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Article

Initiation of primary cell cultures from embryonic *Haemaphysalis bispinosa* ticks

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Abstract

Tick cell cultures have been widely used as an important tool for the study of tick-associated microorganisms, specifically for medically important bacteria or viruses that may be difficult to isolate or culture in axenic conditions. In this study, primary embryonic tick cell cultures were initiated separately from each of the egg batches laid by 10 female ticks belonging to the hard tick genus *Haemaphysalis*. All cultures were maintained at 28°C. After 10 months, 4 healthy cultures were identified with the potential for developing into continuous tick cell lines. These cultures comprise large cells predominantly forming floating cell clumps with multicellular vesicles, which are morphologically similar to cell lines derived from the soft tick *Ornithodoros moubata*. Subculture has not yet been performed due to the low cell density at the time of writing. Amplification and sequencing of a fragment of the 16S rRNA gene from DNA extracted from the parent ticks showed 99%-100% similarity to published sequences of *Haemaphysalis bispinosa*. This is the first report of the initiation of embryonic cell cultures from *Haemaphysalis* ticks found in Malaysia. Such tick cell cultures will be useful for studies of tick-borne pathogens in this region, where recent studies have shown that *Haemaphysalis* ticks are highly represented and harbor medically important bacteria.

Key words: *Haemaphysalis*, tick, primary cell culture, tick-borne pathogens

Introduction

Ticks are important vectors of diseases worldwide. Being able to feed on the blood of multiple hosts throughout their lifespan, ticks are capable of transmitting infectious diseases between the hosts. Ticks are a healthcare threat particularly to human populations at high risk of exposure to tick bites (Parola and Raoult 2001; Wu *et al.* 2013). These include the human populations who live or work near forested or rural areas, such as farmers and forest rangers. Furthermore, tick infestation of livestock animals such as cattle, sheep and goats affects animal health and consequently the yields of dairy products, meat, hides and wool, which leads to huge economic losses (Duh *et al.* 2008; Mehlhorn 1985). Hence, surveillance of and research into the transmission of tick-borne infections are important in safeguarding human health and economic gains of livestock farmers, especially in economically disadvantaged populations (Hotez *et al.* 2015).

Tick cell lines play a significant role in research involving tick biology, tick control, tick-borne pathogens and endosymbionts (Bell-Sakyi and Attoui 2013). There are now over 50 cell lines derived from 14 species of hard ticks (Ixodidae) and two species of soft ticks (Argasidae) (Bell-Sakyi *et al.* 2007). Tick cell cultures have been used in the propagation and study of many tick-associated

pathogens of medical importance, including *Anaplasma*, *Ehrlichia*, *Rickettsia*, tick-borne encephalitis virus (TBEV) and Crimean-Congo hemorrhagic fever virus (CCHFV) (Barbet *et al.* 2003; Bell-Sakyi *et al.* 2012; Lawrie *et al.* 2004; Policastro *et al.* 1997; Singu *et al.* 2006). Furthermore, tick cell cultures allow for investigation of vector-pathogen interactions, which is important for understanding pathogen infectivity and transmission by tick vectors (de la Fuente *et al.* 2002). The antiviral response in ticks and regulation of gene expression by infecting pathogens are also being increasingly studied using *in vitro* cultured tick cells (Ciota *et al.* 2015; Schnettler *et al.* 2014; Weisheit *et al.* 2015).

To date, most of the available tick cell lines are derived from *Amblyomma*, *Dermacentor*, *Hyalomma*, *Ixodes* and *Rhipicephalus* spp. ticks (Bell-Sakyi *et al.* 2007). Previous studies have described the establishment of cell lines from Indian *Haemaphysalis spinigera* and *Haemaphysalis obesa* ticks (Banerjee *et al.* 1977; Guru *et al.* 1976) and primary cultures from Japanese *Haemaphysalis longicornis* ticks (Kamio *et al.* 1986). However, as there have been no more recent reports of application of *Haemaphysalis* spp. cell lines, it is unlikely that any are currently available (Bell-Sakyi *et al.* 2012). This report describes the initiation and prolonged maintenance, with a view to cell line establishment, of primary tick cell cultures from embryonic tissues of *Haemaphysalis bispinosa* ticks collected from the field in Malaysia.

