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Establishment and characterization of two new cell lines from the mosquito *Armigeres subalbatus* (Coquillett) (Diptera: Culicidae)

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Abstract *Armigeres subalbatus* (Coquillett) is a medically important mosquito and a model species for immunology research. We successfully established two cell lines from the neonate larvae of *A. subalbatus* using two different media. To our knowledge, this is the first report of an established *Armigeres* mosquito cell line. The cell lines, designated as Ar-3 and Ar-13, consist of adherent and diploid cells with compact colonies. Both these cell lines grow slowly after passage at a split ratio of 1:5 and a population doubling time of 2.7 and 3.0 d, respectively. Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) was used to confirm that these lines correspond to the species of origin and are clearly distinct from seven other insect cell lines. Furthermore, reverse-transcription PCR was used to demonstrate that the Ar-3 cell line is susceptible to the Japanese encephalitis virus and two insect flaviviruses associated with *Culex* and *Aedes* mosquitoes but relatively insensitive to dengue virus. These data indicate that the newly established cell lines are cellular models of *A. subalbatus* as well as beneficial tools for the propagation of viruses associated with the *Armigeres* mosquito.

Keywords Cell line · Mosquito · *Armigeres subalbatus* · Flavivirus

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Introduction

Mosquito cell lines offer unique pathogen susceptibilities making them advantageous for medical studies (Pudney *et al.* 1982; Mitsuhashi 1994). Notably, mosquitoes include many species known to be principal vectors that facilitate pathogenic viral transmission. Over the past several years, mosquito cell culture has significantly progressed with respect to virological research (Kuwata *et al.* 2012). However, the variety of mosquito species from which the available cell lines are derived is limited, depending upon the specific vector–virus association. C6/36 cells cloned from Singh's line from the *Aedes albopictus* mosquito (Igarashi 1978) have been used for experiments with viruses, such as the Japanese encephalitis virus (JEV), which are mainly transmitted by the *Culex* mosquitoes. This limited variety of mosquito cell lines is partly attributed to the loss of cryopreserved lines. Some of these lost lines have since been recovered through the establishment of new lines, especially from *Culex* (Athawale *et al.* 2002; Segura *et al.* 2012; Kuwata *et al.* 2012). The mosquito *Armigeres subalbatus* (Coquillett) has been recognized as one of the synanthropic and medically important species in Asia (Tanaka *et al.* 1979; Muslim *et al.* 2013) and is also a physiological model species because of its distinct innate immune system (Sasaki *et al.* 2010; Aliota *et al.* 2010). Thus, an *Armigeres* cell line would play a significant role in several studies concerning the biology and virology of *Armigeres* mosquito because no cell line from this species has ever been

reported (Mitsuhashi 2002) although an attempt to culture *A. subalbatus* was noted (Hink 1976).

In this study, we attempted to culture *A. subalbatus* mosquito cells to establish a cell line from the species. However, establishment of mosquito cell lines still depends on the spontaneous growth of primary cultured cells without an efficient derivation method such as the expression of oncogenes in *Drosophila* (Simcox *et al.* 2008). Since the establishment of earlier mosquito lines from the genus *Aedes* (Grace 1966; Singh 1967) using Grace's (Grace 1962) and MM (Mitsuhashi and Maramorosch 1964) media, modification of the media components has facilitated the establishment of cell lines from species belonging to other genera such as *Psorophora* (Bello *et al.* 2001), *Culex* (Kitamura 1970), as well as *Anopheles* (Schneider 1969). Accordingly, we initiated mosquito cultures in media that have been successfully used for culturing various genera of mosquitoes because the genus *Armigeres* is taxonomically different from any genera known from which cell lines have been established (Tanaka 2005; Reinert *et al.* 2009). After establishing the mosquito cell lines, we performed basic characterization of their morphology, growth kinetics, karyotyping, and authentication, and further analyzed their viral susceptibility.

Materials and Methods

Culture media. We prepared four types of media specifically developed for insect cell culture: MM (Mitsuhashi and Maramorosch 1964), VP-12 (adjusted to pH 6.9) (Varma and Pudney 1969), Schneider's (Sigma-Aldrich Japan, Tokyo, Japan) (Schneider 1972), and MGM450 (adjusted to pH 6.3) (Mitsuhashi and Inoue 1988). The mosquito cells were cultured separately in each media and in mixtures of two types of media; thus, a total of ten types of media were tested to select the most suitable for the primary cultures. All the media were supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS), which did not contain any antibiotics.

Mosquito and primary culture. A laboratory colony of *A. subalbatus* established by the US Army 406th Medical General Laboratory was maintained in a controlled insectary at 25°C in 50–70% relative humidity and a light/dark photoperiod of 16/8 h. The eggs deposited on the filter paper were surface-sterilized three times by immersion in 70% ethanol for 3 min and subsequently dried on autoclaved filter paper. The sterilized eggs were then transferred to a new 35-mm plastic dish (Falcon, Oxnard, CA) containing 2 mL of culture medium and allowed to hatch. The hatched neonate larvae were collected in 1.5 mL centrifuge tubes (Greiner Bio-One Co Ltd., Tokyo, Japan), placed on ice, and then washed three times with fresh medium without floating the larvae that settle at the bottom. The sterile larvae were mixed with a drop of medium (80 μL volume, ten larvae per drop) sitting on a new 35-mm surface-modified tissue culture dish (Primaria; Falcon). The larvae were then cut into pieces with a razor blade, and the head portions were removed to avoid their feeding on the other larval pieces. All cultures were maintained by placing a drop of sterile water at the margins of the dish to prevent desiccation of the culture medium, sealed with Parafilm (American National Can, Greenwich, CT), and kept at 25°C. Primary cultures were initiated under the same conditions in triplicate.

