MALAYSIAN JOURNAL OF MICROSCOPY

Journal homepage: https://malaysianjournalofmicroscopy.org

RESEARCH ARTICLE

WOLBACHIA INFECTION IN AN *IXODES SCAPULARIS*-DERIVED TICK CELL LINE

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Abstract. *Wolbachia*, an obligate intracellular bacterial endosymbiont, is gaining recognition for its ability to reduce the transmission of arboviruses by *Aedes aegypti* mosquitoes, leading to its use in dengue virus control programs. Some *Wolbachia* strains can be propagated in vitro in insect or tick cell lines, facilitating the study of host-bacteria interactions. Here, we present findings from a microscopic study of a *Wolbachia* endosymbiont (*w*Cfe) that was isolated from Malaysian *Ctenocephalides felis* fleas, in an *Ixodes scapularis*-derived tick cell line (ISE6). The presence of *Wolbachia* in cultures was visualized using Giemsa staining at 0, 1-, 7-, 14-, and 21 days post-infection. The cytocentrifuge smears were examined under a compound microscope at 1000× magnification, and images were captured using XCamView software. Comparison of Giemsa-stained uninfected and *w*Cfe-infected ISE6 cultures revealed that the proportion of infected cells increased over the 21-day examination period. Bacteria were visible in the cell cytoplasm, often clustering near the nucleus. Heavily infected cells became more fragile and burst, and the total number of cells in the culture reduced following a severe infection. Despite extensive cytopathic effects seen by 29 days post-infection, not all cells died; two months later, the surviving cells appeared to have become persistently infected with *w*Cfe. In conclusion, based on the successful observation of *w*Cfe infection dynamics within the ISE6 cells, the study confirms that this tick cell line is suitable for propagating different strains of *Wolbachia*. Our results also show that light microscopy plays a useful role in monitoring the course of *Wolbachia* infection in arthropod cell lines and could be used to complement electron microscopy and molecular quantification studies.

Keywords: Vector-borne diseases, intracellular bacteria, *Wolbachia*, tick cell lines

Article Info

Received 10 January 2024
Accepted 19 October 2024
Published 6 December 2024

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ISSN: 1823-7010, eISSN: 2600-7444

1. INTRODUCTION

Ticks and tick-borne diseases have continued to cause interest among researchers and public health professionals as a myriad of tick-borne bacteria and viruses have been reported to cause infections in humans and animals in recent times, in addition to the isolation and detection of tick endosymbionts that have piqued the interest of many [1]. Intracellular symbiotic bacteria of ticks such as *Francisella*-like, *Rickettsia*-like and *Coxiella*-like endosymbionts, as well as novel endosymbionts such as *Candidatus* Midichloria mitochondrii have been discovered lately [2]. These microbial symbionts may enhance the tick's fitness and may have a role in pathogen transmission, although the mechanism for the latter remains ambiguous [3].

Wolbachia is an intracellular Gram-negative bacterium known to infect the reproductive tissues of hundreds of arthropod taxa, including insects. Based on their widespread distribution and diverse characteristics, *Wolbachia* are divided into over 20 lineages called 'supergroups', denoted by letters of the alphabet. These ubiquitous bacteria maintain their abundance due to their ability to induce the manipulation of the reproductive ability of the host in a variety of ways which include cytoplasmic incompatibility (CI), male-killing, inducing parthenogenesis, and feminization in the genetic male host, all of which influence arthropod populations [4]. *Wolbachia* has been detected in ticks of the genera *Ixodes*, *Amblyomma*, *Hyalomma* and *Rhipicephalus*, albeit at a comparatively lower prevalence. However, detection of this bacterium in ticks was usually associated with the presence of parasitoid wasps or nematodes carrying the bacteria [5]. Nevertheless, there is a lack of reported studies on its potential to be used as a biological control approach for tick-borne diseases.

