Heliyon 8 (2022) e10674

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Establishing and characterising a new cell line from *Calliphora vicina* (diptera: calliphoridae) fly embryonic tissues

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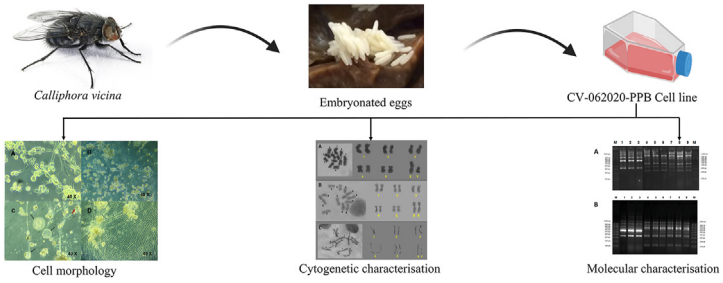
HIGHLIGHTS

- A new *C. vicina* embryonic tissue-derived cell line is here reported.
- Fibroblast-like cells were predominant.
- The cell-line karyotype was 2n = 12 diploid chromosomes.
- The DNA profile enabled discriminating its molecular identity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Calliphora vicina
CV-062020-PPB
Cell morphology
Karyotype
RAPD-PCR

ABSTRACT

Insect cell lines represent a promising and expanding field as they have several research applications including biotechnology, virology, immunity, toxicology, cell signalling mechanisms and evolution. They constitute a powerful tool having a direct impact on human and veterinary medicine and agriculture. Although more than 1000 cell lines have currently been established from various insect species, *Calliphora vicina*-derived fly cell lines are lacking. This study was aimed at establishing a new *C. vicina* embryonic tissue-derived cell line. Adult flies were collected and embryonated eggs were mechanically homogenised and seeded in four types of culture media (L15, Grace's insect medium, Grace's/L15 and DMEM). Cell growth and morphological characteristics were recorded and cytogenetic and molecular patterns were determined. The CV-062020-PPB cell line was established and was shown to have optimal growth in Grace's/L15 medium. CV-062020-PPB cell monolayers that had been sub-cultured over 16 times consisted of firmly adhering cells having different morphologies; a fibroblast-like shape dominated and the karyotype had a 12-chromosome diploid number. RAPD-PCR analysis of the CV-062020-PPB cell line revealed a high similarity index and strong intraspecific relationship with *C. vicina* adult flies and a weaker relationship with the *Lutzomyia longipalpis*-derived cell line (Lulo). The CV-062020-PPB cell line constitutes the first cell line obtained from *C. vicina* embryonic tissues and represents an important basic and applied research tool.

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https://doi.org/10.1016/j.heliyon.2022.e10674
Received 13 January 2022; Received in revised form 25 April 2022; Accepted 9 September 2022

1. Introduction

Calliphora vicina Robineau-Desvoidy, 1830 is a necrophagous and hemisynanthropic fly (Figueroa-Roa and Linhares 2002; Pinilla et al., 2013) that belongs to the Calliphoridae family; *C. vicina* has ecological, animal health and forensic importance (Martinez et al., 2007). This blowfly is geographically distributed in the Holarctic and Neotropical regions (Kosmann et al., 2013). *C. vicina* is found in Colombia's Casanare, Tolima, Santander, Caldas, Valle del Cauca, Meta and Cundinamarca regions. *C. vicina* is particularly adapted to the Savanna of Bogotá (2,500 m above sea level) (Amat et al., 2008; Florez and Wolff 2009).

Insect cell lines have contributed significantly to the development of physiological studies of the insect species from which they are derived (Goodman et al., 2021). These cell cultures also represent important biotechnological tools in the fields of immunology, molecular biology, genetics and biopesticide research (Arunkarthick et al., 2017); they have even been used in studies regarding parasite-host relationships, the spread of specific pathogens and in the biopharmaceutical industry, particularly concerning recombinant protein expression (Smagghe et al., 2009).

Newly established cell lines require their identities to be validated which includes characterising their predominant cell types through direct observations. However, cell morphology alone is not enough to characterise cell cultures due to changes in cell shapes that often occur during growth, particularly in *vitro* culture conditions where physical, nutritional and environmental factors influence such changes. New cell lines consist of cell populations with a variety of morphologies, even in a cloned cell line (Kawai and Mitsuhashi 1997). Reliable characterisation may thus be confirmed by karyological and molecular tests to ensure authenticity. Correct identification is extremely necessary to avoid cross-contamination with other cell lines or mislabelling (Markovic and Markovic 1998).

C. vicina fatty body and haemocyte primary cultures have been used recently (A. Yakovlev et al., 2017), however, the scientific literature contains no record of cell lines derived from this species' embryonic tissue, which seems to be a completely unexplored field. Developing *C. vicina* cell lines will facilitate virology-, parasitology-, biochemistry-, immunology- and endocrinology-related cellular and molecular studies in human and veterinary biomedicine (Smagghe et al., 2009).

