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RESEARCH ARTICLE

Preliminary short-term establishment and replication kinetics of a flea-derived *Wolbachia* strain in an *Aedes albopictus* cell line

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ABSTRACT

Mosquito-borne arboviral diseases such as dengue and chikungunya have continuously been a major global public health concern. Introducing the intracellular bacterial endosymbiont *Wolbachia* into mosquito populations has been proven to reduce dengue virus transmission, and its broader efficacy against other arboviruses has also been investigated. Several *Wolbachia* strains have been successfully propagated in insect cell lines, highlighting the utility of *in vitro* systems for studying microbial-host interactions under controlled conditions. This study investigated the initial establishment and replication kinetics of a flea-derived *Wolbachia* strain (*w*Cfe), which was originally isolated from Malaysian *Ctenocephalides felis* and maintained in the *Ixodes scapularis* tick-derived cell line (IDE8), and then transferred into an *Aedes albopictus*-derived cell line (C6/36). The *w*Cfe strain was semi-purified from IDE8 cultures and inoculated into C6/36 cells in 24-well plates. Replication dynamics were monitored by quantitative real-time PCR targeting the *Wolbachia pipientis* 16S rRNA gene. Following infection, a lag phase was observed at day 0 to 5 days post-infection (d.p.i.), followed by exponential growth from 6 d.p.i. after which *Wolbachia* levels remained relatively stable until the end of the observation period at 12 d.p.i. Overall, a 25.30-fold increase in *Wolbachia* density was detected relative to 0 d.p.i. Across replicates, the estimated generation time of *w*Cfe in C6/36 cells ranged from 1.7 to 2.5 days. These results demonstrate successful initial establishment and replication of the flea-derived *Wolbachia* strain in the *Aedes* mosquito cell line. However, longer-term *in vivo* studies will be necessary to determine the persistence of *w*Cfe infection in C6/36 cells and within the mosquito host.

Keywords: arthropod vectors; intracellular bacteria; *Wolbachia*; mosquito cell line; replication kinetics.

INTRODUCTION

Wolbachia is a genus of maternally inherited, obligate intracellular bacteria that infects a wide range of arthropods, including insects, arachnids, and crustaceans, as well as some nematodes. It is estimated to occur in approximately 60% of insect species worldwide. *Wolbachia* is widely recognised for its ability to manipulate host reproductive biology and, critically, for its capacity to interfere with the replication and transmission of arboviruses such as dengue and chikungunya (Dobson *et al*., 2002; Frentiu *et al*., 2010; Lu *et al*., 2012). Notably, *Wolbachia*-mediated pathogen blocking can be profound, especially in transinfected mosquito cell lines (Bian *et al*., 2013). For example, the *w*AlbB strain, originally isolated from *Aedes albopictus* and subsequently introduced into *Ae. aegypti* has demonstrated a marked reduction in dengue virus transmission, as evidenced by field deployments in Malaysia (Nazni *et al*., 2019). Similarly, *Wolbachia* strains derived from non-native insect hosts have also shown strong antiviral effects. For instance, *Aedes* mosquitoes transinfected with *w*Mel (a *Wolbachia* strain originally derived from *Drosophila melanogaster*) exhibited a drastic reduction in dengue virus transmission, with only a small fraction of mosquitoes harbouring infectious virus in their salivary glands compared to wild-type controls (Walker *et al*., 2011). These findings underpin the successful deployment of *Wolbachia*-based mosquito control programmes using transinfected strains in several endemic regions and highlight the feasibility of using *Wolbachia* strains originating from non-mosquito hosts to suppress mosquito-borne disease transmission (Paz-Bailey *et al*., 2025).

In vitro cell culture systems provide a controlled and reproducible platform for investigating *Wolbachia*-host interactions, enabling detailed analysis of bacterial replication dynamics, host compatibility, and cellular responses without the use of animal models (Fallon, 2019; Fallon, 2021). To date, several *Wolbachia* strains, predominantly from supergroups A and B, have been

successfully propagated in mosquito-derived cell lines, such as Aa23 and C6/36 derived from *Ae. albopictus* which naturally harbors the *Wolbachia* strains *w*AlbA and *w*AlbB (O'Neill *et al*., 1997; Dobson *et al*., 2002; Khoo *et al*., 2013). These established systems have been instrumental in advancing our understanding of *Wolbachia* biology and its antiviral effects at the cellular level (Fenollar *et al*., 2003; Raquin *et al*., 2015).

Wolbachia strains are phylogenetically classified into supergroups (A-U), yet the majority of these lineages remain poorly characterized in mosquito hosts (Beliavskaia *et al*., 2023). The *Wolbachia* strain examined in this study, *w*Cfe, is a recently described flea-derived isolate originating from *Ctenocephalides felis* in Malaysia. Importantly, *w*Cfe comprises a mixed infection of two phylogenetically distinct strains belonging to supergroups F (*w*CfeF) and V (*w*CfeJ) (Beliavskaia *et al*., 2023). This strain, therefore, represents a phylogenetically divergent *Wolbachia* lineage relative to the strains commonly studied in mosquitoes. *w*Cfe was previously isolated and maintained in an *Ixodes scapularis* tick-derived cell line, IDE8, demonstrating its capacity to persist and replicate in an arthropod cell culture system outside its natural flea host (Khoo *et al*., 2020).