Establishment of cell lines. One week after initiation of primary cultures, 1.5 mL of each medium was added to the samples after removal of the water drop. The medium (0.2–0.5 mL) was exchanged or added regularly. During this process, both migrations and divisions of the cells in each sample were observed by phase contrast microscopy (IX71, Olympus). When the cells reached confluency, they were suspended by pipetting and subcultured in 12.5-cm^2 culture flasks (3018, Falcon). Thereafter, the cells were subcultured at intervals of approximately 2 wk, with a split ratio of 1:1 to 1:2, leading to the establishment of two cell lines designated as Ar-3 and Ar-13.

Characterization of the cell lines. The morphology of the Ar-3 and Ar-13 cells was observed by phase contrast microscopy. For construction of growth curves, the initial cell density was set at 4.0×10^5 cells/mL, and the number of cells was counted in triplicate every 2 d using a Thoma's hemocytometer. The growth curves of the Ar-3 and Ar-13 cell lines were analyzed at the 100th and 70th passages, respectively. Next, the karyotypes of the Ar-3 and Ar-13 cells were analyzed at the 100th and 88th passages, respectively, as previously described (Mitsuhashi 2002). Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (Mitsuhashi 2002) was then performed on the following samples: Ar-3 and Ar-13; adult *A. subalbatus* mosquitoes; the three mosquito cell lines C6/36 (Health Science Research Resources Bank, Osaka, Japan), NIAS-AeAl-2 (Mitsuhashi 1981) from *A. albopictus*, and NIID-CTR from *Culex tritaeniorhynchus* (Kuwata *et al.* 2012); the dipteran S2 cell line from *Drosophila melanogaster* (Schneider 1972); two lepidopteran cell lines, Sf21 from *Spodoptera frugiperda* (Vaughn, *et al.* 1977) and GaMe-LF1 from *Galleria mellonella* (Eguchi and Iwabuchi 2006); and the coleopteran cell line PC-1 from *Plagionotus christophi* (Hoshino *et al.* 2009a). DNA was extracted from the cell pellets or mosquito legs using the DNeasy blood and tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The eluted DNA solution was washed and concentrated using the ethanol precipitation method. Both the concentration and the purity of the samples were confirmed using Nanodrop (Thermo Fisher Scientific, Wilmington, DE).

The ratio of absorbance at 260 and 280 nm (260/280) ranges from 1.61 to 1.86. The samples were stored at −80°C until further use. PCR was conducted using Quick Taq HS Dyemix (Toyobo Co. Ltd., Tokyo, Japan). Two 12-mer oligonucleotides (5′-CCGCAGTTAGAT-3′ and 5′-ACTGGCCGAGGG-3′) were used for RAPD-PCR analysis. PCR was performed in duplicate, according to the manufacturer's instructions using 50 ng DNA in a 50-μL reaction volume under the following PCR conditions: 2 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 42°C, and 2 min at 68°C. The amplicons were electrophoresed in 1.5% agarose gel and stained with ethidium bromide, followed by comparative analysis of the images captured under UV light.

Viral susceptibility. Ar-3 cultured in VP12 medium was selected for large-scale culture based on the medium cost. The following four inoculated flaviviruses were selected: dengue virus (DENV); JEV, isolated from wild-caught *A. subalbatus* (Weng *et al.* 1999; Chen *et al.* 2000; Feng *et al.* 2012; Liu *et al.* 2013); and *Culex* flavivirus (CXFV) and *Aedes* flavivirus (AEFV), insect flaviviruses associated with the *Culex* and *Aedes* mosquitoes, respectively (Hoshino *et al.* 2007, 2009b), but never isolated from the *Armigeres* species. To test the viral susceptibility of Ar-3 cells, the following four flaviviral strains were prepared from our virus stocks: JEV strain Mie/41/2002 (Nerome *et al.* 2007; GenBank accession no. AB241119), DENV strain NIID02-20 (Tajima *et al.* 2006; GenBank accession no. AB178040), CXFV strain NIID-21 (Hoshino *et al.* 2007; GenBank accession no. AB377213), and AEFV strain Narita-21 (Hoshino *et al.* 2009b; GenBank accession no. AB488408). The copy number of insect flaviviral genomic RNA in the stock solution was determined by quantitative reverse-transcription PCR (RT-PCR) using primers specific for CXFV (Isawa *et al.* 2012) and AEFV (sequence position 156–363 in strain Narita-21). To prepare the viral RNA standards, in vitro-transcribed RNAs from CXFV and AEFV cDNA were generated according to a previously described method (Isawa *et al.* 2012). The resultant transcripts were purified using the RNeasy MinElute Cleanup kit (QIAGEN), quantified spectrophotometrically using Nanodrop (Thermo Fisher Scientific) to determine the copy number of the RNA standard, and then used as a template for standard curve analysis. Each viral stock solution in VP12 medium (1 mL) containing 2% FBS was inoculated onto 80% confluent cell monolayers of Ar-3 cells seeded in 25-cm^2 flasks (3014, Falcon) at 10^2–10^3 plaque-forming units (PFU)/mL for JEV and DENV (titered with standard plaque assay method described by Tajima *et al.* 2010) or 10^2–10^3 RNA copies/mL for CXFV and AEFV. The inoculum was removed after 2 h of viral adsorption. Finally, the flasks were filled with 5 mL fresh medium containing 2% FBS after washing three times with Dulbecco's phosphate-buffered saline (D8662, Sigma-Aldrich Japan). The culture medium was sampled on days 0, 1, 2, 3, 5, and 7 after the viral inoculation. RNA was extracted from the samples using QIAamp Viral RNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Viral detection was conducted by RT-PCR using PrimeScript One Step RT-PCR Kit Ver.2 (Takara Bio., Shiga, Japan). PCR was performed according to the manufacturer's instructions under the following conditions: 30 min at 50°C for reverse transcription, 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. The primers used were specific for each viral envelope region as follows: JEV-E31F, 5′- TTC ATA GAA GGA GCC AGT GGA GC -3′ and JEV-E287R, 5′-AAG CCT TGT TTG CAC AC -3′ for JEV; DEN-F, 5′-AAG GAC TAG AGG TTA KAG GAG ACC C-3′ and DEN-R, 5′-GGC GYT CTG TGC CTG GAW TGA TG-3′ for DENV (Callahan *et al.* 2001); CXFV-E417F, 5′- CTC AGT GGA TGA CGT CCA GCA ACT C -3′ and CXFV-E963R, 5′- TCC AGG TCC GAA CAC CAT CTT CGT C -3′ for CXFV; and AEFV-E156F, 5′- AAC GTT GCT AAC ATC ATG TGA GAT -3′ and AEFV-E363R, 5′- ATT TAC CAT GGT CAG GCA TTG GA -3′ for AEFV. The amplified products were separated on 2% agarose gels and visualized with ethidium bromide. For the infective virus quantification of JEV and DENV, small aliquots of the medium were recovered on days 1, 3, and 7 after the viral inoculation and titrated by a standard plaque assay on Vero cells described by Tajima *et al.* 2010. Briefly, Vero cells were fixed with 3.7% (*v*/*v*) formaldehyde solution in PBS for 1 h, the methylcellulose overlay was removed, and the cells were stained with methylene blue solution.