Many strains of *Wolbachia* from different insect species have been isolated and propagated in insect cell lines, enabling researchers to observe bacteria-host cell interactions within a controlled environment [6]. Several *Wolbachia* strains have also been propagated in tick cell lines [7-9], including the cell line ISE6 derived from the tick *Ixodes scapularis.* The ISE6 cell line has been shown to be permissible to many types of tick-borne microbes, such as pathogenic bacteria from the genera *Rickettsia*, *Anaplasma* and *Ehrlichia* [10]; thus, this cell line has served as an advantageous tool in understanding vector-pathogen interactions.

In recent years, the interest in controlling vector-borne diseases has intensified, particularly through the study of bacterial endosymbionts such as *Wolbachia*. These intracellular bacteria have shown promise in reducing the transmission of arboviruses, including dengue, by the mosquito *Aedes aegypti* [11]. While extensively studied in mosquitoes, the potential of *Wolbachia* in ticks remains underexplored. The present study investigates the infection dynamics of *Wolbachia* in a tick cell line. By visualizing the interaction between *Wolbachia* and tick cells, this research aims to expand our understanding of potential applications of *Wolbachia* in biological control beyond mosquitoes, possibly paving the way for novel strategies in managing tick-borne diseases. Here, we present the results of a microscopic study of a flea-derived *Wolbachia* (*w*Cfe) infection in the cell line ISE6. The *w*Cfe strain was originally isolated from Malaysian *Ctenocephalides felis* fleas into tick cells [7] and subsequently found to comprise a mixture of two strains belonging to the supergroups F and J, namely *w*CfeF and *w*CfeJ [12]. Our study, carried out with the original mixed *w*Cfe strain, aimed to visualize the presence and effect of the bacterium in the ISE6 cells by examining Giemsa-stained cytocentrifuge smears prepared at 1-, 7-, 14-, and 21-days post-infection (d.p.i).

2. MATERIALS AND METHODS

2.1 Tick Cell Lines and Wolbachia

The tick cell lines ISE6 [13] and IDE8 [14], derived from the North American black-legged tick *I. scapularis*, were maintained in the Tick Cell Biobank Asia Outpost, Universiti Malaya, Malaysia. The ISE6 cells were grown, in L-15B300 medium and the IDE8 cells were grown in L-15B medium as described previously [8,15]; both media were supplemented with 10% tryptose phosphate broth (TPB),

5% fetal bovine serum (FBS), 0.1% bovine lipoprotein (MP Biomedicals, Solon, OH, USA), 2 mM L-glutamine and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). The cell lines were maintained at 32 ℃ in sealed, flat-sided cell culture tubes (Nunc, Thermo Fisher, Loughborough, UK) with ¾ of the medium replaced with fresh medium weekly and subcultured at 1–3 month intervals. The *w*Cfe strain of *Wolbachia* was propagated in IDE8 cells maintained at 28 ℃ as described previously [7].

2.2 Preparation of Bacterial Culture Stock

IDE8 cells infected with *Wolbachia w*Cfe were resuspended and forcibly passed through a 25 G needle 5 times and the resulting suspension was subsequently filtered through a 2.0 µm syringe filter and centrifuged at 1000 x g for 5 min at 4 ℃. The supernatant was inoculated into a naive ISE6 culture. Once a heavy infection was established in which cytopathic effects were observed, 1 ml aliquots of the whole culture were cryopreserved in vapor phase liquid nitrogen with the addition of 10% dimethyl sulfoxide, to be used as a bacterial stock.