Producing a new cell line from *C. vicina* will thus support basic and applied research including the characterisation of novel antimicrobial substances (antimicrobial peptides), and other *C. vicina* molecules involved in tissue regeneration and/or recovery. This will enable a better understanding of the mechanisms underlying the use of larval therapy in patients with chronic wounds, as reported for other species from the Calliphoridae family (Limsopatham et al., 2017; Peck and Kirkup 2012). This study's main objective was thus to establish and characterise, for the first time, a new *C. vicina* embryonic tissue-derived cell line.

2. Materials and methods

2.1. Establishing C. vicina colonies

Adult *C. vicina* fly specimens were collected from the Enrique Olaya Herrera National Park in Bogota (4º37′16″N, 74º03′35″W). Adult flies were attracted by using raw beef liver as bait and captured during morning hours between 9:00 am and 1:00 pm. Taxonomic identification followed the keys and guidelines reported by Amat et al. (2008). Individuals identified as *C. vicina* were kept in 45 × 45 × 45 cm Gerberg insect rearing cages at 21 °C–25 °C, 45%–50% relative humidity and exposed to a natural daily 12 h light and 12 h darkness photoperiodicity (Pérez et al., 2016). The study was approved by the Ethics Committee of the University of La Salle (Bogotá, Colombia), project license number COBIULS-0050-2019, and had National Environmental Licensing Authority (ANLA) permission to collect fly specimens (resolution 1473, December 3rd, 2014).

2.2. Primary culture initiation

Embryonated eggs of the above-mentioned *C. vicina* adult fly colony were collected from raw beef liver. The eggs were surface sterilised using 0.5% sodium hypochlorite for 10 min followed by 70% ethanol for 10 min and washed 3 three times in sterile distilled water containing a penicillin (100 units/mL)/streptomycin (100 mg/mL) mixture for 5 min each. Disinfected eggs incubated in 2 mL of each culture medium were transferred to a Ten Broeck homogenizer (Pyrex-Corning, Arizona, USA) where they were macerated to disintegrate the tissues and release individual embryonic cells (Cruz and Bello 2012). The released cell suspensions were seeded in a 25 cm^2 plastic tissue culture flask containing 8 mL of each tested culture medium. The cultures were incubated at 27 °C without CO_2; cell adaptation and proliferation progress was monitored daily using an inverted microscope (Zeiss, Oberkochen, Southeastern Germany). The cultures were fed weekly by replacing half of the spent medium with fresh medium until confluence, taking primary culture growth and proliferation level into account.

2.3. Culture media

Four different culture media were tested for their suitability for *C. vicina* embryonic cell primary culture and subculture (Leibovitz's L-15 medium, Gibco, Paisley, Scotland, UK), Grace's insect cell medium (Gibco, Paisley, Scotland, UK), Grace's/L-15 (1:1) mixture and Dulbecco's Modified Eagle Medium, DMEM (Sigma-Aldrich, Burlington, MA, USA). Each culture media was supplemented with 20% heat-inactivated foetal bovine serum (FBS) (Sigma-Aldrich, Burlington, MA, USA) (Nims and Harbell 2017) and a mixture of penicillin (100 units/mL), streptomycin (100 mg/mL) (ThermoFisher Scientific, Waltham, USA) and antimycotics (2.5 μg/mL amphotericin B). The media's pH was adjusted to between 6.7 and 6.9.

2.4. Cell morphology and growth curve

The cell cultures were examined daily using a Zeiss Inverted Microscope equipped with phase contrast optics and integrated modulation contrast kit to check the cell cultures' general condition and confluence was recorded using 10x, 20x and 40x object lenses. The growth patterns of the most predominant cell culture morphologies were also recorded. Photographs were taken during different cell growth stages.

A growth curve was produced at passage 15 by seeding around 2 × 10^5 cells/mL in a T25 flask (Falcon, Waltham, Massachusetts, USA) containing Grace's/L15 medium. The amount of cells was counted every 24 h using a haemocytometer. The growth curve was drawn according to average cell density. The amount of generations per time unit (doubling time) was calculated according to the formula reported by Hayflick and Moorhead (1961).

2.5. Karyotype analysis

Two flasks of *C. vicina* cell culture having around 80% confluence were used for each experiment; 0.6 μg/mL colchicine (Sigma-Aldrich, Burlington, MA, USA) was added for 30 min at 27 °C to stop cell replication and determine the karyotype. The cells were obtained from the cultures by using two methods: mechanical detachment using a 1.8 cm blade x 25 cm handle scraper (Sigma-Aldrich, Burlington, MA, USA) for adherent culture and chemical detachment by adding 0.25% trypsin/EDTA solution at 27 °C for 5 min.

The detached cell suspensions were transferred to 15 mL Falcon tubes and centrifuged at 1,000 g for 10 min. The cell pellet was suspended in 3 mL hypotonic (0.075M NaCl) sodium chloride solution and incubated at 27 °C for 30 min. The tubes were centrifuged again using the same conditions and the supernatant discarded.