Experimental transfection of *Wolbachia* into novel or phylogenetically distinct host species or cell lines often produces unpredictable outcomes, with infections frequently being unstable or lost after initial establishment (van Meer & Stouthamer, 1999). However, cell culture-based transfection approaches have been shown to promote *Wolbachia* adaptation and persistence, particularly through serial passages in recipient cells that support intracellular replication (McMeniman *et al*., 2008; Fallon, 2019). Despite growing interest in broadening the diversity of *Wolbachia* strains available for vector biology and disease control research, motivated in part by the success of non-mosquito-derived strains such as *w*Mel (Walker *et al*., 2011), flea-derived *Wolbachia*, especially those belonging to supergroups F and V, have not previously been established in mosquito-derived cell lines. This represents a critical knowledge gap in assessing the broader applicability of phylogenetically diverse *Wolbachia* strains for vector control.

The present study reports the initial establishment and replication kinetics of *w*Cfe in the *Ae. albopictus*-derived C6/36 cell line. The ability of *w*Cfe to establish infection was evaluated by quantifying bacterial growth using quantitative real-time PCR and by visualising intracellular bacterial localisation through Giemsa staining. Establishing a mosquito-derived cell culture system capable of maintaining *w*Cfe provides a critical experimental foundation for characterising phylogenetically divergent *Wolbachia* strains and for evaluating their potential relevance in future studies of *Aedes*-borne pathogen control.

MATERIALS AND METHODS

Cell lines and *Wolbachia*

All procedures involving bacterial infection and cell culture work were conducted in the biosafety level 2 (BSL2) laboratory and were approved by the Universiti Malaya Institutional Biosafety and Biosecurity Committee (IBBC) (Approval No.: UMIBBC/NOI/TNCPNI/TIDREC-015/2025-19112025). The *Ae. albopictus* C6/36 cell line (CRL-1660), purchased from the American Type Culture Collection (ATCC), was maintained at the Tropical Infectious Diseases Research & Education Centre, Universiti Malaya, Malaysia. The cells were maintained in 25 cm^2 tissue culture flasks and sub-cultured at 3-5 day intervals. C6/36 cells were maintained in Eagle's Minimum Essential Medium (EMEM) containing Earle's salts and L-glutamine (Ref. 61100061) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin and 100 µg/mL streptomycin at 28°C in 5% CO_2 incubator.

The tick-derived cell line IDE8, containing *Wolbachia* strain *w*Cfe and originally derived from the North American black-legged tick, *I. scapularis* (Munderloh *et al*., 1994), was maintained at the Tick Cell Biobank Asia Outpost, Universiti Malaya, Malaysia. The IDE8 cells were grown in L-15B medium (Munderloh & Kurtti, 1989) supplemented with 10% tryptose phosphate broth, 5% FBS, 0.1% bovine lipoprotein (MP Biomedicals, USA), 2mM L-glutamine and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin), as described previously (Bell Sakyi *et al*., 2021; Husin *et al*., 2021). The *w*Cfe-infected IDE8 cultures were maintained at 28 °C as previously described (Khoo *et al*., 2020).

Preparation of *w*Cfe suspension from IDE8 cells and inoculation into C6/36 cells

C6/36 cells (passages 83 and 88) at ~95% confluency were seeded at a density of 2 × 10^5 cells/mL into 24-well tissue culture plates (Corning, USA) and incubated at 28°C with 5% CO_2 for 24 h before infection with *w*Cfe with minor modifications (Bell Sakyi *et al*., 2021). Briefly, multiple tubes of IDE8 cells infected with *w*Cfe (passages 100 and 101) were resuspended and pooled. The pooled suspension was centrifuged at 2 000 × g for 5 min, and the supernatant was subsequently filtered through a 0.45 µm syringe filter to remove host cell debris.

An aliquot of 200 µL of the filtered inoculum was collected for DNA extraction and quantitative PCR (qPCR) analysis to represent day 0 post-infection (d.p.i). The remaining filtrate was inoculated in 200 µl aliquots into each well of mosquito cells, followed by the addition of 1 000 µL of C6/36 maintenance medium (EMEM supplemented with 2% FBS) to achieve a final inoculation ratio of 1:5. The cultures were then maintained at 28°C with 5% CO, and monitored daily for cytopathic effects or changes in cell morphology using a BMI-100 inverted microscope (Biobase, China). Images were captured using a digital microscope equipped with DinoCapture 2.0 software (DinoLite, Taiwan), and scale bars were generated using the same software. At designated time points, 200 µL of cell suspension was collected for the preparation of Giemsa-stained cytocentrifuge smears and for qPCR quantification of *Wolbachia* copy numbers.

Molecular confirmation of *w*Cfe and checking of tick cells carry-over

Samples from 0, 2, 5, 6 and 12 d.p.i. and *w*Cfe-infected IDE8 as positive control were subjected to DNA extraction and PCR amplification of the tick-specific partial 16S rRNA gene (Black & Piesman, 1994) to check for the carry-over of tick DNA. The reaction mixture consisted of a final concentration of 400 nM of the primer pairs, 1× MyTaq™ Red Mix Mastermix (Bioline, UK), and 2 µl DNA to give a final volume of 50 µL.

The assay was performed on the SimpliAmp™ Thermal Cycler PCR System (Applied Biosystems, USA), with the following cycling conditions: activation at 94°C for 2 min, 10 cycles of denaturation at 92°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 1 min. This was followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Finally, a final extension was performed at 72°C for 7 min. The amplicons were subsequently observed on 1% AGE with an estimated base pair (b.p.) of 460 if tick DNA was amplified. Bands at 0, 2, and 12 d.p.i., as well as the tick-positive control observed in the gel, whether at target or non-target base pair sizes, were selected for excision and submitted to a third-party service provider for Sanger sequencing. The sequencing result was analysed by Sequence Scanner version 2 (Applied Biosystems, USA) and BioEdit sequence alignment editor version 7.0.5.3 software and subsequently submitted for BLAST analyses at the GeneBank database from the National Centre for Biotechnology Information (NCBI).