2.3 Infection and Monitoring of Cells

Triplicate tubes of naive ISE6 cells were seeded at a density of 3 x 10^6 cells per ml for infection with *Wolbachia w*Cfe. An aliquot of cryopreserved bacterial culture stock was thawed rapidly in a 37 ℃ water bath followed by the addition of 200 µL of the suspension to duplicate ISE6 cultures. The third ISE6 culture was maintained as uninfected control. Cells were monitored by inverted microscope for cytopathic effects and collected at the selected time points within the infection time course for preparation of Giemsa-stained cytocentrifuge smears as follows. The uninfected and *Wolbachia*-infected ISE6 cultures were resuspended by pipetting and 50 µL of each cell suspension was taken for the preparation of cytocentrifuge smears. The cell suspensions were centrifuged onto clean microscope slides at 1000 rpm for 5 min in a Cytospin 4 cytocentrifuge (Shandon, Cheshire, UK) to produce smears. The smears were then air-dried, fixed in methanol for 3 min, stained with Giemsa (Merck, Darmstadt, Germany) for 20 min and rinsed in deionised water buffered to pH 7.2.

2.4 Imaging and Observation by Light Microscopy

Live cultures were examined for presence of cytopathic effects under a BM-100 inverted microscope (Biobase, Shandong, China) with 20x and 40x objectives. The stained smears were examined under a compound microscope (Nikon Eclipse Si Upright Microscope, Tokyo, Japan) at 1000× magnification to visualize the bacteria in the tick cells. A digital camera (BestScope BHC4-4K8MPB, Beijing, China) and XCamView software were used to capture images of the cells. The scale bar for calibration was synthesized by using the same software.

3. RESULTS AND DISCUSSION

3.1 Bacterial Infection Rate in Giemsa-stained Infected ISE6 Cells

The *w*Cfe strain of *Wolbachia* was originally isolated and continuously cultivated in the *I. scapularis*-derived tick cell line IDE8 [7]. Here, we report successful transfer of the *w*Cfe infection from IDE8 cells into another *I. scapularis* cell line, ISE6. After one passage through ISE6 cells, duplicate naïve ISE6 cultures were inoculated with cell-free *w*Cfe bacteria and sampled on 1, 7, 14 and 21 d.p.i. by preparation of Giemsa-stained cytocentrifuge smears. The percentage of visibly infected cells was calculated by examining 300 cells/smear for presence of intracellular bacteria at each time point (Figure 1).

The infected cultures showed progressive infection from 1 d.p.i to 21 d.p.i. At 1 d.p.i., the infection rate was observed to be approximately 20% and 16% in Cultures 1 and 2, respectively. As the

inoculum comprised cryopreserved, already-infected ISE6 cells and were added to the naïve cells in a v/v ratio of 1:10, up to 10% of the infected cells seen at 1 d.p.i were likely to be derived from the inoculum, rather than freshly infected cells from the recipient cultures. At 7 d.p.i, Cultures 1 and 2 showed infection rates of approximately 40% and 55%, increasing to 78% and 98% respectively at 14 d.p.i. This increase showed that most infected cells seen from 7 d.p.i represented new infections.

By 21 dp.i., among the 300 cells counted, 100% infection rates were seen in both cultures. This contrasted favorably with the 25% infection rate reported for *w*Cfe in IDE8 cells at 8 months post infection [7]. Moreover, ISE6 cells appeared to be more susceptible to infection with *Wolbachia* than with other intracellular bacteria of the order Rickettsiales. *Ehrlichia ruminantium* infected 5-10% of ISE6 cells on day 7 after addition of semi-purified bacteria [16]. A ruminant strain of *Anaplasma phagocytophilum* infected 15% of ISE6 cells by 6 weeks following infection from sheep blood [17]. *Rickettsia buchneri* was isolated from an *I. scapularis* tick failed to grow in ISE6 cells at 28 °C and grew slowly at 32 °C reaching ~75% infection rate at 35 d.p.i [18].

Counting infected cells in stained smears provides valuable information on the susceptibility of individual cells in a culture to infection with a particular bacterium (infection rate) but is not suitable for quantifying the exact number of bacteria per cell or per culture (infection level). The infection level can be determined by quantification through real-time PCR amplification of a target gene in DNA extracted from the culture. Ideally, both methods of analysis should be combined to obtain a complete picture of the growth kinetics of the bacteria in the cells [15].