Methods

Preparation of primary cell cultures

Engorged female *Haemaphysalis* sp. ticks were collected from goats at a goat farm in the Perak state (4.5921° N, 101.0901° E), Malaysia with the approval of the farm owner. Ten ticks were chosen for the establishment of primary cell cultures. The method followed for the preparation of primary tick cell cultures was as described previously with slight modification (Bell-Sakyi 1991). Briefly, the engorged female ticks were surface-sterilized for 5 min in 0.1% benzalkonium chloride followed by 1 min in 70% ethanol and 1 min in sterile deionized water to remove environmental contaminants. The ticks were then incubated at 28ºC, 100% relative humidity for oviposition. Developing embryos were clearly visible in the eggs after 14 days. Eggs from each female tick were transferred into separate wells of a 6-well plate, washed with 70% ethanol for 1 min and rinsed with Hanks balanced salt solution (HBSS). The eggs were crushed in 1 mL of HBSS to release the embryonic tissues, which were then transferred to a flat-sided tube (Nunc, USA) in 3 mL of complete tick cell culture medium consisting of L-15B medium (Munderloh and Kurtti 1989) supplemented with 10% tryptose phosphate broth (DIFCO BD, USA), 20% fetal bovine serum (FBS) (GIBCO, USA), 0.1% bovine lipoprotein (MP Biomedicals, USA), 2 mM L-glutamine (Sigma Aldrich, USA), 100 units/mL penicillin, 100 µg/mL streptomycin and 25 µg/mL amphotericin (Lonza, Switzerland). No attempt was made to remove the egg shells from the suspension of embryonic tissues. The primary embryo-derived cell cultures were incubated at 28ºC and half of the cell culture medium was replaced weekly. The cultures were examined weekly for signs of cell growth and absence of contamination using an inverted microscope (Nikon Eclipse TE-2000E). The parent female ticks whose egg batches had been used to initiate primary cell cultures were stored at -80ºC for subsequent DNA extraction to confirm species identity.

DNA extraction and polymerase chain reaction (PCR) amplification

DNA was extracted from female *Haemaphysalis* ticks whose eggs had been used for culture initiation using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. For molecular identification of the ticks, PCR amplification of a fragment of the ticks'

mitochondrial 16S rRNA gene was performed using primers and protocols as previously described (Black and Piesman 1994). PCR amplicons were separated by 1.0% agarose gel electrophoresis and stained with SYBR® Safe nucleic acid stain (Invitrogen Life Technologies, USA). Amplicons were gel-purified using NucleoSpin Gel and PCR Clean-up (MACHEREY-NAGEL, Germany) according to the manufacturer's protocol. Sequencing of the PCR amplicons was performed by a third party service provider (First BASE Laboratories, Malaysia). Sequences obtained were compared to existing sequences available in the NCBI Genbank database. All sequences obtained were deposited in the European Nucleotide Archive (ENA Accession: LT593135 - LT593138).

Results

Ten separate primary cultures of *Haemaphysalis* cells, each from a single egg batch, were initiated in this study and named HB01 to HB10 respectively. Two weeks post-seeding, all cultures comprised floating cell or tissue clumps. Large adherent, elongated spindle-shaped cells (Figure 1A, filled arrow), rounded non-adherent cells (Figure 1A, open arrow) and dumbbell-shaped guanine crystals (Figure 1B, bar arrow) could be seen. Recognizable fragments of developing embryonic limbs were also visible in the culture. Cell clumps, fibroblast-like cells (Figure 2A, filled arrows) and spherical cells (Figure 2B, open arrow) were clearly visible in the cultures after 1 month of culture. After 3 months, most of the fibroblast-like cells had detached from the surface in all the cultures. Large clumps of cells containing hollow multicellular vesicles and vacuolated rounded cells became evident (Figure 3A, B and C). Finger-like protrusions were observed on some of the cells (Figure 3D, arrows). Pulsating cells were observed in one of the cultures (HB06). Two cultures (HB09 and HB10) were terminated and discarded in the 5th month due to fungal contamination.

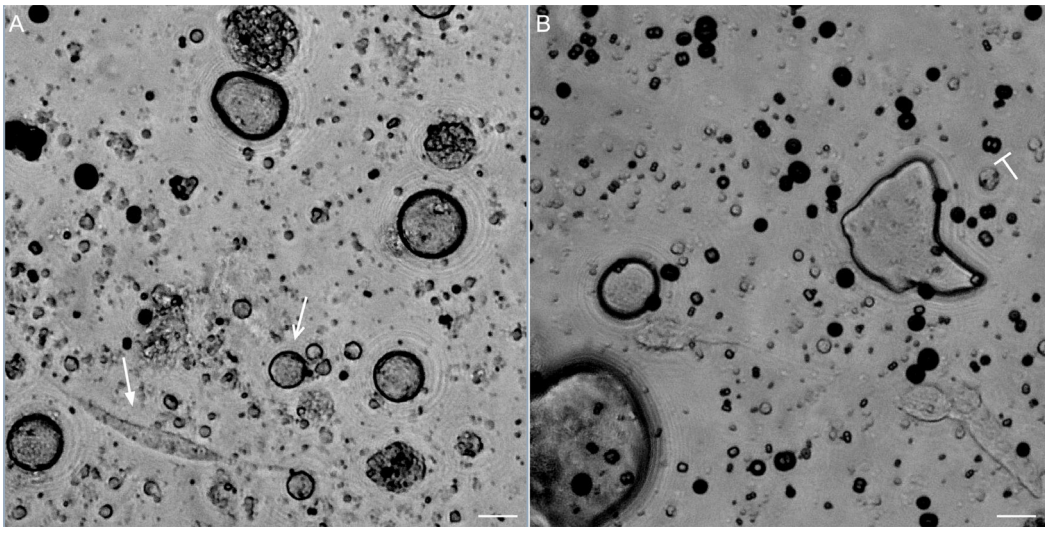

FIGURE 1. *H. bispinosa* embryonic cells at 2 weeks post-initiation. Fibroblast-like cells (filled arrow) and spherical cells (open arrow) observed in the *H. bispinosa* primary cell culture HB02, 2 weeks post-initiation (A). Guanine crystals (bar arrow) were visible in the HB06 culture (B). Live, inverted microscope; scale bar = 10 µm.