Additionally, the inoculum was also amplified by PCR to confirm the presence of *w*Cfe by targeting the *Wolbachia*-specific 16S rRNA gene (Carvajal *et al*., 2019). Each reaction comprised a

final concentration of 500 nM of the primer pairs, 1× MyTaq™ Red Mix Mastermix (Bioline, UK), and 2 µl DNA to give a final volume of 50 µL. The PCR cycling conditions consisted of: 95°C for 2 min for activation, followed by 2 cycles of denaturation at 95°C for 2 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. Following this, another round of cycles consisting of 35 cycles was performed; denaturation at 95°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 45 s. A final extension at the end of the PCR reaction was performed at 10°C for 10 min. The amplicons were then observed on 1.5% AGE with an estimated size of 850 b.p. (Carvajal *et al*., 2019). The extracted DNA of this inoculum was also used as a template for quantification of *w*Cfe at 0 d.p.i., in the qPCR assay.

Quantification of *w*Cfe by qPCR

Adherent cells were resuspended by scraping, and DNA was extracted from 200 µL of cell suspension collected from each time point using the NucleoSpin Tissue kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. Absolute quantification of *Wolbachia* was performed using a previously published qPCR assay targeting the 99-bp fragment of the *Wolbachia pipientis* 16S rRNA gene (Makepeace *et al*., 2006). qPCR reactions were performed using the CFX Opus 96 Real-Time PCR System (Bio-Rad Laboratories, USA). Each 20 µL reaction contained 1× SensiFAST SYBR No-ROX Master Mix (Meridian Bioscience, USA), 200 nM of each primer, and 2 µL of template DNA. The cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 15 s, with a subsequent melt curve analysis to confirm product specificity.

Ten-fold serial dilutions of a known concentration of *W. pipientis* DNA were used to generate a standard curve for absolute quantification (Makepeace *et al*., 2006). Assuming exponential bacterial growth, the mean generation time of *w*Cfe in each biological replicate was calculated using the equation previously described by Husin *et al*. (2021).

Preparation of cytocentrifuge smears for Giemsa staining

At each time-point except for 0 d.p.i, cells were collected for preparation of Giemsa-stained cytocentrifuge smears. Briefly, 50 µL aliquots of cell suspension from uninfected and *Wolbachia*-infected C6/36 were centrifuged onto clean microscope slides at 1 000 r.p.m. for 5 min using a Cytospin 4 cytocentrifuge (Shandon, UK). The smears were then air-dried, fixed in methanol for 3 min, stained with Giemsa for 20 min, and rinsed with deionised water adjusted to pH 7.2. Stained smears were examined under a compound microscope (Nikon Eclipse Si Upright Microscope, Japan) at 1 000 × magnification to visualise intracellular *Wolbachia* in the infected mosquito cells. Images were captured using a BestScope BHC4-4K8MPB digital camera (BestScope, China) and XCamView software, with scale bars generated using the same software.

RESULTS

Molecular confirmation of *w*Cfe and absence of carry-over of tick cells

The presence of *Wolbachia* in the inoculum was confirmed by sequencing of the *Wolbachia*-specific 16S rRNA gene (Carvajal *et al*., 2019), which showed >99% identity and 100% query coverage (GenBank accession number: CP116768.1). Meanwhile, the sequencing of the PCR amplicon targeting a 460 b.p. fragment of the tick-specific partial 16S rRNA gene (Black & Piesman, 1994), performed on the selected samples (tick-positive control, 0, 2 and 12 d.p.i.), detected *I. scapularis* DNA at the earlier time points at 0 and 2 d.p.i. showing >99% identity and 100% query cover to *Ixodes scapularis* (GeneBank accession number: KT821598.1). However, no high coverage matches to *I. scapularis* DNA were detected at 12 d.p.i., with BLAST analysis instead showing 100% identity to *Ae. albopictus* with a lower query coverage at 13% (GenBank accession number: XM_029871899.2), indicating a non-specific alignment. AGE at 12 d.p.i. revealed bands of approximately 800 b.p. (Figure 1).