Results

Primary culture and establishment of cell lines. Cell migration was significantly induced in the primary cultures when VP12, MM+MGM450, and Schneider's media were used (Fig. 1*A*). Three independent cultures grown in each of these three media were found to show enough growth for subculturing within 2–3 mo and were then transferred to new flasks. During subculturing, cell growth continued with frequent appearance of vacuolated cells in the earlier phase (Fig. 1*B*, *C*). However, as the passage number increased, the cells became uniformly adherent (Fig. 1*D*). Finally, two cell lines grown in VP12 and MM+MGM450 media and stably maintained without any crisis period were selected and designated as Ar-3 and Ar-13, respectively. These lines have now been subcultured for more than 200 passages over a period of 7 yr. Both cell lines formed many compact colonies with high densities, even if dissociated during passaging (Fig. 2*A*, *B*). Unfortunately, all the cell lines grown in Schneider's medium

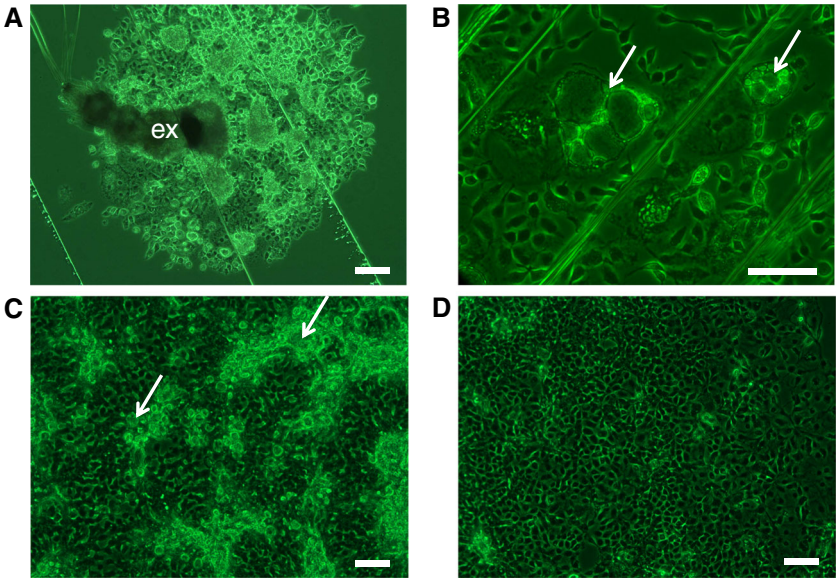

Figure 1 Phase contrast micrographs of *A. subalbatus* cells in culture. *A* Representative image of cell migration from an explant (*ex*) of a neonate larval piece. *B* Surviving and vacuolated cells (*arrows*) observed at 3 mo after the initiation of primary culture. Lines on the surface of the culture dish seen in *A* and *B* are scars made by razors used to cut the neonate larvae. Cells growing after the seventh and eighth passages and vacuolated cells (*arrows*) in the *C* Ar-3 and *D* Ar-13 cell lines. *Bars* indicate 50 μm.

were lost during subculturing due to severe crystallization of the medium. Among the other media samples, some cell migration was observed in the lines grown in VP12+MM [Tra medium in Kuno (1980)], VP12+MGM450, and VP12+ Schneider's media, while MM medium promoted the formation of several hollow spherical vesicles. However, none of these media enabled continuous cell growth.