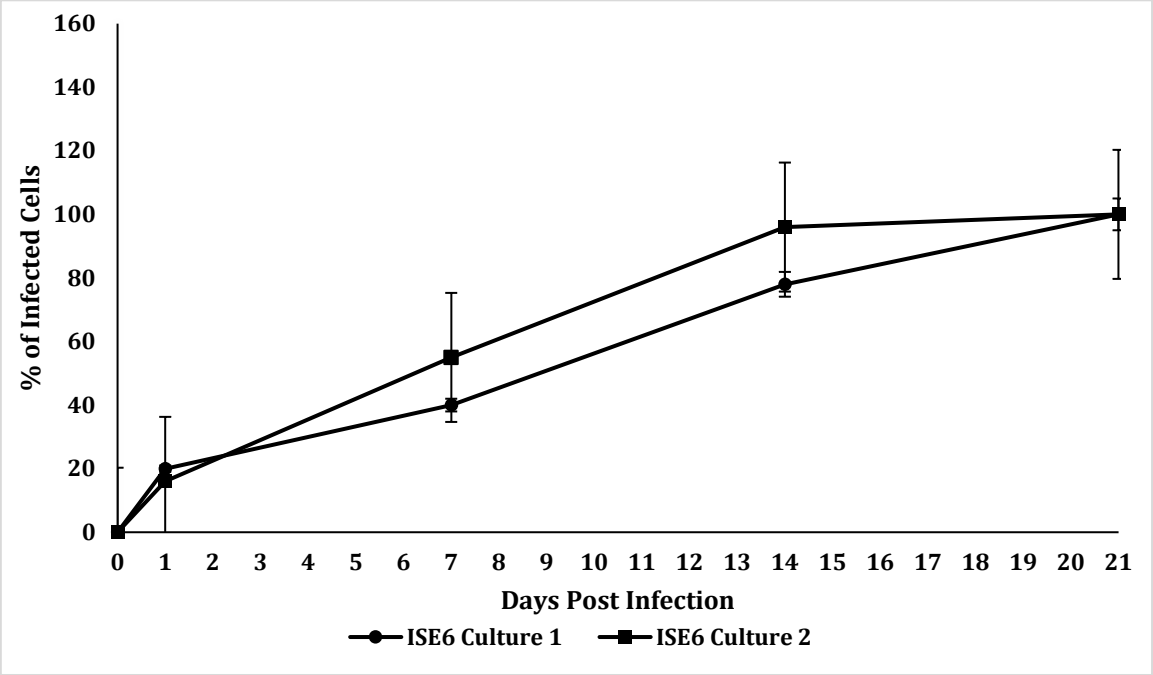

Figure 1: Growth of *Wolbachia w*Cfe in duplicate cultures of the *Ixodes scapulari*s cell line ISE6 over 21 days post-infection, determined by examination of Giemsa-stained cytocentrifuge smears. The percentage of visibly infected cells was determined by examining 300 cells per culture per time point. Error bars indicate standard error.

3.2 Observation of Microscopic Images of Uninfected and Wolbachia-infected ISE6 Cultures

In Giemsa-stained cytocentrifuge smears, the *w*Cfe bacteria appeared pleomorphic, consistent with previous findings [7]. At 1 d.p.i., bacteria were observed in the cytoplasm of some of the ISE6 cells but it was not possible to distinguish between infected cells from the inoculum and cells newly-infected with bacteria that had actively entered or been phagocytosed. At this stage the bacteria were a mixture of rods, cocci and small, pycnotic forms (Figure 2(a)). By 7 d.p.i., the bacteria showed vigorous growth, although not all cells were infected. At this time, the bacteria were predominantly rod-shaped and, in some cells, were observed to concentrate near the nucleus (Figure 2(b)).