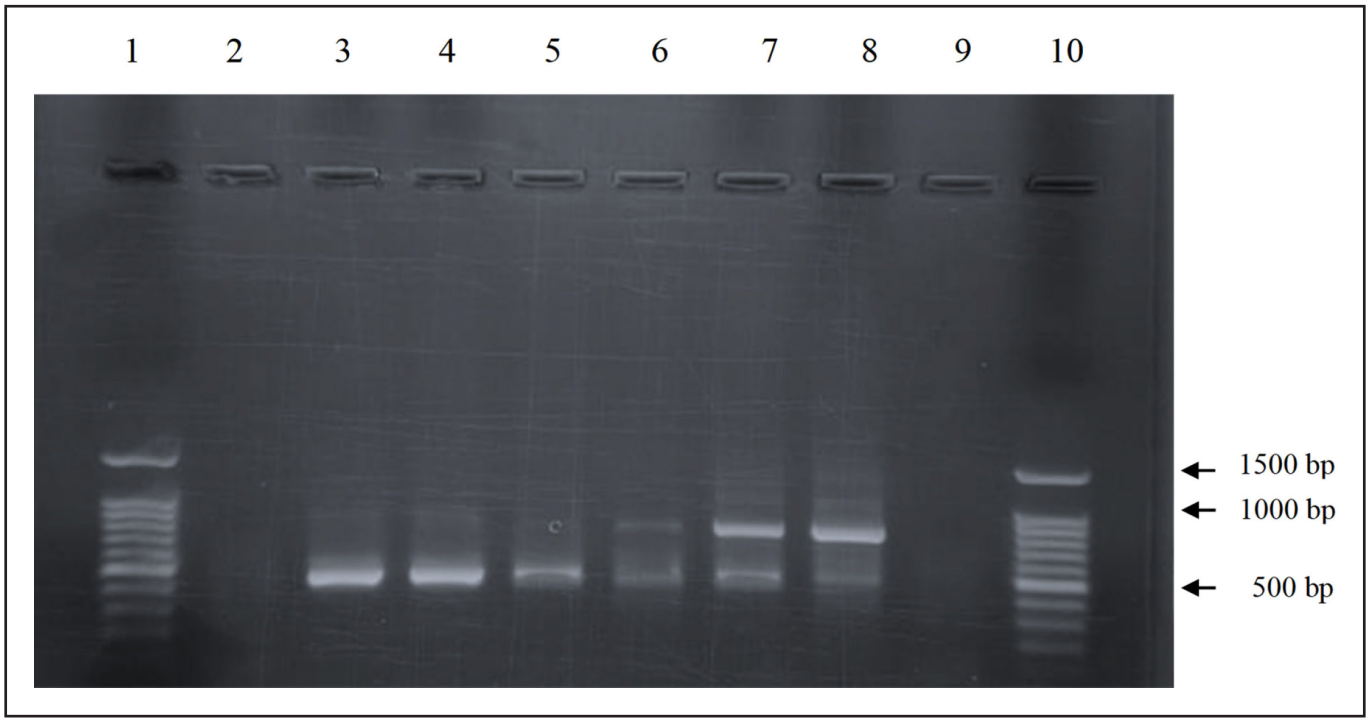

Figure 1. Gel electrophoresis image showing the amplification of the tick-specific partial 16S rRNA gene. Lane 1: 100 bp DNA ladder, Lane 2: Negative control, Lane 3: Tick-positive control, Lane 4: 0 d.p.i. inoculum, Lane 5: 2 d.p.i., Lane 6: 5 d.p.i., Lane 7: 6 d.p.i., Lane 8: 12 d.p.i., Lane 9: Not available, Lane 10: 100 bp DNA ladder.

Cell morphology changes during *w*Cfe infection of C6/36 cells

Based on the phase contrast microscopy images used to assess the morphology of C6/36 cells following exposure to *w*Cfe (Figure 2), the infected cells at 2 d.p.i. displayed a comparable homogeneity in cell size and distribution to that of uninfected controls. No overt cytopathic effects were evident at this stage. By 6 d.p.i., the infected cells appeared denser and more compact, showing pronounced clumping with reduced intercellular spaces and less distinct cell boundaries, whereas uninfected control cells retained the characteristic rounded or spindle-like morphology typical of C6/36 cells (Singh, 1967).

Observation of infected cells in Giemsa-stained cytocentrifuge smears

At 2 and 3 d.p.i., examination of around 100 C6/36 cells from infected cultures revealed coccoid or rod-shaped forms of *Wolbachia* to be scarce or absent. From 5 d.p.i. onwards, intracellular bacteria were clearly visible in around 50% of cells (Figure 3A). Heavily-infected cells were visible by 12 d.p.i, with some cells beginning to die and rupture, releasing bacteria (Figure 3B). At this terminal time point, almost 90% of the cells were infected based on the observation of 100-200 cells per smear. No bacteria were seen in uninfected control cells (Figure 3C).

Replication kinetics of *w*Cfe in C6/36 cells

The number of *w*Cfe bacteria in C6/36 cells was quantified as the copy number of the *Wolbachia* 16S rRNA gene target determined by qPCR to assess the growth of the endosymbiont upon infection of naïve C6/36 cells. The replication kinetics were assessed in three independent infection experiments (n = 3) from 0, 2, 3, 5, 6, and 12 d.p.i., with bacterial load expressed as DNA copy number per µL, with data presented as mean ± SE (Figure 4). To allow fair comparison with later time points, the inoculum value at 0 d.p.i. was adjusted for the 1:5 dilution of the inoculum. Based on the adjusted mean copies/µL across biological replicates, 5.93×10^4 copies/µL of *w*Cfe were used to initiate infection at 0 d.p.i. From 2 to 5 d.p.i., the copy number remained relatively stable, consistent with a lag phase following establishment. A modest increase was observed by 6 d.p.i., followed by pronounced growth thereafter, indicative of exponential growth, with a maximum copy number reaching 1.50×10^6 copies/µL at 12 d.p.i. Overall, this corresponded to a 25.30-fold increase relative to 0 d.p.i. The generation time of *w*Cfe in C6/36 cells across all the replicates was between 1.7 and 2.5 days.