Ten months after initiation, four of the cultures, HB02, HB03, HB05 and HB06, remained healthy. Change in the color of the cell culture medium from pink to yellow due to increasing acidity was observed after the weekly medium change, indicating the presence of metabolic activity in these

cultures. The remaining cultures did not exhibit any change in the medium color, suggesting that metabolic activity had ceased. The previously observed fibroblast-like cells had mostly disappeared in the remaining healthy cultures. The predominant cell type in these cultures comprised irregularly shaped clumps of a heterogeneous population of cells growing on top of one another (Figure 4A to D). These cell clumps existed as rafts of connected cells floating on or in the cell culture medium or lightly attached to the bottom of the tube. Large multicellular vesicles ranging from 20µm to 150µm in diameter were clearly visible among these cell clumps (Figure 4C). Some of these cells appeared to be vacuolated (Figure 4D, arrow). Although it is now twelve months since the initiation of the cultures, increase in cell density is not yet obvious, suggesting a slow rate of cell multiplication. Cell counting and passaging have not been performed at the time of writing to avoid any disturbance to cell growth.

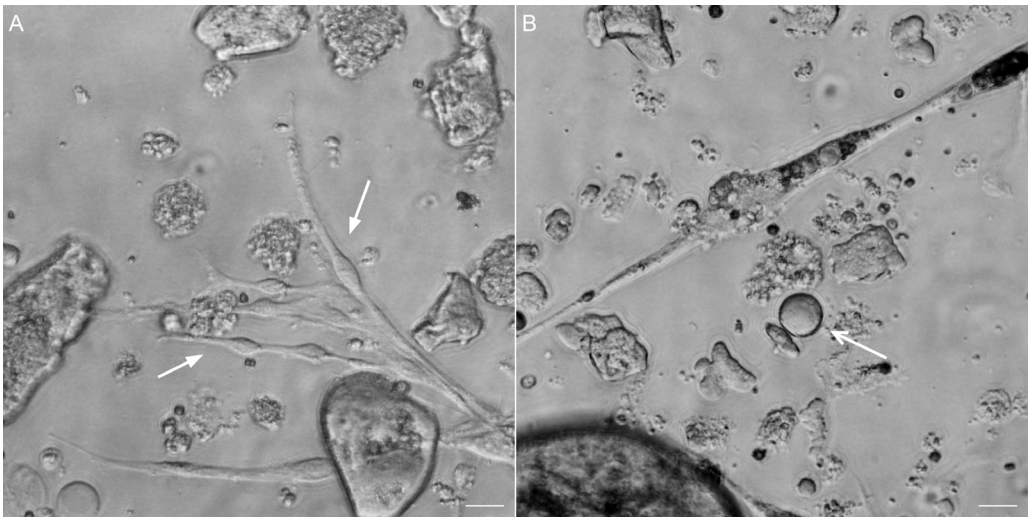

FIGURE 2. *H. bispinosa* embryonic cells at 1 month post-initiation. Fibroblast-like cells (filled arrows) and spherical cells (open arrow) observed in the *H. bispinosa* primary cell culture HB10, 1 month post-initiation. Live, inverted microscope; scale bar = 10 µm.

Comparison of the partial 16S rRNA sequences amplified from the female ticks, from whose eggs the HB02, HB03, HB05 and HB06 cell cultures were derived, with existing sequences in NCBI Genbank showed 100% similarity to *Haemaphysalis bispinosa* (NCBI Accession: KC853419.1 and KC853418.1) and 99% to *Haemaphysalis bispinosa* (NCBI Accession: KC853420.1) These results confirmed that we have successfully initiated primary cell cultures from *H. bispinosa* ticks.