The cell pellet was then fixed with 2 mL Carnoy's solution (3:1 methanol:glacial acetic acid) for 30 min, repeated twice. The fixed cells

were then suspended in 1 mL Carnoy's solution and around 0.5–1.0 mL of this cell suspension were dropped onto clean glass slides using a Pasteur pipet. The slides were dried at room temperature and stained with 10% Giemsa for 30 min. *C. vicina* cell separated chromosomes were analysed by light microscope (Zeiss, 100× object lens); photographs of the best 20 metaphases were taken. Image Pro Plus 5.0 software was used for chromosome measurements according to guidelines reported by others (Levan et al., 1964; Zapata et al., 2005), i.e. discriminating each pair of chromosomes, total chromosome length (TL), relative length (RL), centromere index (CI), short (p) and long (q) arms, the arms ratio (q/p and p/q) and the average absolute value of length (AAVL).

2.6. Molecular characterisation

2.6.1. Genomic DNA extraction

A GeneJET genomic DNA purification kit (ThermoFisher Scientific, Waltham, USA) was used for extracting genomic DNA (gDNA) from adult *C. vicina* fly, CV-062020-PPB cell line and the control Lulo cell line, established from *Lutzomyia longipalpis* (Diptera: Psychodidae) embryonic tissue (Rey et al., 2000) which enables rapid, efficient and high-quality gDNA purification (according to the company's protocol). Purified gDNA from each type of sample was incubated at 37 °C for 10 min, quantified by NanoDrop ™ 2000 spectrophotometer (ThermoFisher Scientific, Waltham, USA) and then kept at 4 °C until use.

2.6.2. RAPD-PCR

Invitrogen PCR SuperMix (ThermoFisher Scientific, Waltham, USA) was used for PCR amplification of random amplified polymorphic DNA fragments from *C. vicina* flies' gDNA CV-062020-PPB confluent monolayers and the control Lulo cell line (20 μL per reaction tube volume). The PCR reaction consisted of 2.5 μL 1x buffer, 2.0 μL dNTPs (0.25 mM), 1.6 μL $MgCl_2$ (1 mM), 0.7 μL primer (4 μM), 0.125 μL Taq DNA polymerase (2 U/μL), 9.86 PCR-grade water and 3 μL DNA template from adult *C. vicina* fly primary culture (37.3 ng/μL) (14 ng/μL) or the Lulo control cell line (22 ng/μL). The *Invitrogen synthesised* random primers' nucleotide sequences were as follows: A2=(5′-TGCCGAGCTG-3′), A10=(5′-ACGGCGTATG-3′) and A20=(5′- GTTGCGATCC- 3′). The PCR programme involved a denaturation step at 95 °C for 5 min, 45 cycles of DNA amplification that consisted of denaturation at 95°C/1 min, annealing at 36°C/2 min, extension at 72°C/2 min and a final extension step at 72°C/5 min.

2.6.3. Agarose gel electrophoresis

The amplified PCR products from *C. vicina* cell culture, adult *C. vicina* flies and Lulo control cell gDNA were separated on 1.5% agarose gel electrophoresis on Tris-Glycine gels or TAE buffer at 150V for 90 min. Two μL of PCR products from each reaction tube were mixed with 4 μL Orange DNA Loading Dye and seeded in each well. The gel was stained with 3 μL HydraGreen Safe DNA Dye (ACTGene, Inc), visualised and photographed under UV light.

Amplified polymorphic DNA band patterns were scored as being present or absent and compared using the Nei and Li's similarity coefficient, according to the following formula: SAB=(2 NAB)/((NA + NB)), where NAB refers to the number of shared bands and NA represents the total number of bands shown by individual A and NB by B (Léry et al., 2003).

2.7. Mycoplasma test

A PCR Mycoplasma Test Kit (PanReac AppliChem, Barcelona, Spain) was used for detecting *Mycoplasma* in the cell culture obtained from *C. vicina* embryo tissue, following the manufacturer's instructions.

2.8. Cryopreservation

CV-062020-PPB cell line semiconfluent (80% confluence) monolayers were used to make cryopreserved cell stocks. The cells were mechanically detached with a rubber scraper, adjusted to 5×10^6/mL with fresh medium (50%) containing 40% foetal bovine serum (Gibco, Paisley, Scotland, UK) and 10% DMSO (ThermoFisher Scientific, Waltham, USA).

Nunc 1.8 mL CryoTube cryogenic vials (Sigma-Aldrich, Burlington, MA, United States) (previously labelled with the culture name, passage number and freezing date) were loaded with 1.5 mL cell suspension. Cooling and freezing were carried out slowly as follows: cooling at 4°C/15 min, freezing at -20°C/1 h and freezing at -70°C/12 h. The cryogenic vials were then transferred to a tank containing liquid nitrogen (−196 °C) and stored indefinitely (Zapata et al., 2005).

2.9. Statistical analysis

Morphometric data obtained from karyotype analysis was listed in an Excel table (long arm and short arm dimensions and total *C. vicina* chromosome length); STATA12 software was used for descriptive analysis. Descriptive parameters included sample size (n), mean and standard deviation (SD), with 95% confidence interval. The Pearson chi-squared test was used for comparing culture medium effect on cell growth.