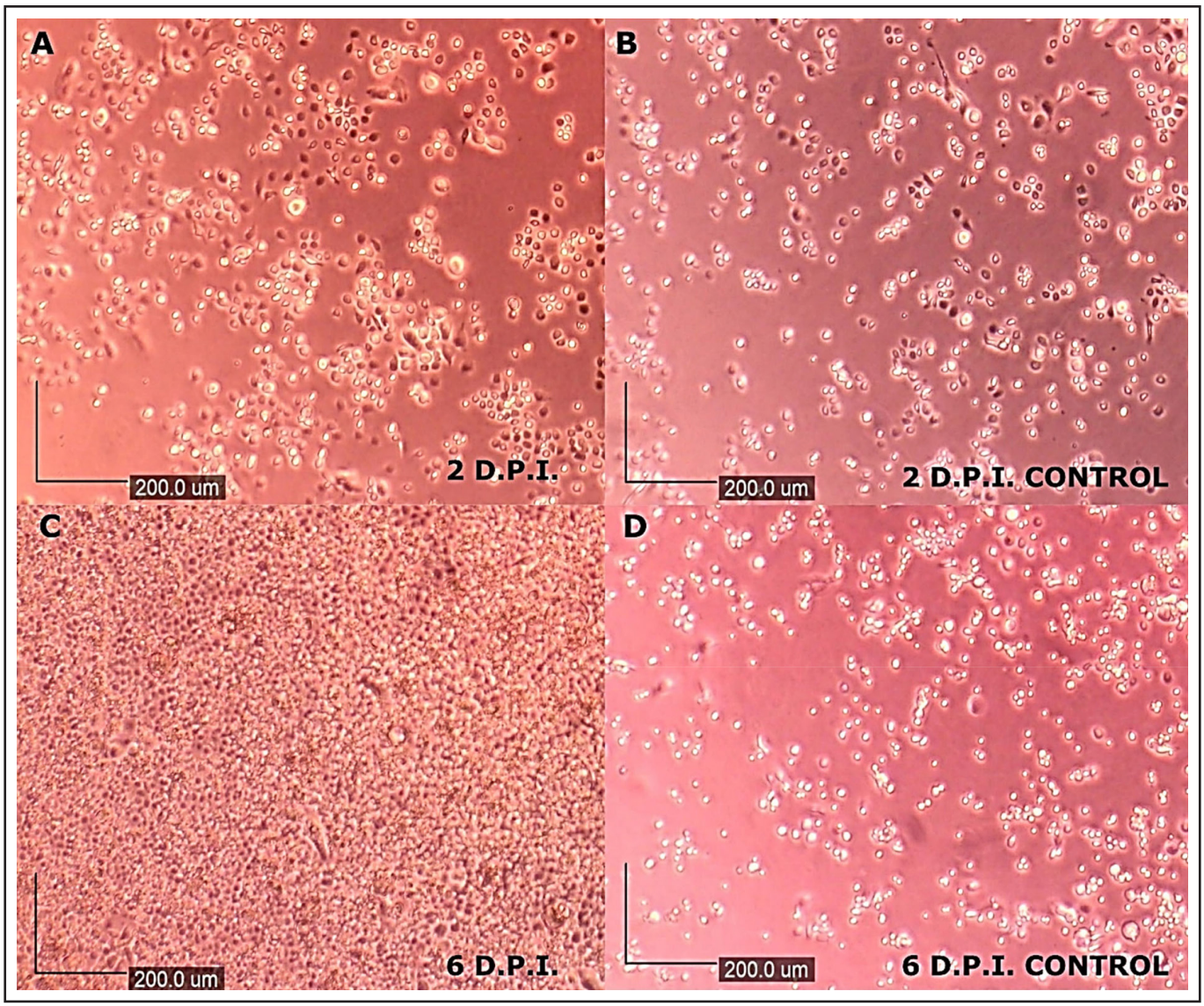

Figure 2. Phase-contrast images of the live C6/36 culture infected with *Wolbachia* strain *w*Cfe (A & C) and uninfected controls (B & D) at 2- and 6-days post-infection (d.p.i.) demonstrating increased cell clustering and density (Scale bar represents 200 µm).