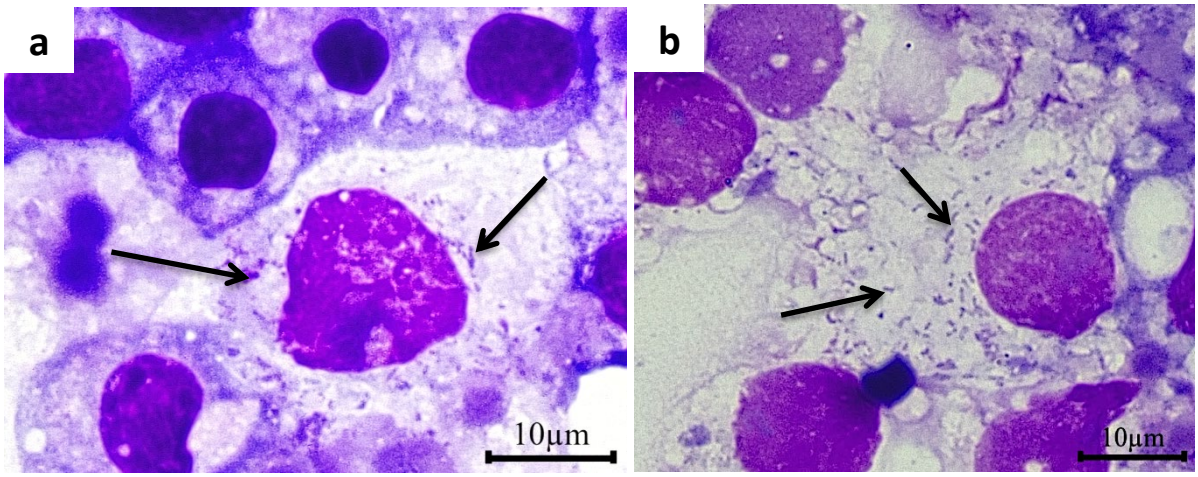

Figure 2: Giemsa-stained cytocentrifuge smear images of *Wolbachia w*Cfe in the *Ixodes scapularis* cell line ISE6 prepared at (a) 1 d.p.i and (b) 7 d.p.i. Arrows indicate the bacteria.

Figure 3 shows Giemsa-stained cytocentrifuge smear images of *Ixodes scapularis* ISE6 cells. On 14 d.p.i., the cytoplasm of the cells appeared to be more vacuolated (Figure 3(a)), and in some cases, the membranes of infected cells had ruptured, possibly because of the process of resuspension for preparation of the smears. Visualization of the cells by Giemsa-staining on day 21 d.p.i showed that the plasma membranes of many of the host cells had ruptured and cell-free bacteria were present (Figure 3(b)). A considerable number of cells showed large, clear vacuoles in the cytoplasm, a possible indicator of exposure to heavy infection with bacteria (Figure 3(c)). On the other hand, the increase in cytoplasmic vacuolation could be a response to infection of the cells with the tick-only St Croix River virus, which persistently infects IDE8 cells but is absent from ISE6 cells [19]. This virus would have been transferred from IDE8 to the ISE6 cells alongside the *w*Cfe bacteria.

From the overall observation of the Giemsa-stained smears, the number of cells was observed to have reduced as compared to the uninfected Giemsa-stained ISE6 culture, although total cell counts were not carried out. A general decrease in cell numbers over time was also observed during infection of three tick cell lines with the intracellular bacterium *Rickettsia raoultii* [15]. In the present study, it was not possible to determine the cause of death of the infected cells by morphological observation; one possibility was that infected cells were undergoing apoptosis. In some insects, *Wolbachia*-induced apoptosis increases fecundity, which is essential for oogenesis progression [20]. Here, the presence of a heavy infection with *w*Cfe clearly caused death of the infected cells, but at the same time, lightly infected cells apparently entering mitosis, and therefore presumably unaffected by presence of small numbers of bacteria, were observed in the ISE6 cultures at 21 d.p.i. (Figure 3(c)), while typical apoptotic nuclei were not seen. Uninfected ISE6 cells stained with Giemsa were bound by membranes stained in an intense purplish-blue. The nucleus was stained in intense purple, while the cytoplasm was vacuolated and stained in shades of pale blue (Figure 3(d)).