Discussion and Conclusions

The establishment of tick cell lines is a time-consuming and labor-intensive process, in which the overall success rate is very low (Bell-Sakyi *et al.* 2007). It may take up to several years before a continuous cell line can be established. To date, the most widely used tick cell lines for the propagation and studies of tick-borne pathogens are derived from *Amblyomma*, *Ixodes* and *Rhipicephalus* spp. ticks (Bell-Sakyi *et al.* 2007). Although a previous study described the initiation of tick cell lines from *H. spinigera* and *H. obesa* in India (Guru *et al.* 1976), it is unlikely that these cell lines still exist. Cell lines from *Haemaphysalis* ticks will be important for the study of tick-borne

pathogens especially from the South-East Asian region, as *Haemaphysalis* ticks are highly represented among the ticks commonly found here in areas inhabited by humans and animals, and have been shown to harbour pathogenic bacteria such as *Bartonella*, *Rickettsia* and *Coxiella* (Kho *et al.* 2015; Khoo *et al.* 2016; Lerdthusnee *et al.* 2008; Vongphayloth *et al.* 2016). Moreover, *Haemaphysalis* ticks have been incriminated as vectors of the virus causing the emerging human disease, severe fever with thrombocytopenia syndrome (SFTS), currently causing outbreaks in China, Korea and Japan (Kim *et al.* 2013; Liu *et al.* 2012; Takahashi *et al.* 2014). One of the cell lines derived by Guru et al. (1976) from *H. spinigera* was shown to be able to support the propagation of tick-borne viruses such as Kyasanur Forest disease and Bhanja viruses (Banerjee *et al.* 1977). Hence, the establishment of cell lines from *Haemaphysalis* ticks will be of great importance for further research on viruses such as SFTSV. Here, we have described our attempts to initiate cell lines from *H. bispinosa* ticks collected from goats on a farm in Malaysia.

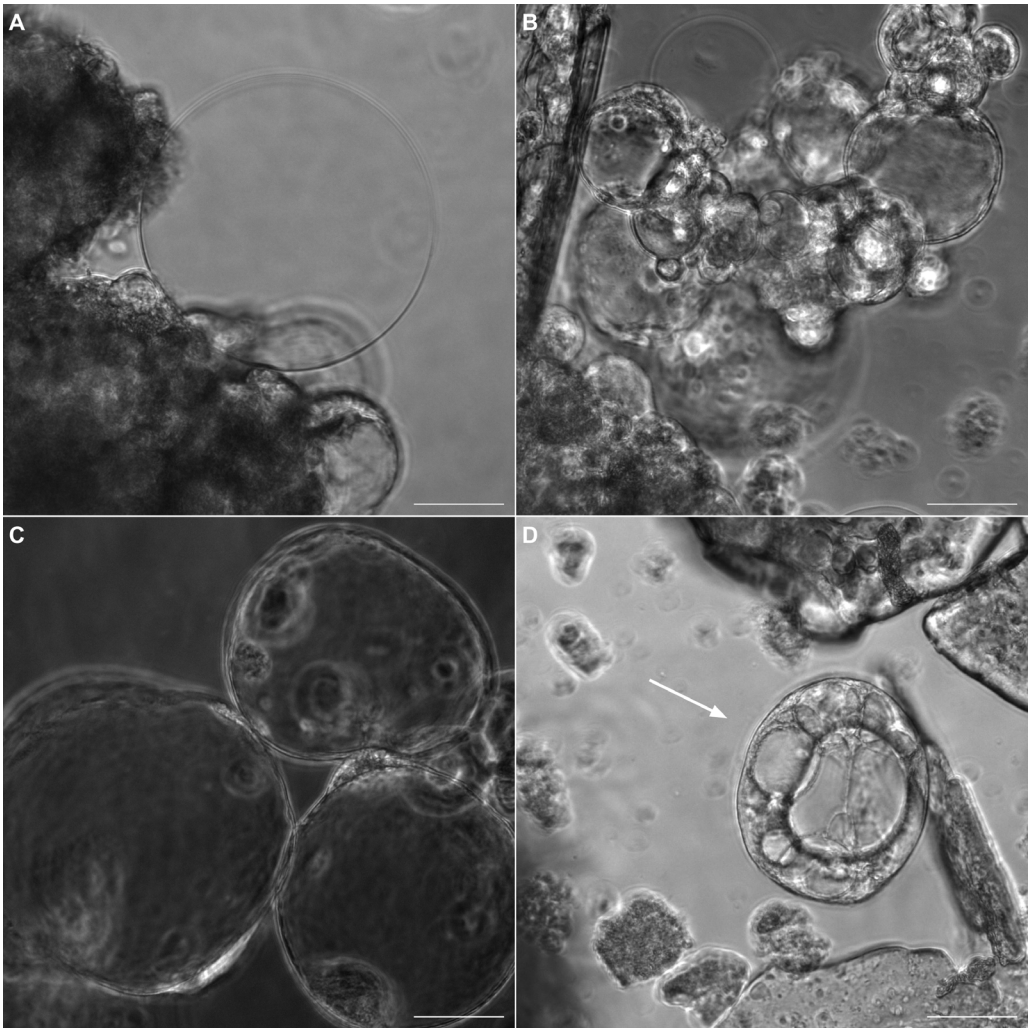

FIGURE 3. *H. bispinosa* embryonic cells at 3 months post-initiation. Cell clumps with multicellular hollow vesicles and large vacuolated rounded cells as seen in HB03 (A and B) and HB05 cultures (C). Finger-like protrusions (arrows) were visible in the HB05 culture (D). Live, inverted microscope; scale bar = 50 µm.