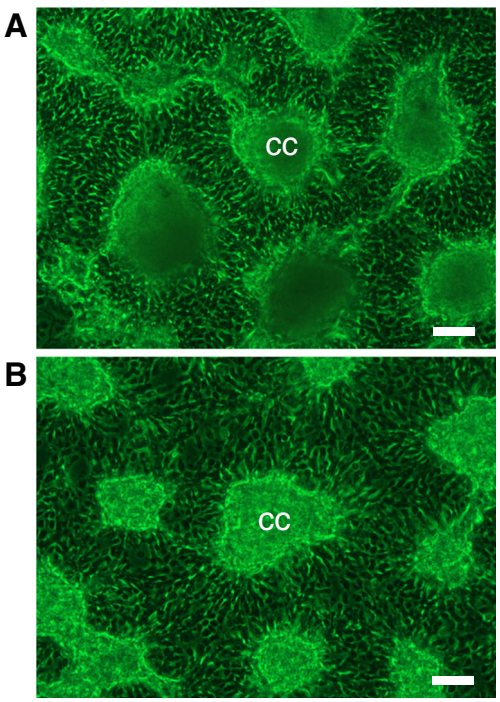

Figure 2 Cellular morphology of the established *A. subalbatus* cell lines. Both *A* Ar-3 and *B* Ar-13 cell lines contain adherent cells (appearing black due to phase contrast microscopy) and compact colonies (*cc*) with a patch-like distribution. Bar indicates 50 μm.

Cell growth, karyotypic analysis, and authentication by RAPD-PCR. The split ratio during subculturing was fixed at 1:5 after passage 100. Both the Ar-3 and Ar-13 cell lines showed similar growth curves consisting of a clear but flat lag phase, an exponential phase that slowly increased for 6–7 d, and a confluent plateau phase that remained stable for at least 4 d (Fig. 3). The population doubling time (PDT) at the most rapid growth period during the exponential phase was calculated to be 2.7 and 3.0 d for the Ar-3 and Ar-13 lines, respectively. The chromosome number was counted in the Ar-13 line and determined to be 6 (Fig. 4), which corresponds to that of diploid ($2n=6$) in the species of origin, *A. subalbatus* (Clements 1992; Marquardt 2004), with $N=36$ and $N=30$ for Ar-3 and Ar-13 cells, respectively. RAPD-PCR was performed to further confirm that both Ar cell lines are novel. The electrophoresis band patterns obtained after RAPD-PCR on the DNA extracted from Ar-3, Ar-13, *A. subalbatus*, and seven additional insect cell lines were compared. The band pattern was confirmed by each of the two primers to correspond to Ar-3, Ar-13, and *A. subalbatus*, although some differences in the intensity of the bands were detected (Fig. 5). Both the Ar-3 and Ar-13 lines showed band patterns that were distinct from those of the other seven insect cell lines. In contrast, the two cell lines that originated from the same species (C6/36 and AeAl-2) did not show the same band patterns with each of the two primers. There was no difference between the resultant band patterns of RAPD-PCR performed in duplicate; one representative electrophoretic image for each primer is shown in Fig. 5.

Susceptibility to four flaviviruses. Viral susceptibility was demonstrated through molecular analysis of the supernatant of the virus-inoculated Ar-3 cells using RT-PCR (Fig. 6). The

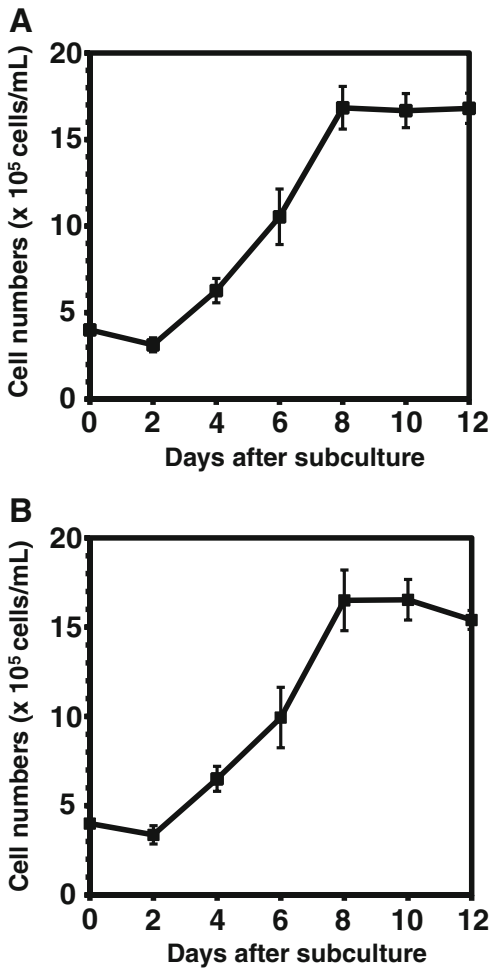

Figure 3 Growth curves of the *A. subalbatus* cell lines. *A* Ar-3 cells at the 100th passage. *B* Ar-13 cells at the 70th passage. Each point is the mean of three independent cultures. *Bars* represent standard deviation.

specific band at the expected amplicon size for JEV was detected from 3 d post-inoculation (p.i.), and intense staining of the band was observed 5 d p.i. In contrast, DENV was not detected until 15 d p.i. (data not shown). The insect

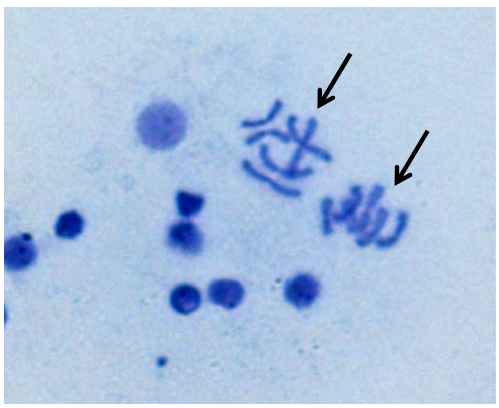

Figure 4 Micrograph of the chromosomes from Ar-13 cells stained with Giemsa (*arrows*). The number of chromosomes is 6, consistent with that of the diploid cells of the species of origin.