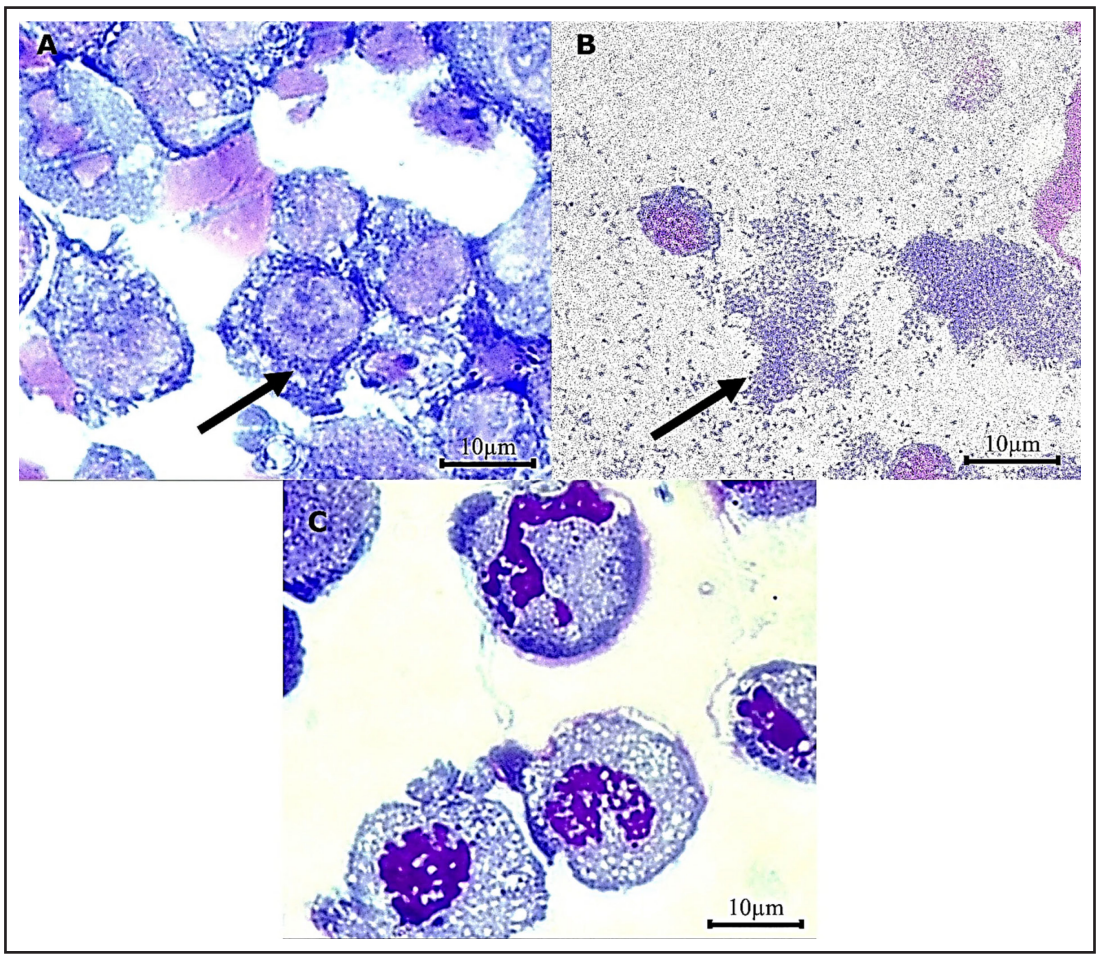

Figure 3. Giemsa-stained cytocentrifuge smears prepared from C6/36 cells with *Wolbachia* strain *w*Cfe. Infected cells at 5 days post-infection (d.p.i.) (A), and at 12 d.p.i. (B), with the uninfected control cells (C), Arrows indicate the presence of bacteria. Scale bars represent 10 µm.

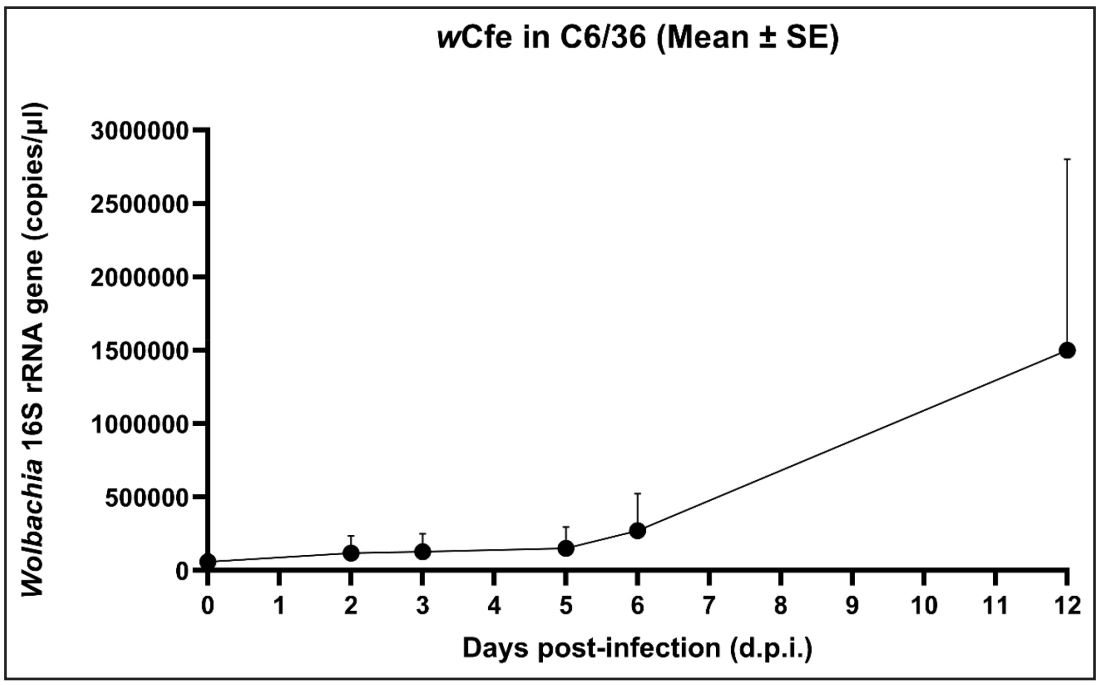

Figure 4. Replication kinetics of *w*Cfe in the *Aedes albopictus*-derived cell line C6/36 based on the copy number of the *Wolbachia* 16S rRNA gene determined from three independent experiments (*n*=3) of three different *w*Cfe passages in the inoculum. The bacterial load was expressed as DNA copy number per µL. Points represent the mean ± SE of the three biological replicates.

DISCUSSION

In the present study, infection of C6/36 cells with *w*Cfe was successfully initiated, as confirmed by qPCR analysis. These findings demonstrate that phylogenetically divergent *Wolbachia* strains can be propagated in the mosquito-derived cell line, consistent with previous observations for other *Wolbachia*–host cell systems (Fallon, 2021). The replication kinetics analysis was limited to a preliminary study in 24-well plates, which undeniably reduced the possible observation time and limited the total number of infected cells available for analysis, and infection using larger culture flasks would have allowed a more extensive characterisation of the novel endosymbiont in the mosquito cells. Nevertheless, we have demonstrated the possibility of using C6/36 for the propagation of *w*Cfe as the bacteria were shown to continue to proliferate (Figure 4) during the early observation period. Based on the BLAST analysis, the use of tick-specific 16S rRNA primers on the samples from the last time-point yielded a low query cover, indicating a non-specific amplification rather than a true, reliable detection of tick DNA. No high coverage that matched the tick-derived sequences was detected at 12 d.p.i. compared to the tick-positive control, and at the earliest time points. This suggests that the tick DNA, which was initially present as carry-over from the inoculum, was progressively lost and became undetectable below the detection limit as the infection progressed. Therefore, the observed proliferation was most likely driven by the endosymbiont after it had successfully adapted to the C6/36 cells.