3. Results

3.1. Evaluating the culture media

C. vicina embryonic cells grew satisfactorily in Grace's/L15 and L-15 media; cell replication began in a relatively short period of 3 days for Grace's/L15 and 4 days for L-15 media. A confluent monolayer began to form by days 15–19 after the explants were seeded in each medium. By contrast, *C. vicina* embryonic cells did not grow in DMEM and Grace's media. The statistical test revealed significant differences ($p < 0.05$) between Grace's insect medium/L15 and L-15; several subcultures corroborated that Grace's/L15 media provided suitable and necessary conditions for cell adaptation and replication. Although slight bacterial contamination was seen in a few *C. vicina* primary cell culture flasks containing Grace's/L15 medium, this was followed by self-control of bacterial contamination, thereby favouring cell adhesion, proliferation and growth (Table 1).

3.2. Cell growth curve

The CV-062020-PPB embryonic cell line growth curve determined at passage 15 in Grace's/L15 medium had a stationary phase on day 1, then a logarithmic exponential growth phase from day 3 to day 7; cell growth reached a plateau phase on day 8 (Figure 1). CV-062020-PPB embryonic cell population doubling time was around 37.7 h.

3.3. Initiating primary cell culture and subcultures

C. vicina embryonic tissue cell replication in L15 medium was demonstrated on day 4; individual cell colonies initially adhered to the flasks' surface (Figure 2). As the cells continued to grow and proliferate they occupied larger areas of the flask surface until a confluent monolayer was formed. Two subcultures were made in this medium; nonetheless, the cells gradually lost viability between passages until the new subculture become non-viable.

The main source of cell growth in Grace's/L15 medium was observed around embryo fragments and also from groups of individual cells that had adhered to the surface of the flasks (Fig. 3A-B). The embryonic cell colonies progressed after some days and had favourable growth in Grace/L15 medium. Groups of vesicles were another source of cell attachment and proliferation (Figure 3C); they were initially observed floating on the medium's surface and they represented a source of cell release contributing to cell adherence and proliferation after being dispersed by means of vigorous pipetting.

The contractile movements noted in the primary cell culture's proliferation areas were a consistent finding supporting *C. vicina* cells' vigorous

Table 1. Evaluating different culture media to support in vitro *C. vicina* embryonic cell adaptation and growth.

Culture media	Source	Number of tissue explants	Number of viable cell cultures	Start of cell growth (days)	Number of subcultures	Monolayer formation	Features of cell culture progression
L15	Eggs	35	1*	3–8	2	Yes 19 days	Cells became detached from flask surface and died after two successful subcultures
GRACE	Eggs	20	0	-	-	No	Did not develop
GRACE/ L15	Eggs	87	52*	1–3	16	Yes 15 days	Optimum cell growth and proliferation through continuous successful subcultures
DMEM	Eggs	20	0	-	-	No	Did not develop
GRACE/ L15	Larvae	20	0	-	-	No	Did not develop

* $p = 0.000$.

growth in this medium. Confluent monolayers were obtained after 15 days of tissue explant seeding (Figure 3D). Sixteen CV-062020-PPB embryonic cells' serial subcultures have been obtained; these had slow growth at the beginning, having characteristics similar to primary cultures with low cell proliferation rates; nevertheless, cell division increased significantly from the fifth passage on, occurring at 1:5 split ratio once per week. There was no evidence of cell contamination with mycoplasma.

The ability of cells to be cryopreserved was tested by storing them in liquid nitrogen for four months, thawing them, and then determining their viability two days after. Likewise, cell recovery occurred within ten days after thawing. Percentage viability after thawing cells in high passages was 75% on average.

3.4. Cell morphology

Cell cultures in initial growth stages had consistent heterogeneous morphology, represented by spherical, elongated and irregular cell shapes (as well as giant cell shapes in a few cases). It is worth noting that cell monolayers that had reached confluence were characterised by cell types having predominant fusiform morphology, as were subsequent subcultures, and also by cells having highly ramified cell shapes resembling fibroblast and neuron-like cells having long dendrites (Figure 4A). However, a significant amount of *C. vicina* cells having shapes resembling epithelial cells were also recorded from the fourth subculture onwards (Figure 4B).

3.5. Cytogenetic characterisation

Multiple extended cell cultures were performed to obtain the *C. vicina* embryonic cell karyotype; some metaphases (Fig. 5A-B) and prometaphases (Figure 5C) were displayed. *C. vicina* embryonic cells had 5 pairs of autosomal chromosomes and a pair of sex chromosomes, resembling the same diploid number ($2n = 12$) observed during metaphase. The position of the centromere in each pairs of chromosomes was taken as reference for designating the chromosomes as being metacentric, sub-metacentric, sub-telocentric or telocentric (Table 2) according to the guidelines reported by Levan et al. (1964). The karyotype remained stable with the species' diploid chromosome number, following the different subcultures.

3.6. Molecular characterisation

The presence of genetic material was qualitatively verified by electrophoresis after DNA extraction; the DNA was quantified, having an average 37.3 ng/μL *C. vicina* cell culture and 14 ng/μL for adult flies.