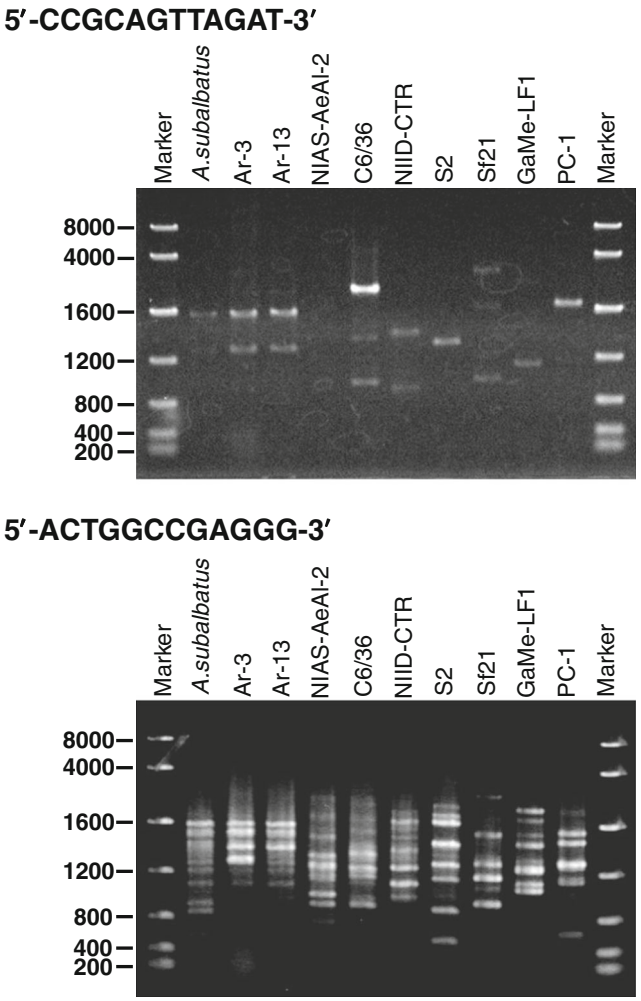

Figure 5 Electrophoretic images of random amplified polymorphic DNA (*RAPD*) profiles obtained by polymerase chain reaction (*PCR*) with two different 12-mer oligonucleotides (5′-CCGCAGTTAGAT-3′; 5′-ACTGGCCGAGGG-3′). *Marker* molecular marker; the *numbers on the left* represent the molecular size (bp). *A. sabalbatus* mosquito, the original source of the new cell lines; *Ar-3* the new cell line from *A. subalbatus*, cultured in VP12 medium; *Ar-13* the new cell line from *A. subalbatus*, cultured in MM+MGM450 medium. *NIAS-AeAl-2, C6/36* cell line from *A. albopictus* mosquito; *NIID-CTR* cell line from *C. tritaeniorhynchus* mosquito; *S2* dipteran *D. melanogaster* cell line; *Sf21* lepidopteran *S. frugiperda* cell line; *GaMe-LF1* lepidopteran *G. mellonella* cell line; and *PC-1* coleopteran *P. christophi* cell line. The band patterns were confirmed to correspond to Ar-3, Ar-13, and *A. subalbatus* with some differences in band intensity. Both Ar lines showed band patterns that were distinct from those of the seven other insect cell lines.

flaviviruses CXFV and AEFV were only faintly detected at 3 d p.i. and then increased in staining intensity as the days p.i. progressed. Negative results for all viruses during the early phase (0–2 d p.i.) indicate that the remnant of the inoculum was low enough to test the susceptibility of cells to each virus. We occasionally observed cytopathic effect (CPE) in the JEV-inoculated cells 7 d p.i., whereas no CPEs in the CXFV-, and

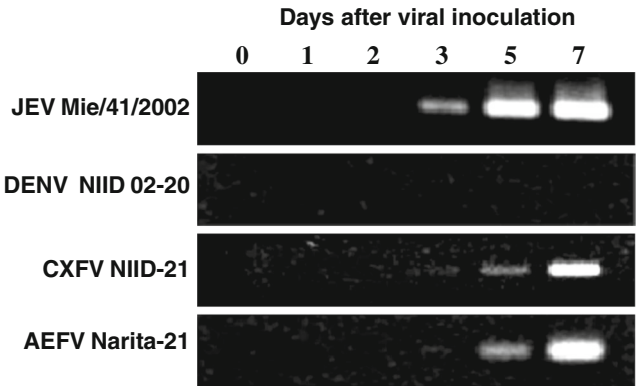

Figure 6 RT-PCR detection of viral RNAs from the cultured medium of Ar-3 cells at 0, 1, 2, 3, 5, and 7 d after viral inoculation. The amplified products were separated on 2% agarose gels and visualized with ethidium bromide, and the portion of the expected amplicon size of each virus is shown. *JEV* Japanese encephalitis virus, *DENV* dengue virus, *CXFV Culex* flavivirus, *AEFV Aedes* flavivirus. The strain name appears after each abbreviated virus name.