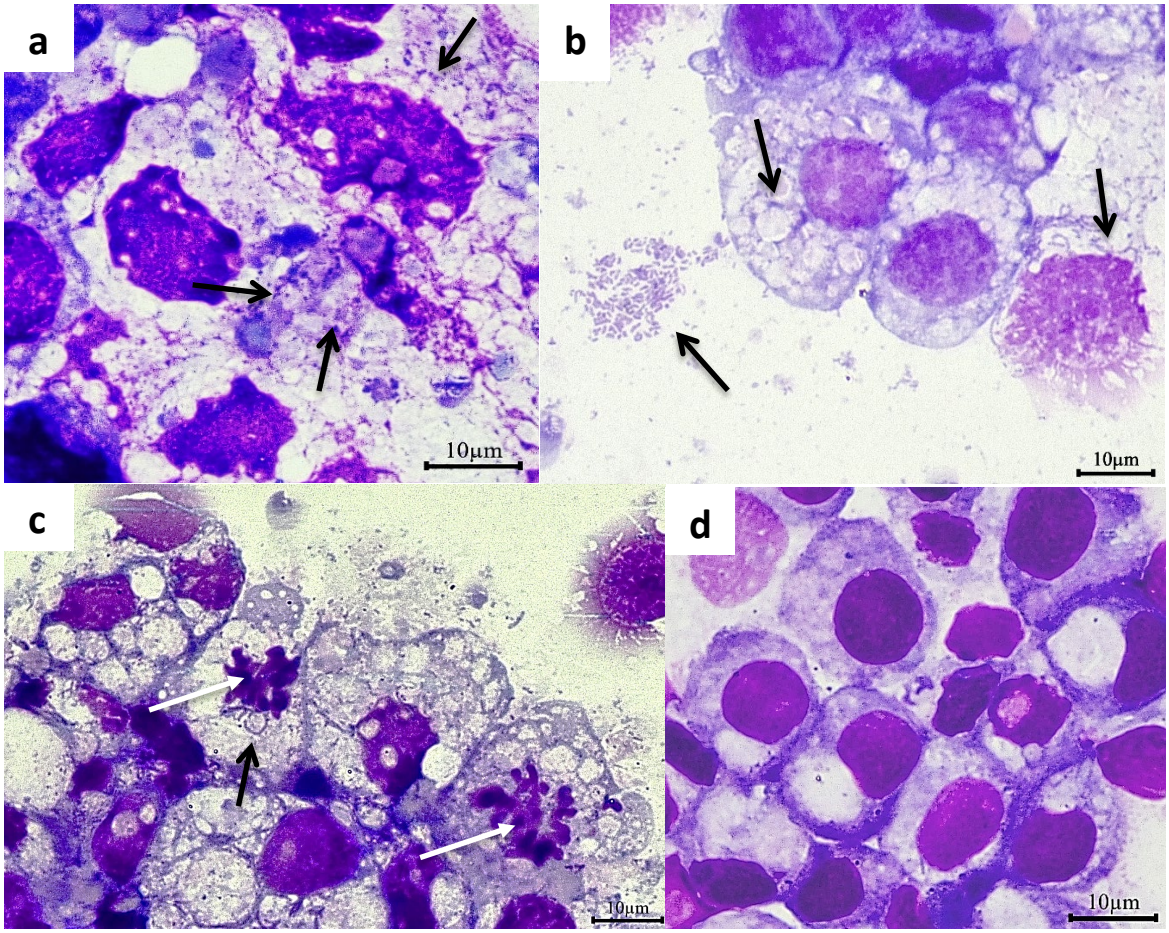

Figure 3: Giemsa-stained cytocentrifuge smear images of *Ixodes scapularis* ISE6 cells. Cells infected with *Wolbachia* *w*Cfe at (a) 14 d.p.i., (b) 21 d.p.i. and (c) 21 d.p.i showing infected cells possibly entering mitosis (white arrows). (d) Uninfected ISE6 cells. Black arrows indicate the bacteria; Scale bars represent 10 µm.