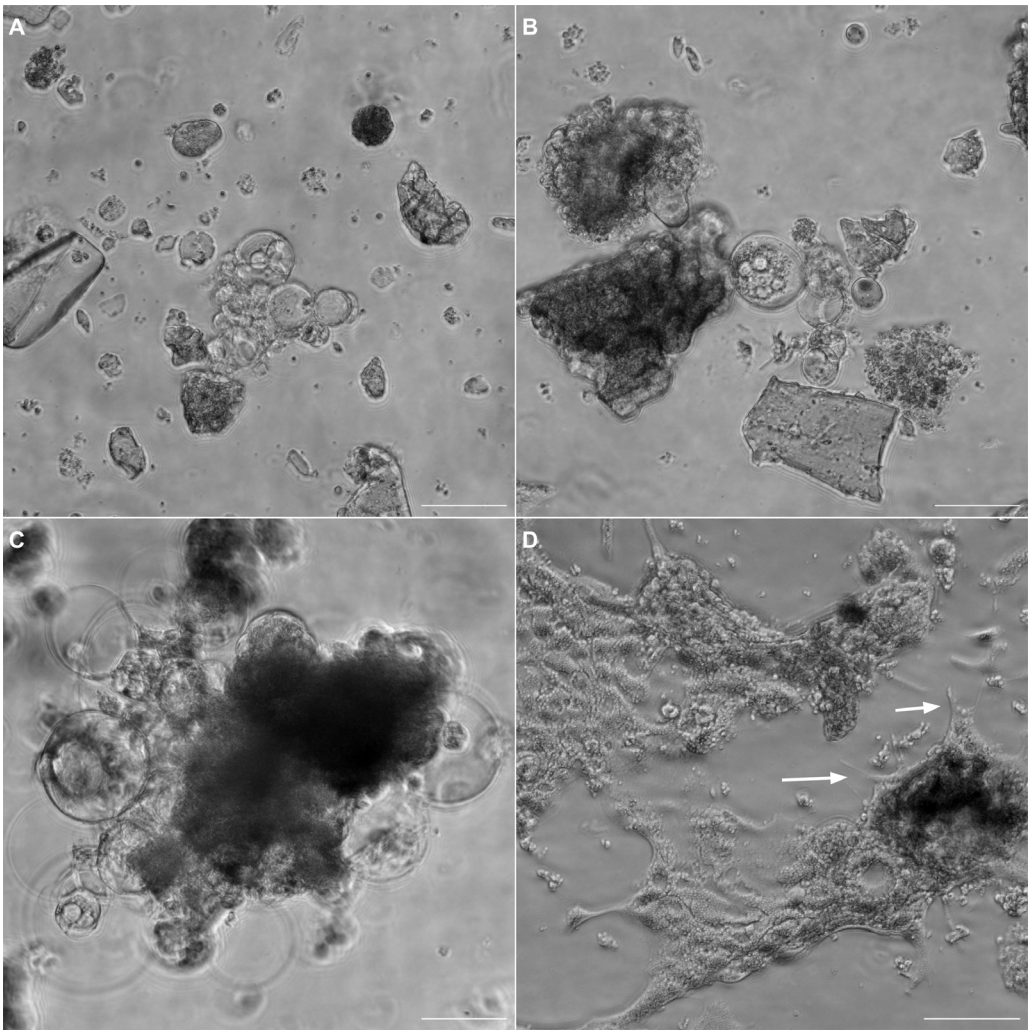

FIGURE 4. *H. bispinosa* embryonic cells at 10 months post-initiation. Cell clumps with multicellular hollow vesicles and vacuolated rounded cells (D, arrow) as seen in culture HB03. Live, inverted microscope; scale bar = 50 µm.

We observed that the *H. bispinosa* primary cell cultures appeared to be morphologically similar to the OME/CTVM21 tick cell line derived from *Ornithodoros moubata*, a soft tick (Bell-Sakyi *et al.* 2009). Floating cell clumps, multicellular vesicles and large, vacuolated cells are the morphological characteristics of OME/CTVM21 cells. Our primary cultures appear quite different to the morphologies of the *Haemaphysalis* tick cell cultures described in previous reports. H. *longicornis* primary cultures appeared to be dominated by fibroblasts or epithelial-like cells which formed a monolayer network of cells in less than 7 days post-seeding (Kamio *et al.* 1986). Cell cultures derived from *H. spinigera* and *H. obesa*, on the other hand, featured a mix of multicellular clumps together with fibroblasts or epithelial cells that could be separated after subculture (Guru *et al.* 1976). During the first month post-initiation, the *Haemaphysalis* cells in the present study appeared morphologically similar to the *H. spinigera* cells of Guru *et al.* (1976). However, progressive loss of the fibroblast-like cells could be observed in our cultures between 1 and 10 months post-initiation.