RAPD-PCR results showed a higher number of amplified DNA fragments using the A20 primer compared to A2 and A10 primers, although all primers produced DNA fragments ranging from 100 to 1,500 bp (Figure 6). The similarity coefficient was higher (0.96) between the RAPD-PCR profile obtained from the CV-062020-PPB embryonic cell line and *C. vicina* adult fly gDNA when the A10 primer was used, whereas a

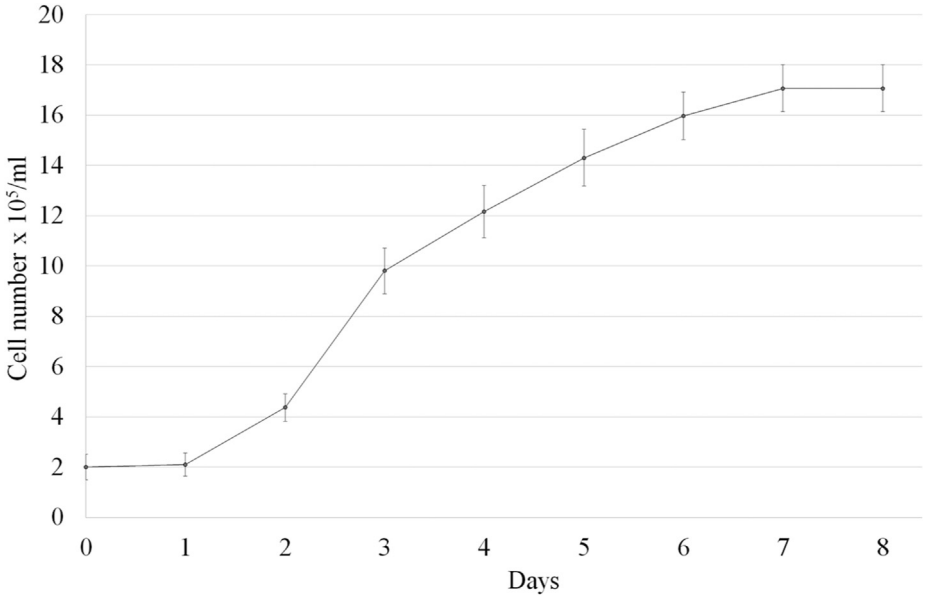

Figure 1. *Calliphora vicina* CV-062020-PPB cell line growth curve at passage 15. Each point is the mean of three replicate cultures. Bars represent one standard deviation.

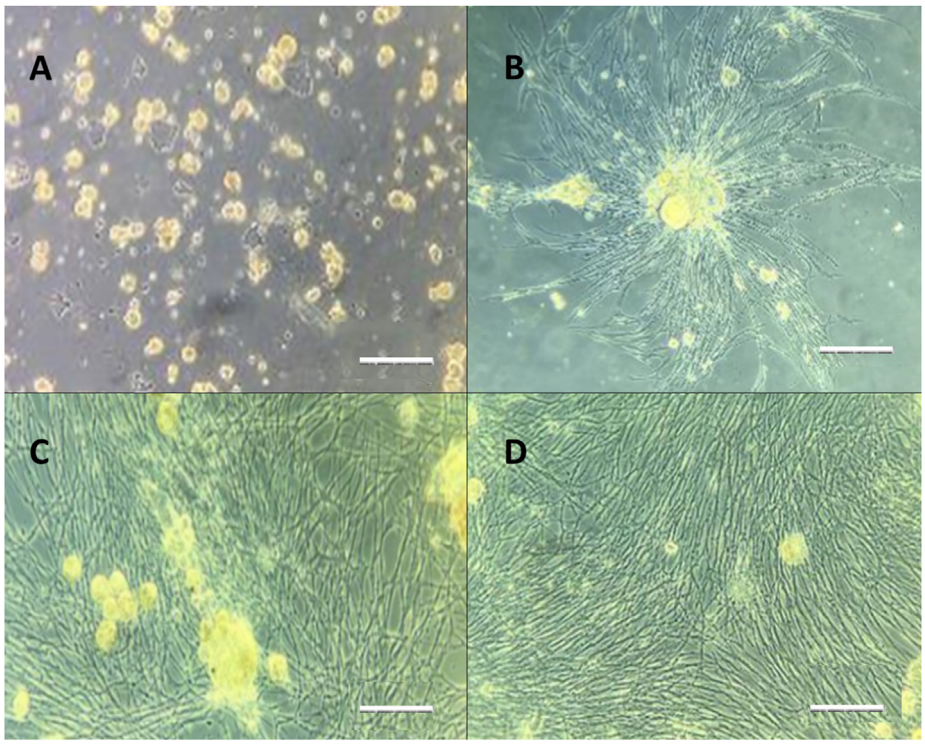

Figure 2. *Calliphora vicina* embryonic tissue cell growth in L15 medium. A. Collected and seeded cells on day 0. B. Initiation of cell growth on day 3. C. Cell growth on day 15. D. Cell growth on day 19. Scale bar = 200 μM.

