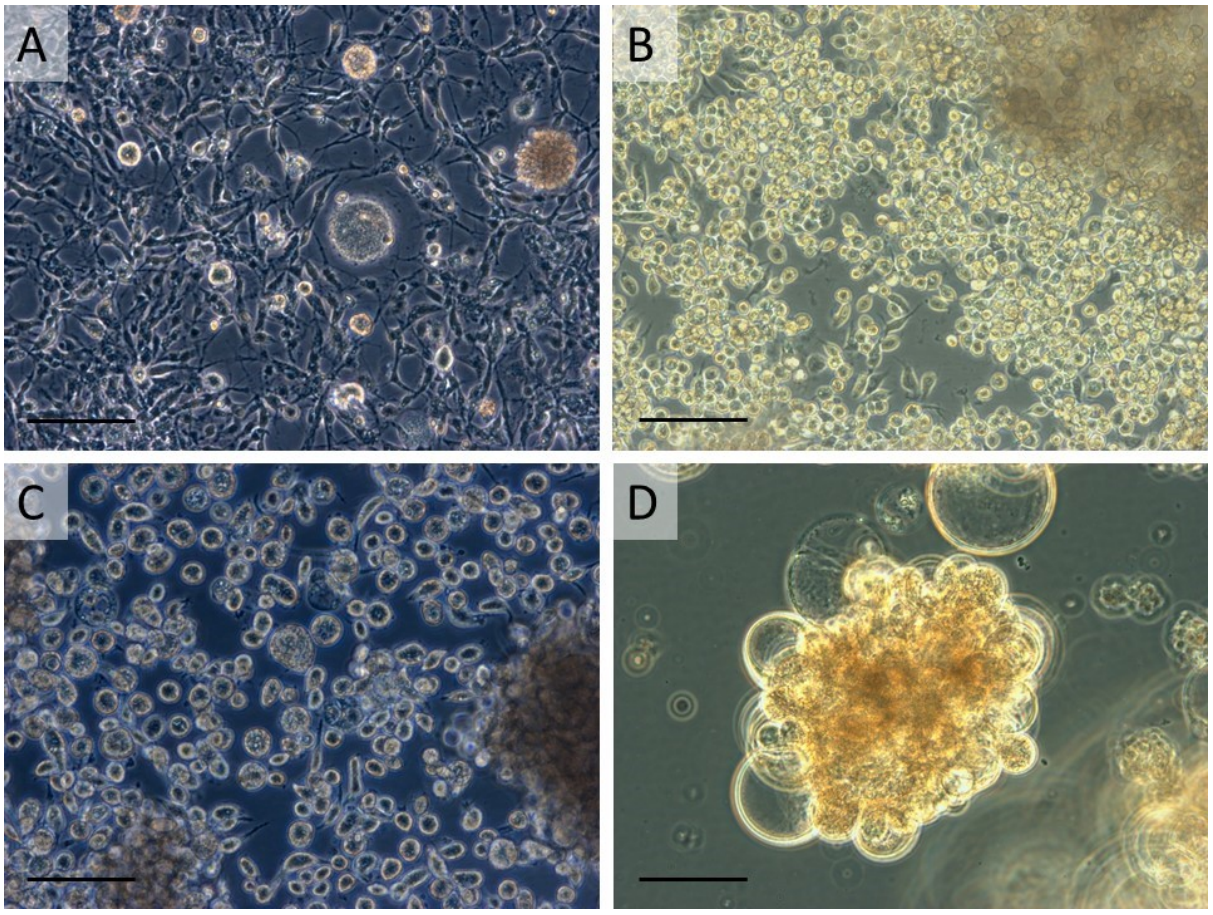

Figure 2. Light micrographs of tick cell lines illustrating the diversity of cell phenotypes and dimensions within and between lines. (A) *Rhipicephalus appendiculatus* cell line RAE/CTVM1 [42]; (B) *Rhipicephalus microplus* cell line BME/CTVM6 [42]; (C) *Amblyomma variegatum* cell line AVL/CTVM17 [42]; (D) *Ornithodoros moubata* cell line OME/CTVM21 [54]. Live, phase-contrast inverted microscope (Zeiss Axiovert); scale bars = 100 µm.