AEFV-inoculated cells, even after 7 d p.i. (data not shown). The titers of JEV and DENV in Ar-3 cells were determined by a standard plaque assay. JEV was detected from 3 d p.i. (8.67×10^3 PFU/mL) and then reached the high titer of 3.80×10^8 PFU/mL 7 d p.i., but DENV did not increase, which is in accordance with the RT-PCR results.

Discussion

In this study, we initiated primary cultures and established two cell lines from the mosquito *A. subalbatus*. In the primary culture, cell migration occurred in three different media, among which VP12 is unequivocally one of the best for culturing a wide range of mosquito species (Varma and Pudney 1969; Kuwata *et al.* 2012), including *Armigeres*, as confirmed by this study. The MM medium also promoted cell migration, as reported for many aedine mosquito cell lines (Hink 1976); when mixed with the MGM450 medium, this combination efficiently established the *Armigeres* mosquito cell line. Although these two media differ considerably in component richness and Na/K ratio (Mitsuhashi 1989), it is possible that mixing the media may offer some favorable culture conditions as observed previously (Kuno 1980; Bello *et al.* 2001). When grown in Schneider's medium, the primary cells themselves possibly changed salt concentrations to form crystals, as the frequently formed vesicles are known to function as ion-exchange cell layers, similar to renal cells (Lynn *et al.* 1998). Thus, by employing combinations of different media, the optimal culture conditions were obtained, which likely facilitated continuous growth by inhibiting the vesicle formation observed in MM medium alone.

Despite being cultured in different media, the two established cell lines (Ar-3 and Ar-13) were composed of similar types of cells that were adherent and karyotypically normal. Both cell lines formed distinct, compact colonies reminiscent of those formed in mammalian embryonic stem cell lines (Englund *et al.* 2010; Aran *et al.* 2010). Similar colonies have been observed in other mosquito cell lines, including AeAl-2 (Mitsuhashi 1981) and ATC-10 (Singh 1967). Therefore, this characteristic may arise in cell lines of mosquito neonate larval tissue origin. RAPD-PCR results confirmed that both Ar lines originated from *A. subalbatus*; thus, these cell lines are the first available in vitro cell models for studying the biology of this species. Comparative analysis of RAPD-PCR results demonstrates slight differences between Ar-3, Ar-13, and *A. subalbatus*, suggesting that genetic alterations may have been generated. Cryopreservation at −80°C in 12% dimethyl sulfoxide for at least 1 yr was confirmed in both cell lines at around the 100th passages (data not shown), allowing storage without accumulation of the genetic and karyotypic changes expected a priori in cultured cells.

The Ar-3 cell line is susceptible to JEV but relatively insensitive to DENV, as demonstrated by the viral isolation results from wild-caught *A. subalbatus* mosquitoes (e.g., Weng *et al.* 1999). Additionally, the viral susceptibilities of this cell line did not tend to be accompanied by CPEs as in many mosquito cell lines (e.g., Peleg 1968; Sudeep *et al.* 2009). Ar-3 is also susceptible to insect flaviviruses with no signs of CPE, suggesting the possibility of persistent infection by insect flaviviruses. Insect viruses known to be harbored in mosquito cell lines include the first reported insect flavivirus, a cell fusing agent isolated from Peleg's cell line from *Aedes aegypti* (Stollar and Thomas 1975), other insect flaviviruses, and other insect viruses (Scott *et al.* 2010). Accordingly, the Ar cell lines should be examined for persistent viral infections that could potentially be related to derivation of a cell line that generates CPE-like vacuolation in early subcultures. In general, viral susceptibility in vitro is restricted by the host range of the virus (Kuno 2007); therefore, Ar-3 will be extremely valuable for the detection of *Armigeres*-associated viruses such as *Armigeres* totivirus (Zhai *et al.* 2010). The Ar-3 line is expected to improve efficient isolation of viruses from *A. subalbatus* mosquitoes, as C6/36 cells exhibit serious cellular damage, including cell death, in almost all cases of inoculation with *A. subalbatus* mosquito homogenates. Thus, the two new cell lines established from *A. subalbatus* in this study are cellular models for this species and may serve as beneficial tools for the propagation of viruses associated with the *Armigeres* mosquito.

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References

Aliota MT, Fuchs JF, Rocheleau TA, Clark AK, Hillyer JF, Chen CC, Christensen BM (2010) Mosquito transcriptome profiles and filarial worm susceptibility in *Armigeres subalbatus*. PLoS Negl Trop Dis 4(4):e666

Aran B, Rodríguez-Pizà I, Raya A, Consiglio A, Muñoz Y, Barri PN, Izpisúa JC, Veiga A (2010) Derivation of human embryonic stem cells at the Center of Regenerative Medicine in Barcelona. In Vitro Cell Dev Biol Anim 46(3–4):356–66

Athawale SS, Sudeep AB, Barde PV, Jadi R, Mishra AC, Mourya D (2002) A new cell line from the embryonic tissues of *Culex tritaeniorhynchus* and its susceptibility to certain flaviviruses. Acta Virol 46:237–240

Bello FJ, Rodríguez JA, Escovar J, Olano VA, Morales A, González M, Rey G (2001) A new continuous cell line from the mosquito *Psorophora confinnis* (Diptera: Culicidae) and its susceptibility to infections with some arboviruses. Mem Inst Oswaldo Cruz 96:865–873