The *w*Cfe growth curve across all replicates exhibited an initial lag-stationary phase followed by an exponential phase, with no distinct declining phase. Bacterial growth phase is generally divided into four classical phases: initial lag phase, log (exponential) growth phase, stationary phase and death phase (Fenollar *et al*., 2003). The absence of the declining phase may be due to the bacterial culture not entering the death phase within the observed timeframe. The exponential phase of *w*Cfe, which only commenced at approximately 6 d.p,i., contrasts with a rather fast and favourable growth of *W. pipientis* in Aa23 within 24 hours, as reported by Fenollar *et al*. (2003). This difference is unsurprising, given that *W. pipientis* was already stably maintained in its natural mosquito host cell line prior to experimentation. *w*Cfe in our study originated from cat fleas and was subsequently propagated in tick cells before introduction into C6/36 cells; such sequential host transitions may impose physiological constraints, resulting in a prolonged lag prior to efficient replication. Although, like IDE8, C6/36 can be maintained in Leibovitz L-15-based medium (Abrao & da Fonseca, 2016; Santana-Román *et al*., 2021), the inoculum was diluted with EMEM to reduce the risk of the C6/36 cells suffering stress from the residue of tick cell medium and to aid better adaptation, since C6/36 in our laboratory had never been routinely cultured in L-15B. At 2 d.p.i., the infected cells appeared relatively normal compared to the controls, thereby providing a stable cell environment for growth (Figure 2A). Further optimisation of the working medium and seeding volume will be required to determine whether *w*Cfe can be maintained in C6/36 cells for long-term passages. Regardless, the inoculum was infectious enough to initiate and promote progressive growth, which is a crucial consideration in establishing a successful infection (Fallon, 2019).

In the context of mosquito cells, the delayed development of *Wolbachia* transinfected into C6/36 was similarly reported for *w*Pip isolated from the naturally-infected *Culex pipiens* cell line CPE/LULS50 (Bell Sakyi *et al*., 2021) and a novel *Wolbachia* strain, *w*ChemPL3, isolated into *Drosophila* S2 cells from the bedbug *Cimex hemipterus* (Laidoudi *et al*., 2020), while the infection of *Wolbachia* strains sourced from diverse insect hosts was confirmed after about 3 passages in the *Ae. albopictus*-derived cell line NIAS-AeA1-2 (Furukawa *et al*., 2008). Likewise, *w*Mel, a strain naturally inhabiting *D. melanogaster*, was successfully cultured long-term in naïve C6/36 up to 45 passages, whilst remaining unstable in the tetracycline-treated Aa23 cell line, which initially hosted *Wolbachia* strain *w*AlbB (Voronin *et al*., 2010). Comparable slow development has also been observed outside mosquito cultures, as demonstrated by *w*CfeJ – the same strain also used in this study, which showed delayed growth following introduction into a newly established *Glossina morsitans morsitans*–derived cell line (GMA/LULS61), in contrast to the more rapid proliferation of *w*AlbB and *w*Str1 in the same cell line (Bell-Sakyi *et al*., 2024). Although replication kinetics were not reported in these studies for those strains, the findings showcased the complexity of *Wolbachia* adaptation and maintenance following transinfection into heterologous recipient cell lines. Most of these studies also maintained the *Wolbachia*-infected cultures at 28°C, similar to the temperature used to maintain *w*Cfe in this study; therefore, incubation temperature alone is unlikely to explain the discrepancies observed across the cell lines. Overall, the successful establishment of *Wolbachia* into a host cell line may depend on the differences between *Wolbachia* strains and the variable nature of the recipient host cell lines prior to infection (Fallon, 2019). Fluctuations in *Wolbachia* levels are also influenced by host cell density, which can depend on the passaging approach and the state of confluency of the culture (Khoo *et al*., 2013).

The morphological assessment of the cells following transinfection of *w*Cfe into C6/36 was carried out to complement the replication kinetic assessment. *Wolbachia* infection has been known to induce changes in host phenotypes, including altering the intracellular structures and host cell metabolism (Brennan *et al*., 2008; White *et al*., 2017; Jacobs *et al*., 2025). Many studies have reported cellular morphology changes in the transinfected host, although some *Wolbachia* strains did not impose any aberrant effect on the infected host cells (Fenollar *et al*., 2003; Khoo *et al*., 2020), highlighting the variability of host responses to *Wolbachia* infection. In the study reported by Khoo *et al*. (2013), both *w*AlbB and *w*Ri caused an increase in infected cell size correlated with the infection. Similarly, *Wolbachia*-infected AeA1-2 cells also resulted in cell clumping (Noda *et al*., 2002). More severe cytopathic effects have been reported for *w*Stri and *w*Cfe in the ISE6 (*I. scapularis*), a tick-derived cell line, where infection led to enlarged cells and cell lysis (Khoo *et al*., 2020; Bahrain *et al*., 2024). These observations are in agreement with the findings of the present study. In this study, progressive morphological changes became apparent at later time points post-infection (Figure 2C), coinciding with the increase in bacterial load observed in the kinetic analysis (Figure 4). Additionally, floating cells were also observed in the infected cells at the later time points, which suggests the occurrence of potential cellular stress induced by the infection.

Giemsa staining can facilitate clearer visualisation of the intracellular bacteria and the infected host cellular structure, which was not readily discernible by phase-contrast microscopy. At 12 d.p.i., Giemsa-stained preparations revealed heavy *w*Cfe infection, with some cells exhibiting membrane rupture and release of cell-free *Wolbachia* – features typically associated with cytopathic effects. However, similar observations have been reported in other studies where cells heavily-infected with *Wolbachia* co-exist with subpopulations of uninfected cells that remain viable (Fenollar *et al*., 2003; Khoo *et al*., 2020; Bell-Sakyi *et al*., 2021; Bahrain *et al*., 2024), and therefore may contribute to the longevity of the infected culture. This highlights the need to quantify *Ae. albopictus* host cell copy number to determine whether host cell proliferation would be able to continue under high *w*Cfe burden. This would also help to distinguish whether the cytopathic effect was induced by other cellular environment factors, such as nutrient deficiency or growth arrest associated with high cell density. Overall, this suggests that C6/36 cells may possess a degree of tolerance to *w*Cfe infection, although further investigation is required to confirm this capacity.