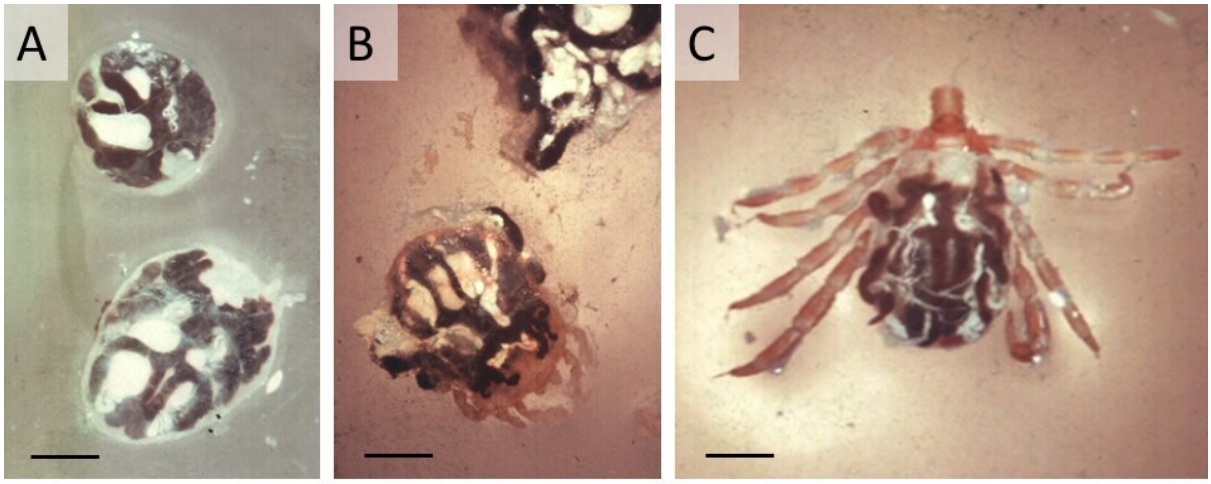

Figure 3. Types of whole-body tick organ cultures, prepared as described previously [56,58]. (A) developing adult *Hyalomma anatolicum* explant two days after culture initiation; (B) developing adult *Rhipicephalus appendiculatus* explant 5 months after culture initiation; (C) unfed backless adult female *H. anatolicum* two days after culture initiation. Scale bars = 1 mm.

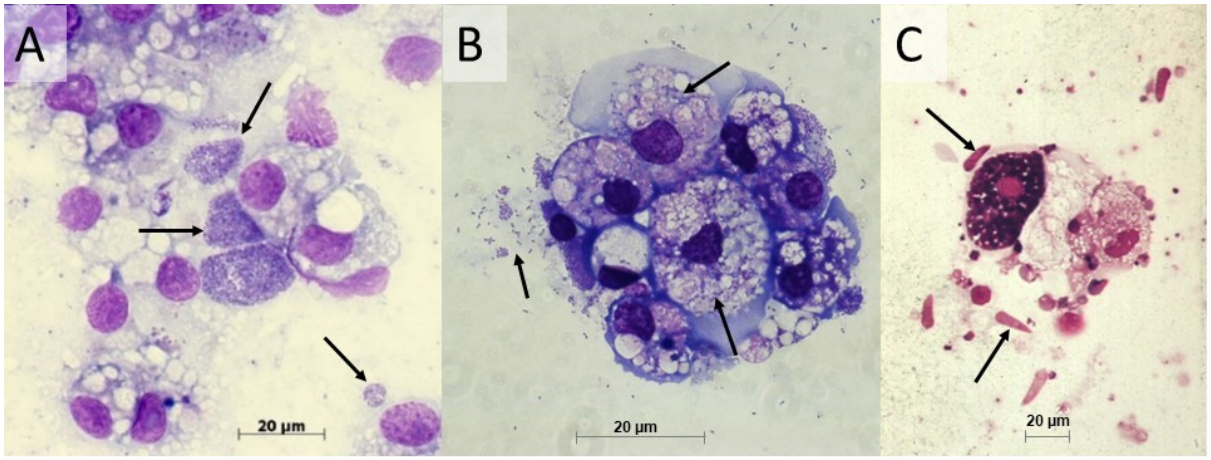

Figure 4. Tick-borne bacteria and protozoa in tick cell cultures, prepared as described previously [47,56,111]. (A) Membrane-bound colonies (morulae, arrows) of *Anaplasma marginale* in IDE8 cells. (B) *Rickettsia raoultii* bacteria (arrows) in the cytoplasm of BME/CTVM23 cells. (C) *Theileria annulata* kinetes (arrows) and tick haemocytes in the supernate of a *Hyalomma anatolicum* developing adult explant culture. Giemsa-stained centrifuge smears; scale bars = 20 µm.