In vitro, uninfected ISE6 cells grow morphologically as round or spindle-shaped cells, often in clusters and clumps and the diameter of the stained cells can range between 10 and 20 µm. Examination of the cell culture by inverted microscope found that uninfected ISE6 grew abundantly and densely in the cell culture tubes. In contrast, *w*Cfe-infected ISE6 cells remained attached until around 29 d.p.i after which they started to detach from the bottom of the tube and floated in the supernatant medium, indicating the occurrence of heavy infection with the bacteria. When examining the cultures two months following the 21 d.p.i time point, it was observed that the cells had not completely succumbed to the infection, unlike the rapid cytopathic effect caused by *R. raoultii* in IDE8 cells [15]. At this time, the cultures comprised apparently healthy infected cells (data not shown) metabolising at a rate like that of uninfected ISE6 cultures, as judged by the similar colour of the pH indicator (phenol red) in the culture medium. Survival of these cells could be attributed to the original presence of a subpopulation of ISE6 cells that were able to tolerate or control the *Wolbachia* infection. Persistent infection with *w*Cfe was also reported in IDE8 cells [7-8]. The phenotypes of the two *I scapularis* cell lines used may also be a factor affecting how they respond to infection with different intracellular bacteria. The ISE6 cell line has previously been used to propagate and study a wide variety of intracellular bacterial and viral pathogens [10], as well as symbionts including species of the genera *Cardinium* [13], *Rickettsia* [18], and *Wolbachia* [8-9]. The effect of the *Wolbachia* infection on ISE6 cells varied depending on the strain; like *w*Cfe, *w*Pip and *w*Pap both caused heavy infections and cytopathic effects in ISE6 cells [8], while *w*AlbB produced only low-level infection and eventual loss after 16 passages in ISE6 [9].

4. CONCLUSIONS

In this study, we presented the morphological appearance of tick (ISE6) cells infected with *Ctenocephalides felis*-derived *Wolbachia* bacteria (*w*Cfe) at different time points based on examination of Giemsa-stained cytocentrifuge smears. ISE6 cells were able to support the growth of the *w*Cfe endosymbiont and many cells showed severe infection from 21 d.p.i, which may suggest that *w*Cfe was able to overcome the host tick cell immune system. Light microscopic data can provide information on the infection rate and severity of infection of the cells, in which the morphology of the cells can be examined to denote the characteristics of the microorganism infecting the cell. However, transmission electron microscopy should be utilized to further explain and reveal the ultrastructure of the endosymbiont and its interaction with the intracellular components of the infected cells at high resolution.

Acknowledgements

This research was supported by the Fundamental Research Grant Scheme (FRGS), Ministry of Higher Education, under grant number: FRGS/1/2022/STG03/UM/02/9. We would like to thank the Tick Cell Biobank Asia Outpost, Universiti Malaya, partly funded by Wellcome Trust grant number 223743_Z_21_Z, for providing us with the facilities and the tick cell lines used in this study. The ISE6 and IDE8 cell lines were used by kind permission of Prof. Ulrike Munderloh, University of Minnesota. Special thanks to Dr. Lesley Bell-Sakyi and Dr. Jing Jing Khoo from the Department of Infection Biology and Microbiomes, Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool for providing valuable comments that helped improve this manuscript.

Author Contributions

All authors contributed to the drafting, conceptualization, data analysis and revision of the manuscript. All authors have agreed to the published version of the manuscript.

Disclosure of Conflict of Interest

The authors declared no conflict of interest.

Compliance with Ethical Standards

The work does not involve any animal or human subjects. All experiments using cell cultures and the rest of the work were conducted in compliance with relevant ethical standards.

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