Callahan JD, Wu S-L, Dion-Schultz A, Mangold BE, Peruski LF, Watts DM, Porter KR, Murphy GR, Suharyono W, King C-C, Hayes CG, Temenak JJ (2001) Development and evaluation of serotype- and group-specific fluorogenic reverse transcriptase PCR (TaqMan) assays for dengue virus. J Clin Microbiol 39:4119–4124

Chen WG, Dong CF, Chiou LY, Chuang WL (2000) Potential role of *Armigeres subalbatus* (Diptera: Culicidae) in the transmission of Japanese encephalitis virus in the absence of rice culture on Liu-Chiu islet. Taiwan J Med Entomol 37(1):108–113

Clements AN (1992) The biology of mosquitoes. Volume 1: development, nutrition and reproduction. CABI Publishing, Wallingford, pp 1–532

Eguchi D, Iwabuchi K (2006) A new cell line from the wax moth *Galleria mellonella* Linne (Lepidoptera: Pyralididae). In Vitro Cell Dev Biol Anim 42(1–2):1–3

Englund MC, Caisander G, Noaksson K, Emanuelsson K, Lundin K, Bergh C, Hansson C, Semb H, Strehl R, Hyllner J (2010) The establishment of 20 different human embryonic stem cell lines and subclones; a report on derivation, culture, characterisation and banking. In Vitro Cell Dev Biol Anim 46(3–4):217–30

Feng Y, Fu S, Zhang H, Li M, Zhou T, Wang J, Zhang Y, Wang H, Tang Q, Liang G (2012) Distribution of mosquitoes and mosquito-borne viruses along the China–Myanmar border in Yunnan Province. Jpn J Infect Dis 65(3):215–221

Grace TDC (1962) Establishment of four strains of cells from insect tissues grown in vitro. Nature 195:788–789

Grace TDC (1966) Establishment of a line of mosquito (*Aedes aegypti* L.) cells grown in vitro. Nature 211:366–367

Hink WFA (1976) Compliation of invertebrate cell lines and culture media. In: Maramorosch K (ed) Invertebrate tissue culture. Academic Press, New York, pp 319–369

Hoshino K, Isawa H, Tsuda Y, Yano K, Sasaki T, Yuda M, Takasaki T, Kobayashi M, Sawabe K (2007) Genetic characterization of a new insect flavivirus isolated from *Culex pipiens* mosquito in Japan. Virology 359:405–414

Hoshino K, Hirose M, Iwabuchi K (2009a) A new insect cell line from the longicorn beetle *Plagionotus christophi* (Coleoptera: Cerambycidae). In Vitro Cell Dev Biol Anim 45:19–22

Hoshino K, Isawa H, Tsuda Y, Sawabe K, Kobayashi M (2009b) Isolation and characterization of a new insect flavivirus from *Aedes albopictus* and *Aedes flavopictus* mosquitoes in Japan. Virology 391:119–129

Igarashi A (1978) Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. J Gen Virol 40:531–544

Isawa H, Kuwata R, Tajima S, Hoshino K, Sasaki T, Takasaki T, Kobayashi M, Sawabe K (2012) Construction of an infectious cDNA clone of Culex flavivirus, an insect-specific flavivirus from *Culex* mosquitoes. Arch Virol 157:975–979

Kitamura S (1970) Establishment of cell line from *Culex* mosquito. Kobe J Med Sci 16(1):41–50

Kuno G (1980) A continuous cell line of a nonhematophagous mosquito. Toxorhynchites Amboinensis In Vitro 16(11):915–917

Kuno G (2007) Host range specificity of flaviviruses: correlation with in vitro replication. J Med Entomol 44(1):93–101

Kuwata R, Hoshino K, Isawa H, Tsuda Y, Tajima S, Sasaki T, Takasaki T, Kobayashi M, Sawabe K (2012) Establishment and characterization of a cell line from the mosquito *Culex tritaeniorhynchus* (Diptera: Culicidae). In Vitro Cell Dev Biol Anim 48:369–376

Liu H, Lu HJ, Liu ZJ, Jing J, Ren JQ, Liu YY, Lu F, Jin NY (2013) Japanese encephalitis virus in mosquitoes and swine in Yunnan province, China 2009–2010. Vector Borne Zoonotic Dis 13(1):41–49

Lynn DE, Oberlander H, Porchebon P (1998) Tissues and cells in culture. In: Harrison FW, Locke M (eds) Microscopic anatomy of invertebrates, vol 11C. Wiley-Liss, New York, pp 1119–1141

Marquardt WC (2004) Biology of disease vectors. Academic Press, Burlington, MA, pp 1–785

Mitsuhashi J (2002) Invertebrate tissue culture methods. Springer, Japan, pp 1–446

Mitsuhashi J (1981) A new continuous cell line from larvae of the mosquito *Aedes albopictus* (Diptera, Culicidae). Biomed Res 2(6):599–606

Mitsuhashi J (1989) Nutritional requirements of insect cells in vitro. In: Mitsuhashi J (ed) Invertebrate cell system applications, vol 1. CRC Press, Boca Raton, pp 3–20

Mitsuhashi J (1994) Mosquito cell lines. In: Maramorosch K, McIntosh AH (eds) Arthropod cell culture systems. CRC Press, Boca Raton, pp 19–36

Mitsuhashi J, Maramorosch K (1964) Leafhopper tissue culture: embryonic, nymphal, and imaginal tissues from asptic insects. Contrib Boyce Thompson Inst 22(8):435–460