Apart from the different tick species involved, possible factors responsible for the differences in morphology between our *H. bispinosa* cultures and the *Haemaphysalis* spp. cultures reported previously include the tissue of origin and culture conditions. Both Guru *et al.* (1976) and Kamio *et al.* (1986) used developing adult tissues dissected out of the moulting nymphal integument, whereas we used embryonic tissues. Guru *et al.* (1976) used multiwell plates, plastic flasks and glass Leighton tubes or bottles, and incubated their cultures at 30 °C, Kamio *et al.* (1986) used glass culture tubes and incubated their cultures at 25 °C, whereas we used flat-sided plastic culture tubes incubated at 28 °C. All three studies used L-15 (Leibovitz) based media, but the two previous studies used 10% FBS whereas we used 20% FBS.

There was no obvious increase in cell numbers at twelve months after initiation, which made subculture and further characterization impossible at the time of writing. This was similar to *O. moubata* cell cultures, which were reported to be slow-growing, with the first subcultures possible only after 14.5–24 months (Bell-Sakyi *et al.* 2009). Similarly, primary cultures derived from embryonic *Rhipicephalus evertsi*, a hard tick, could not be successfully subcultured until they were between 22 and 36 months old (L. Bell-Sakyi, unpublished observations). In contrast, cell lines from *H. spinigera* and *H. obesa*, as well as other hard ticks such as *Hyalomma anatolicum*, *Ixodes scapularis*, *Rhipicephalus microplus* and *Dermacentor andersoni* were able to undergo first subculture in less than 12 months after initiation (Esteves *et al.* 2008; Guru *et al.* 1976; Kamio *et al.* 1986; Munderloh *et al.* 1994; Simser *et al.* 2001).

Culture medium supplements and incubation temperature are likely to affect the growth rate of tick cells (Bell-Sakyi *et al.* 2009). *O. moubata* tick cultures appeared to benefit from supplementation with 0.5% lactalbumin hydrolysate, which provides a high level of amino acids to the medium (Bell-Sakyi *et al.* 2009). The L-15-based tick cell culture medium L-15B, designed by Munderloh and Kurtti (1989), has been beneficial in the establishment of numerous cell lines from different tick species (Mattila *et al.* 2007; Munderloh *et al.* 1994; Simser *et al.* 2001; Singu *et al.* 2006). Unfortunately, we were unable to evaluate the use of different culture media supplements and incubation conditions due to insufficient resources and incubation facilities. Patience will be the key to ensure on-going maintenance of the existing cultures until significant cell multiplication is obtained and the first subcultures can be performed.

To summarize, we have described the initiation of primary tick cell cultures from *H. bispinosa* ticks. Currently, these cell cultures are morphologically similar to *O. moubata* tick lines, with a very slow growth rate. Perseverance in the maintenance of these primary cultures is now crucial to ensure success in obtaining a continuous *H. bispinosa* cell line.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contributions

FSL, JJK, LBS, LYC and SAB drafted the manuscript. LBS provided intellectual input into the study design. FSL, JJK, FC and CSK performed the sampling and cell cultures. SAB is the principle investigator of this study. All authors read and approved the final manuscript.

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References

Banerjee, K., Guru, P.Y. & Dhanda, V. (1977) Growth of arboviruses in cell cultures derived from the tick *Haemaphysalts spinigera*. *Indian Journal of Medical Research*, 66, 530–536.

Barbet, A.F., Meeus, P.F.M., Bélanger, M., Bowie, M.V., Yi, J., Lundgren, A.M., Alleman, A.R., Wong, S.J., Chu, F.K., Munderloh, U.G. & Jauron, S.D. (2003) Expression of multiple outer membrane protein sequence variants from a single genomic locus of *Anaplasma phagocytophilum*. *Infection and Immunity*, 71, 1706–1718.
https://doi.org/10.1128/IAI.71.4.1706-1718.2003

Bell-Sakyi, L. (1991) Continuous cell lines from the tick *Hyalomma anatolicum* anatolicum. *The Journal of Parasitology*, 77, 1006–1008.
https://doi.org/10.2307/3282757

Bell-Sakyi, L. & Attoui, H. (2013) Endogenous tick viruses and modulation of tick-borne pathogen growth. *Frontiers in Cellular and Infection Microbiology*, 3, 25.
https://doi.org/10.3389/fcimb.2013.00025

Bell-Sakyi, L., Kohl, A., Bente, D.A. & Fazakerley, J.K. (2012) Tick cell lines for study of Crimean-Congo hemorrhagic fever virus and other arboviruses. *Vector-Borne and Zoonotic Diseases*, 12, 769–781.
https://doi.org/10.1089/vbz.2011.0766

Bell-Sakyi, L., Růžek, D. & Gould, E.A. (2009) Cell lines from the soft tick *Ornithodoros moubata*. *Experimental and Applied Acarology*, 49, 209–219.
https://doi.org/10.1007/s10493-009-9258-y