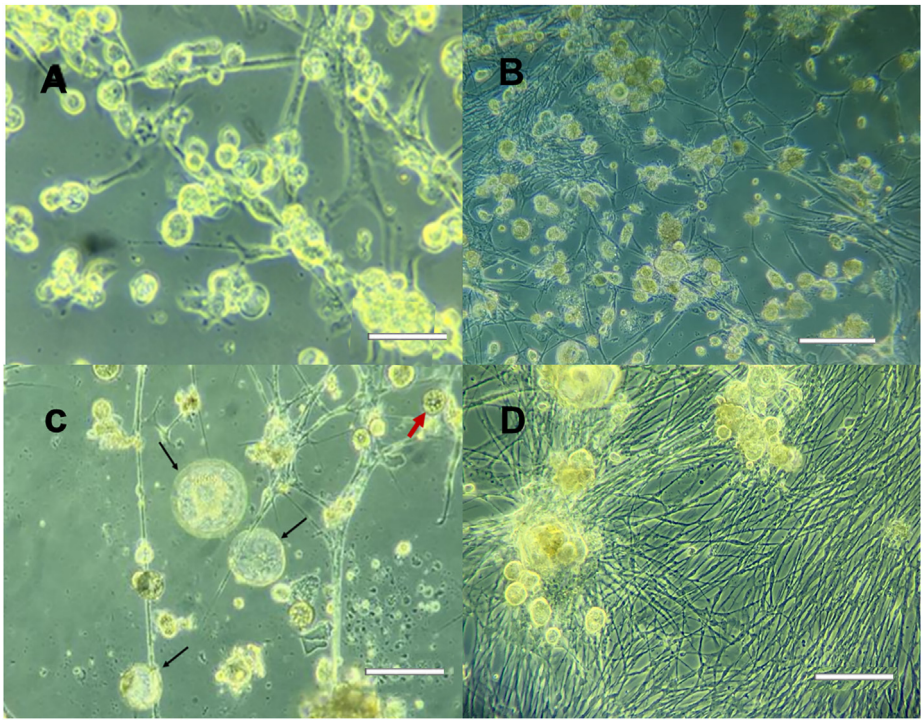

Figure 3. *Calliphora vicina* embryonic tissue cell culture growth in Grace's/L15 medium. A-B. Cell growth around embryonic tissue fragments. C. Vesicle formation in *C. vicina* primary culture in Grace's/L15 medium. The black arrows show empty vesicles and the red arrow indicates a vesicle having spherical cells. D. Confluent monolayer 15 days after tissue explant culture. Scale bar = 200 μM.

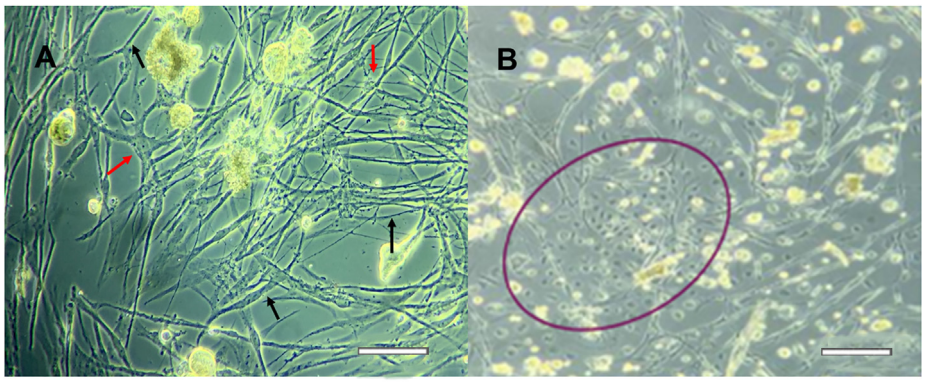

Figure 4. CV-062020-PPB cell line cell morphology in Grace's/L15 medium. A. Fibroblast-like morphology (Black arrow) and neuron-like cells with long dendrites (red arrow) can be seen. B. CV-062020-PPB cell line having epithelioid morphology (purple circle). Scale bar = 200 μM.