Shell vial techniques remain a common approach for introducing *Wolbachia* into new host cell lines (Dobson *et al*., 2002; Ghosh *et al*., 2019; Skinner *et al*., 2022), typically relying on vigorous

centrifugation to deposit the bacteria onto the cell monolayer and possibly forcing them into the cells. More recently, it has been demonstrated that gently collecting semi-purified *Wolbachia* prepared from infected culture supernatants using mild pipetting and low-speed centrifugation and then simply adding them to the recipient culture is also effective (Bell-Sakyi *et al*., 2021) and is deemed highly infectious (Fallon, 2019) for establishing infection in host cell lines. Therefore, the infection technique described in this study could be considered for future *Wolbachia* introduction into novel host cells.

Based on the qPCR-inferred growth kinetics, the apparent doubling time of *w*Cfe in C6/36 cells was longer compared to that observed for *W. pipientis* in Aa23 cells by Fenollar *et al*. (2003), which was estimated to be 14 hours. There are limited studies describing *Wolbachia*'s doubling rate and a detailed analysis of replication kinetics following transinfection into a cell line, with most studies focusing on the overall trend in bacterial density. *Wolbachia*'s replication and transmission across generations in its host is widely known to be temperature-sensitive (Mouton *et al*., 2005; Wiwatanaratanabutr & Kittayapong, 2009); however, the replication rates of *Wolbachia* and adaptation across different cell lines *in vitro* that are suggested to be influenced by parameters such as maintenance temperature, including culture medium remain scarcely studied. In a study on the transinfection of several *Wolbachia* strains into a *Haematobia irritans*-derived cell line, HIE-18, certain temperatures and culture media affected the density of *w*AlbB, *w*Mel, and *w*MelPop (Madhav *et al*., 2020). Other studies have also suggested that variations in the host cell background, such as cell morphologies (Ghosh *et al*., 2019), cell karyotypes (Fallon, 2019) and the dividing state of the host cells (Fallon, 2022), may influence the efficient proliferation of a *Wolbachia* strain within a cell line. Overall, studies suggest that differences in host cell types and physicochemical culture conditions may influence the apparent replication rate of *Wolbachia* under *in vitro* conditions, which in turn may be reflected in the differences in generation time. In the present study, it is possible that *w*Cfe exhibited a prolonged generation time due to its transition from tick cells to mosquito-derived cell lines, in contrast with Fenollar *et al*. (2003), where their selected strain had already been accustomed to the target cell line. It is not yet known whether C6/36 morphologies, the rate of cell division, or culture conditions contributed to the slower doubling time observed for *w*Cfe.

Admittedly, in this study, a higher culture passage number was used for the infection study. Higher-passage cell lines would have undergone changes in genetic and phenotypic drift and protein expression levels that may subsequently alter their permissiveness, immune response to a pathogen infection, and the transinfection efficiency. Differences in the host cell culture age may have affected *Wolbachia*'s growth dynamics, although this was less investigated as an independent driver. Fallon (2019), however, studied the effect of host cell age on infectivity quality of *Wolbachia* that was released from the cell into the medium, in which the resuspended cells were subsequently used as inoculum. The study revealed optimal infectious units could be obtained in the inoculum released from moderately aged and replenished cultures that had reached confluency, formed adherent clusters, and begun detaching, with limited apoptosis. This indicates that host cell age, and potentially a passage number, can influence *Wolbachia* infectivity and should therefore be considered when interpreting differences in transinfection efficiency and the endosymbiont growth dynamics. Apart from this, there is a less widely reported range of host passage numbers used for *Wolbachia*'s maintenance. One reported example used C6/36 cells at passages 11 to 16 (Ha *et al*., 2021) for transient *Wolbachia* infection, whereas tick cell lines at higher passage numbers (approximately at 80 to 100 passages) can be utilised for *Wolbachia* strain infection as well as other intracellular bacteria such as *Anaplasma* (Almazán *et al*., 2020; Bell-Sakyi *et al*., 2021). Ultimately, most studies emphasised maintenance conditions rather than a defined cell line passaging number before *Wolbachia* infection. Nevertheless, we have taken a few considerable steps to ensure the cells were in a good physiological condition prior to the infection experiment, importantly by observing the cellular morphology that revealed normal and consistent growth rates after thawing and during subsequent routine passaging. Additionally, routine mycoplasma screening had also been performed in the cells maintained in the laboratory, including C6/36, to further ensure the reliability of the cells. Overall, it is recommended to reassess the *w*Cfe doubling time once its infection in the mosquito-derived cell lines has stabilised and been maintained over several passages. Thereafter, the effect of variable mosquito host cell passage numbers on doubling time of *w*Cfe and their growth kinetics is worth a future investigation, as this factor has not been considered a strict experimental parameter in many *Wolbachia in vitro* studies.

CONCLUSION

The present study conducted a preliminary investigation of infection by *C. felis*-derived *Wolbachia* (*w*Cfe) in C6/36 cells across multiple time points, with bacterial load quantified using qPCR. Our results indicate that C6/36 cells can support the growth of *w*Cfe, with the bacterium continuing to proliferate beyond 6 days post-infection. However, long-term studies are required to assess the persistence of *w*Cfe in C6/36 cells over extended periods. Despite successful inoculation of *w*Cfe by following this technique, more validation is needed through continuous passaging when its establishment is scaled up in the future. Additionally, Fluorescence *In Situ* Hybridization (FISH) can be employed alongside qPCR to provide complementary data for quantifying and visualising infection levels within the host cells.

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Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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