Bell-Sakyi, L., Zweygarth, E., Blouin, E.F., Gould, E.A. & Jongejan, F. (2007) Tick cell lines: tools for tick and tick-borne disease research. *Trends in Parasitology*, 23, 450–457.
https://doi.org/10.1016/j.pt.2007.07.009

Black, W.C. & Piesman, J. (1994) Phylogeny of hard-and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. *Proceedings of the National Academy of Sciences*, 91, 10034–10038.
https://doi.org/10.1073/pnas.91.21.10034

Ciota, A.T., Payne, A.F. & Kramer, L.D. (2015) West Nile virus adaptation to ixodid tick cells is associated with phenotypic trade-offs in primary hosts. *Virology*, 482, 128–132.
https://doi.org/10.1016/j.virol.2015.03.033

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., Saliki, J.T. & Kocan, K.M. (2002) Infection of tick cells and bovine erythrocytes with one genotype of the intracellular ehrlichia *Anaplasma marginale* excludes infection with other genotypes. *Clinical and Diagnostic Laboratory Immunology*, 9, 658–668.
https://doi.org/10.1128/cdli.9.3.658-668.2002

Duh, D., Punda-Polić, V., Trilar, T. & Avšič-Županc, T. (2008) Molecular detection of *Theileria* sp. in ticks and naturally infected sheep. *Veterinary Parasitology*, 151, 327–331.
https://doi.org/10.1016/j.vetpar.2007.11.004

Esteves, E., Lara, F.A., Lorenzini, D.M., Costa, G.H.N., Fukuzawa, A.H., Pressinotti, L.N., Silva, J.R.M.C., Ferro, J.A., Kurtti, T.J., Munderloh, U.G. & Daffre, S. (2008) Cellular and molecular characterization of an embryonic cell line (BME26) from the tick *Rhipicephalus (Boophilus) microplus*. *Insect Biochemistry and Molecular Biology*, 38, 568–580.
https://doi.org/10.1016/j.ibmb.2008.01.006

Guru, P.Y., Dhanda, V. & Gupta, N.P. (1976) Cell cultures derived from the developing adults of three species

of ticks, by a simplified technique. *Indian Journal of Medical Research*, 64, 1041–1045.
Hotez, P.J., Bottazzi, M.E., Strych, U., Chang, L.-Y., Lim, Y.A.L., Goodenow, M.M. & AbuBakar, S. (2015) Neglected tropical diseases among the Association of Southeast Asian Nations (ASEAN): Overview and update. *PLoS Neglected Tropical Diseases*, 9, e0003575.
https://doi.org/10.1371/journal.pntd.0003575
Kamio, T., Mitsuhashi, J. & Fujisaki, K. (1986) Primary culture of developing adult tissue in a nymphal tick, *Haemaphysalis longicornis* (Acarina: Ixodidae). *Applied Entomology and Zoology*, 21, 394–398.
Kho, K.-L., Koh, F.-X., Jaafar, T., Nizam, Q.N.H. & Tay, S.-T. (2015) Prevalence and molecular heterogeneity of *Bartonella bovis* in cattle and *Haemaphysalis bispinosa* ticks in Peninsular Malaysia. *BMC Veterinary Research*, 11, 1.
https://doi.org/10.1186/s12917-015-0470-1
Khoo, J.J., Chen, F., Kho, K.L., Ahmad Shanizza, A.I., Lim, F.-S., Tan, K.K., Chang, L.Y. & AbuBakar, S. (2016) Bacterial community in *Haemaphysalis* ticks of domesticated animals from the Orang Asli communities in Malaysia. *Ticks and Tick-borne Diseases*, 7, 929–937.
https://doi.org/10.1016/j.ttbdis.2016.04.013
Kim, K.-H., Yi, J., Kim, G., Choi, S.J., Jun, K.I., Kim, N.-H., Choe, P.G., Kim, N.-J., Lee, J.-K. & Oh, M. (2013) Severe fever with thrombocytopenia syndrome, South Korea, 2012. *Emerging Infectious Diseases*, 19, 1892–1894.
https://doi.org/10.3201/eid1911.130792
Lawrie, C.H., Uzcátegui, N.Y., Armesto, M., Bell-Sakyi, L. & Gould, E.A. (2004) Susceptibility of mosquito and tick cell lines to infection with various flaviviruses. *Medical and Veterinary Entomology*, 18, 268–274.
https://doi.org/10.1111/j.0269-283X.2004.00505.x
Lerdthusnee, K., Nigro, J., Monkanna, T., Leepitakrat, W., Leepitakrat, S., Insuan, S., Charoensongsermkit, W., Khlaimanee, N., Akkagraisee, W., Chayapum, K. & Jones, J.W. (2008) Surveys of rodent-borne disease in Thailand with a focus on scrub typhus assessment. *Integrative Zoology*, 3, 267–273.
https://doi.org/10.1111/j.1749-4877.2008.00100.x
Liu, Y., Li, Q., Hu, W., Wu, J., Wang, Y., Mei, L., Walker, D.H., Ren, J., Wang, Y. & Yu, X.-J. (2012) Person-to-person transmission of severe fever with thrombocytopenia syndrome virus. *Vector-Borne and Zoonotic Diseases*, 12, 156–160.
https://doi.org/10.1089/vbz.2011.0758
Mattila, J.T., Burkhardt, N.Y., Hutcheson, H.J., Munderloh, U.G. & Kurtti, T.J. (2007) Isolation of cell lines and a rickettsial endosymbiont from the soft tick *Carios capensis* (Acari: Argasidae: Ornithodorinae). *Journal of Medical Entomology*, 44, 1091–1101.
https://doi.org/10.1093/jmedent/44.6.1091
Mehlhorn, H. (1985) The piroplasms: life cycle and sexual stages. *Advances in Parasitology*, 23, 37–103.
https://doi.org/10.1016/S0065-308X(08)60285-7
Munderloh, U.G. & Kurtti, T.J. (1989) Formulation of medium for tick cell culture. *Experimental & Applied Acarology*, 7, 219–229.
https://doi.org/10.1007/BF01194061
Munderloh, U.G., Liu, Y., Wang, M., Chen, C. & Kurtti, T.J. (1994) Establishment, maintenance and description of cell lines from the tick *Ixodes scapularis*. *The Journal of Parasitology*, 533–543.
https://doi.org/10.2307/3283188
Parola, P. & Raoult, D. (2001) Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. *Clinical Infectious Diseases*, 32, 897–928.
https://doi.org/10.1086/319347
Policastro, P.F., Munderloh, U.G., Fischer, E.R. & Hackstadt, T. (1997) *Rickettsia rickettsii* growth and temperature-inducible protein expression in embryonic tick cell lines. *Journal of Medical Microbiology*, 46, 839–845.
https://doi.org/10.1099/00222615-46-10-839
Schnettler, E., Tykalová, H., Watson, M., Sharma, M., Sterken, M.G., Obbard, D.J., Lewis, S.H., McFarlane, M., Bell-Sakyi, L., Barry, G., Weisheit, S., Best, S.M., Kuhn, R.J., Pijlman, G.P., Chase-Topping, M.E., Gould, E.A., Grubhoffer, L., Fazakerley, J.K. & Kohl, A. (2014) Induction and suppression of tick cell antiviral RNAi responses by tick-borne flaviviruses. *Nucleic Acids Research*, 42, 9436–9446.
https://doi.org/10.1093/nar/gku657
Simser, J.A., Palmer, A.T., Munderloh, U.G. & Kurtti, T.J. (2001) Isolation of a spotted fever group rickettsia,