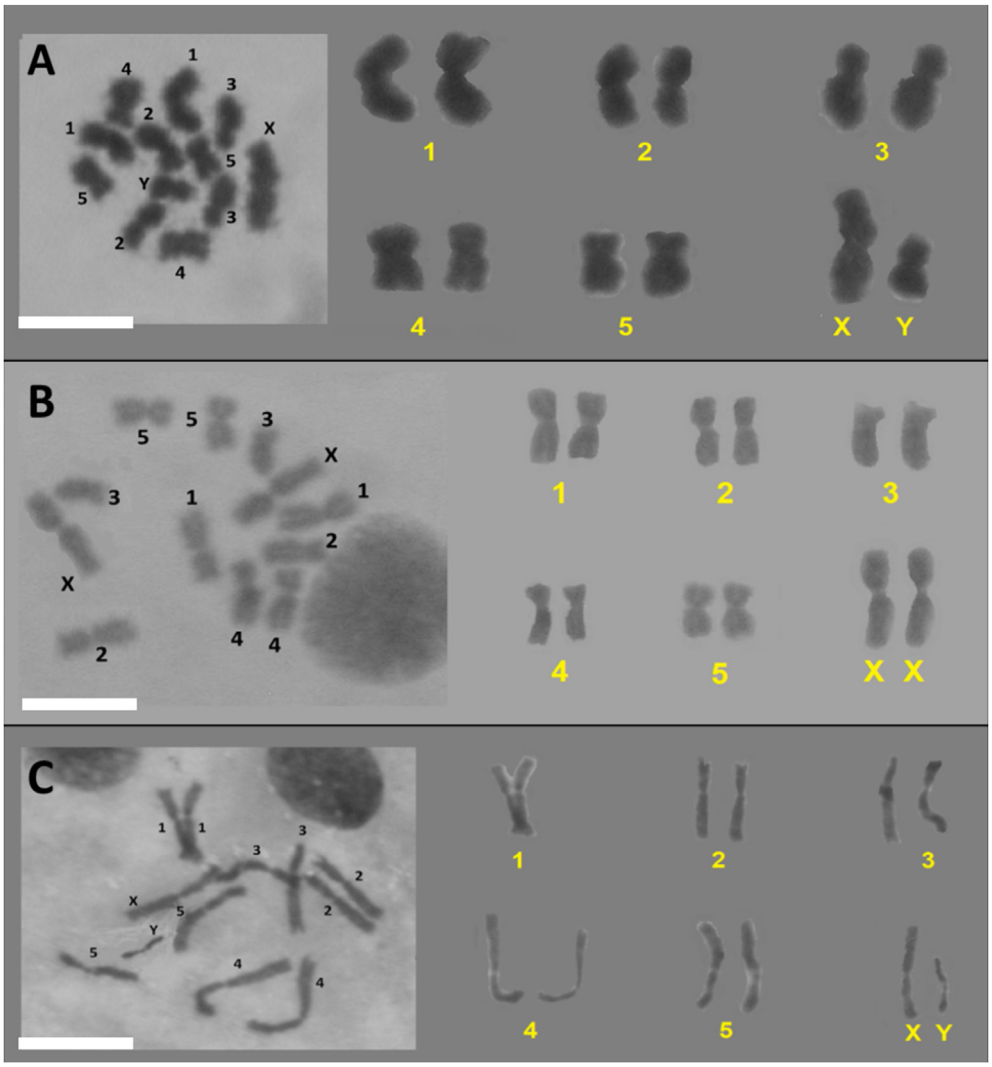

Figure 5. *C. vicina*-derived *Calliphora vicina* CV-062020-PPB cell line karyotype. A. Female metaphase karyotype. B. Male metaphase karyotype. C. Prometaphase karyotype. Scale bar = 200 μM.

lower similarity coefficient (0.37) was found between the RAPD-PCR profile obtained from the CV-062020-PPB embryonic cell line and the Lulo cell control. Coefficient values close to 1 indicated greater similarity (Table 3).

4. Discussion

This work has reported the establishment of a *C. vicina* embryonic tissue-derived cell line (CV-062020-PPB) for the first time. There was a

Table 2. Morphometric parameters for *Calliphora vicina*, CV-062020-PPB cell line autosomal and sex chromosomes.

Chromosome	(μm)		r *q/p*	r *p/q*	TL		RL	CI	AAVL	Classification
	p	*q*			(μm)	SD				
1	6.386	9.579	1.500	0.666	15.965	0.788	0.179	0.400	1.722	M
2	5.527	7.335	1.327	0.753	12.855	0.739	0.144	0.429	1.387	M
3	3.356	7.833	2.334	0.428	11.189	0.651	0.126	0.299	1.207	Sm
4	4.749	5.805	1.222	0.818	10.554	0.858	0.118	0.449	1.138	M
5	4.387	5.817	1.325	0.754	10.204	0.858	0.114	0.429	1.100	M
X	8.245	10.494	1.272	0.785	18.739	1.24	0.211	0.439	2.021	M
Y	3.70	5.568	1.504	0.664	9.268	0.745	0.104	0.399	1	M
Total					88.774					

p: short arm, q: long arm, TL: total length, RL: relative length, CI: centromere index. AAVL: average absolute value of length. M: metacentric chromosome, Sm: submetacentric chromosome. Data collected from 20 metaphases (n = 20).

need to modify and standardise particular conditions regarding this species, despite having used a methodology similar to that conventionally used for insects regarding cell culture initiation, i.e. selecting culture media based on physiochemical composition, the cultures' environmental conditions and their care and maintenance. Such factors enabled cell adaptation, growth and proliferation in the cultures until the cell line had been successfully obtained, being 29 the highest number of passages obtained to date.