Mitsuhashi J, Inoue H (1988) Obtainment of a continuous cell lines from larval fat bodies of the mulberry tiger moth, *Spilosoma imparilis* (Lepidoptera: Arctiidae). Appl Entomol Zool 23:488–490

Muslim A, Fong MY, Mahmud R, Lau YL, Sivanandam S (2013) *Armigeres subalbatus* incriminated as a vector of zoonotic Brugia pahangi filariasis in suburban Kuala Lumpur, Peninsular Malaysia. Parasit Vectors 6:219

Nerome R, Tajima S, Takasaki T, Yoshida T, Kotaki A, Lim CK, Ito M, Sugiyama A, Yamauchi A, Yano T, Kameyama T, Morishita I, Kuwayama M, Ogawa T, Sahara K, Ikegaya A, Kanda M, Hosoya Y, Itokazu K, Onishi H, Chiya S, Yoshida Y, Tabei Y, Katsuki K, Tabata K, Harada S, Kurane I (2007) Molecular epidemiological analyses of Japanese encephalitis virus isolates from swine in Japan from 2002 to 2004. J Gen Virol 88(10):2762–2768

Peleg J (1968) Growth of arboviruses in monolayers from subcultured mosquito embryo cells. Virology 35(4):617–619

Pudney M, Leake CJ, Buckley SM (1982) Replication of arboviruses in arthropod in vitro systems: an overview. In: Maramorosch K, Mitsuhashi J (eds) Invertebrate cell culture applications. Academic Press, New York, pp 159–194

Reinert JF, Harbach RE, Kitching IJ (2009) Phylogeny and classification of tribe Aedini (Diptera: Culicidae). Zool J Linnean Soc 157:700–794

Sasaki T, Hiraoka T, Kobayashi M (2010) Hemolytic activity is mediated by the endogenous lectin in the mosquito hemolymph serum. J Insect Physiol 56(9):1032–9

Schneider I (1969) Establishment of three diploid cell line of *Anopheles stephensi* (Diptera: Culicidae). J Cell Biol 42:603–606

Schneider I (1972) Cell lines derived from late embryonic stages of *Drosophila* melanogaster. J Embryol Exp Morphol 27:363–365

Scott JC, Brackney DE, Campbell CL, Bondu-Hawkins V, Hjelle B, Ebel GD, Olson KE, Blair CD (2010) Comparison of dengue virus type 2-specific small RNAs from RNA interference -competent and -incompetent mosquito cells. PLoS Negl Trop Dis 4:e848

Segura NA, Santamaría E, Cabrera OL, Bello F (2012) Establishment and characterisation of a new cell line derived from *Culex quinquefasciatus* (Diptera: Culicidae). Mem Inst Oswaldo Cruz 107:89–95

Simcox A, Mitra S, Truesdell S, Paul L, Chen T, Butchar JP, Justiniano S (2008) Efficient genetic method for establishing *Drosophila* cell lines unlocks the potential to create lines of specific genotypes. PLoS Genet 4(8):e1000142

Singh KRP (1967) Cell cultures derived from larvae of *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.). Curr Sci 36(19):506–508

Stollar V, Thomas VL (1975) An agent in the *Aedes aegypti* cell line (Peleg) which causes fusion of *Aedes albopictus* cells. Virology 64(2):367–377

Sudeep AB, Parashar D, Jadi RS, Basu A, Mokashi C, Arankalle VA, Mishra AC (2009) Establishment and characterization of a new *Aedes aegypti* (L.) (Diptera: Culicidae) cell line with special emphasis on virus susceptibility. In Vitro Cell Dev Biol Anim 45(9):491–495

Tajima S, Nukui Y, Ito M, Takasaki T, Kurane I (2006) Nineteen nucleotides in the variable region of 3′ non-translated region are dispensable for the replication of dengue type 1 virus in vitro. Virus Res 116:38–44

Tajima S, Nerome R, Nukui Y, Kato F, Takasaki T, Kurane I (2010) A single mutation in the Japanese encephalitis virus E protein (S123R) increases its growth rate in mouse neuroblastoma cells and its pathogenicity in mice. Virology 396:298–304

Tanaka K (2005) Family Culicidae. In: Kawai T, Tanida K (eds) Aquatic insects of Japan: mannual with keys and illustrations. Tokai University Press, Hadano, pp 757–1005

Tanaka K, Mizusawa K, Saugstad ES (1979) A revision of the adult and larval mosquitoes of Japan (including the Ryukyu archipelago and the Ogasawara islands) and Korea (Diptera: Culicidae). Contrib Am Entomol Inst 16:1–987

Varma MGR, Pudney M (1969) The growth and serial passage of cell lines from *Aedes aegypti* (L.) larvae in different media. J Med Entom 6(4):432–439

Vaughn JL, Goodwin RH, Tompkins GJ, McCawley P (1977) The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera; Noctuidae). In Vitro 13(4):213–217

Weng MH, Lien JC, Wang YM, Lin CC, Lin HC, Chin C (1999) Isolation of Japanese encephalitis virus from mosquitoes collected in Northern Taiwan between 1995 and 1996. J Microbiol Immunol Infect 32(1):9–13

Zhai Y, Attoui H, Mohd Jaafar F, Wang HQ, Cao YX, Fan SP, Sun YX, Liu LD, Mertens PP, Meng WS, Wang WS, Liang G (2010) Isolation and full-length sequence analysis of *Armigeres subalbatus* totivirus, the first totivirus isolate from mosquitoes representing a proposed novel genus (*Artirirus*) of the family Totiviridae. J Gen Virol 91:2836–2845