Rickettsia peacockii, in a Rocky Mountain wood tick, *Dermacentor andersoni*, cell line. *Applied and Environmental Microbiology*, 67, 546–552.
https://doi.org/10.1128/AEM.67.2.546-552.2001

Singu, V., Peddireddi, L., Sirigireddy, K.R., Cheng, C., Munderloh, U. & Ganta, R.R. (2006) Unique macrophage and tick cell-specific protein expression from the p28/p30-outer membrane protein multigene locus in *Ehrlichia chaffeensis* and *Ehrlichia canis*. *Cellular Microbiology*, 8, 1475–1487.
https://doi.org/10.1111/j.1462-5822.2006.00727.x

Takahashi, T., Maeda, K., Suzuki, T., Ishido, A., Shigeoka, T., Tominaga, T., Kamei, T., Honda, M., Ninomiya, D. & Sakai, T. (2014) The first identification and retrospective study of severe fever with thrombocytopenia syndrome in Japan. *Journal of Infectious Diseases*, 209, 816–827.
https://doi.org/10.1093/infdis/jit603

Vongphayloth, K., Brey, P.T., Robbins, R.G. & Sutherland, I.W. (2016) First survey of the hard tick (Acari: Ixodidae) fauna of Nakai District, Khammouane Province, Laos, and an updated checklist of the ticks of Laos. *Systematic and Applied Acarology*, 21, 166–180.
https://doi.org/10.11158/saa.21.2.2

Weisheit, S., Villar, M., Tykalová, H., Popara, M., Loecherbach, J., Watson, M., Růžek, D., Grubhoffer, L., de la Fuente, J., Fazakerley, J.K. & Bell-Sakyi, L. (2015) *Ixodes scapularis* and *Ixodes ricinus* tick cell lines respond to infection with tick-borne encephalitis virus: transcriptomic and proteomic analysis. *Parasites & Vectors*, 8, 599.
https://doi.org/10.1186/s13071-015-1210-x

Wu, X.-B., Na, R.-H., Wei, S.-S., Zhu, J.-S. & Peng, H.-J. (2013) Distribution of tick-borne diseases in China. *Parasites & Vectors*, 6, 119.
https://doi.org/10.1186/1756-3305-6-119

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