Culture media evaluation is important because it encourages greater cell adaptation, proliferation and growth. *C. vicina* embryonic tissue-derived cells did not grow in DMEM and/or Grace's media in this study; this pattern was also seen in *Lucilia sericata* cell cultures (Echeverry et al., 2009); the explant lasted 10 days in Grace's medium but the cells did not adhere or proliferate. *C. vicina* cells remained in suspension in the DMEM medium; cells died because they could not adapt to the medium, similarly to that reported by Echeverry et al. (2009) in *L. sericata* cell culture. Cell migration occurred during the first hours after explant in the *L. sericata* cell cultures with L15; the semiconfluent monolayer was obtained after 45 days, indicating that the cell cultures had obtained the necessary nutrients from L15 culture medium, enabling cells to adapt, proliferate and grow. They also observed that the cells did not adhere to the culture flask but remained suspended in Grace's/L15 as cell replication was inhibited and then regressed to a state of apoptosis, unlike the favourable results regarding cell culture growth in our study. These results were obtained when using Grace's/L15 medium and are consistent with that reported by Cruz and Bello (2012) regarding *Sarconesiopsis magellanica* embryo tissue.

Despite the fact that *C. vicina* egg surface sterilisation was considered optimum in this work, a certain microbiological contamination level was observed after two or three days of explant seeding. Since *C. vicina* egg surface sterilisation was rigorously conducted, we suggest that bacterial contaminant might have been caused by an intracellular type of microorganism transmitted by transovarian route, derived from *C. vicina* embryo tissues. That a bacterial contaminant could have originated from the culture media was thus ruled out since microbiological controls were carried out periodically in selective media for bacteria and fungi and no growth was obtained. Likewise, unusual changes in medium pH were not detected, nor was there any increased turbidity or the appearance of suspended particles (Arunkarthick et al., 2017), these being indicators of bacterial contamination.

The bacterial contamination recorded in the primary cultures (precisely in L15 and Grace's/L15 media where there was cell growth) was self-controlled; bacterial activity and amount thus decreased as the culture grew until not being observed; sterility tests demonstrated that the cultures were microorganism-free. The action of cell-produced molecules, such as antimicrobial peptides (AMPs), produces antibacterial activity and controls the aforementioned contamination. The foregoing, along with AMP production in *C. vicina* fat body and haemocyte cell cultures (Yakovlev et al., 2017) and *Sarcophaga peregrina* embryonic tissue-derived cell cultures (Matsuyama and Natori 1988). A more recent illustrative review dealt with the use of insect- and tick-derived cell lines for investigating different aspects of the immune response, i.e. analysing the innate cellular response by stimulating antigens for inducing AMP production (Goodman et al., 2021).

Insect cell culture is often characterised by a heterogeneous cell population and a variety of cell morphologies that may include small, spherical elongated or even epithelial shaped cells (Wang et al., 2011; Cruz and Bello 2012). Insect cells appear to form cytoplasmic projections which were observed amongst cells in *C. vicina* primary and subcultured cultures when using L15 and Grace's/L15 media. Cytoplasmic projections enabled communication between different shaped insect cells in the vicinity and may have enabled the close exchange of growth factors between cells. Such cytoplasmic projections formed very complex networks characterising the cell growth pattern that progressively evolved and formed confluent monolayers made up of highly ramified cells resembling neuron-like cells. This apparently atypical cell growth pattern has also been reported in different insect species, such as *L. sericata* when cultured in L15 medium (Acuña Morera et al., 2011) and *Anasa tristis* (Hemiptera: Coridae) (Goodman et al., 2017), *Spodoptera frugiperda* and *Spodoptera exigua* cell lines (Lepidoptera: Noctuidae) (Reall et al., 2019; Su et al., 2016).

Insect cell cultures are also characterised by large vesicle formation, these being common structures containing a number of spherical cells located on vesicle periphery. Large vesicles are formed during the first days of primary cell culture; they may persist for weeks and remain as suspended large bodies in culture medium in initial subcultures until they eventually become fragmented, releasing a number of proliferating cells. These newly released cells begin fresh cell division when they reach the bottom of the culture plate and progress to form a firmly adhered cell monolayer. *C. vicina* embryo cells that have undergone a series of cell duplication events acquire a fusiform shape that may be called fibroblast-like morphology. This is similar to the morphological characteristics reported for *S. magellanica* insect cells (Cruz and Bello 2012) in lepidopteran species such as *Clostera anachoreta* (Wen et al., 2009), *Papilio demoleus* (Ding et al., 2013) and in Coleoptera species such as *Tribolium castaneum* (Goodman et al., 2012).

The cellular contractile movements observed in *C. vicina* embryonic cell cultures in this study coincided with reports regarding *L. sericata* cell culture (Echeverry et al., 2009) and some lepidopteran species (Ding et al., 2013). Such cell contractions (previously described as pulsating movements) might be mediated by muscle cell progenitor-derived cell-specific contractile proteins; this could indicate that embryonic cells in culture may have pluripotency. However, according to the nutrients in the media, the cells became phenotypically differentiated and adapted to survive environmental culture conditions (Su et al., 2016). Failure to do so may be the most logical reason for explaining cell death and no primary cell culture viability.

Cell cultures offer a tremendous advantage, as better results are obtained during karyotype and morphometric analysis of chromosomes.

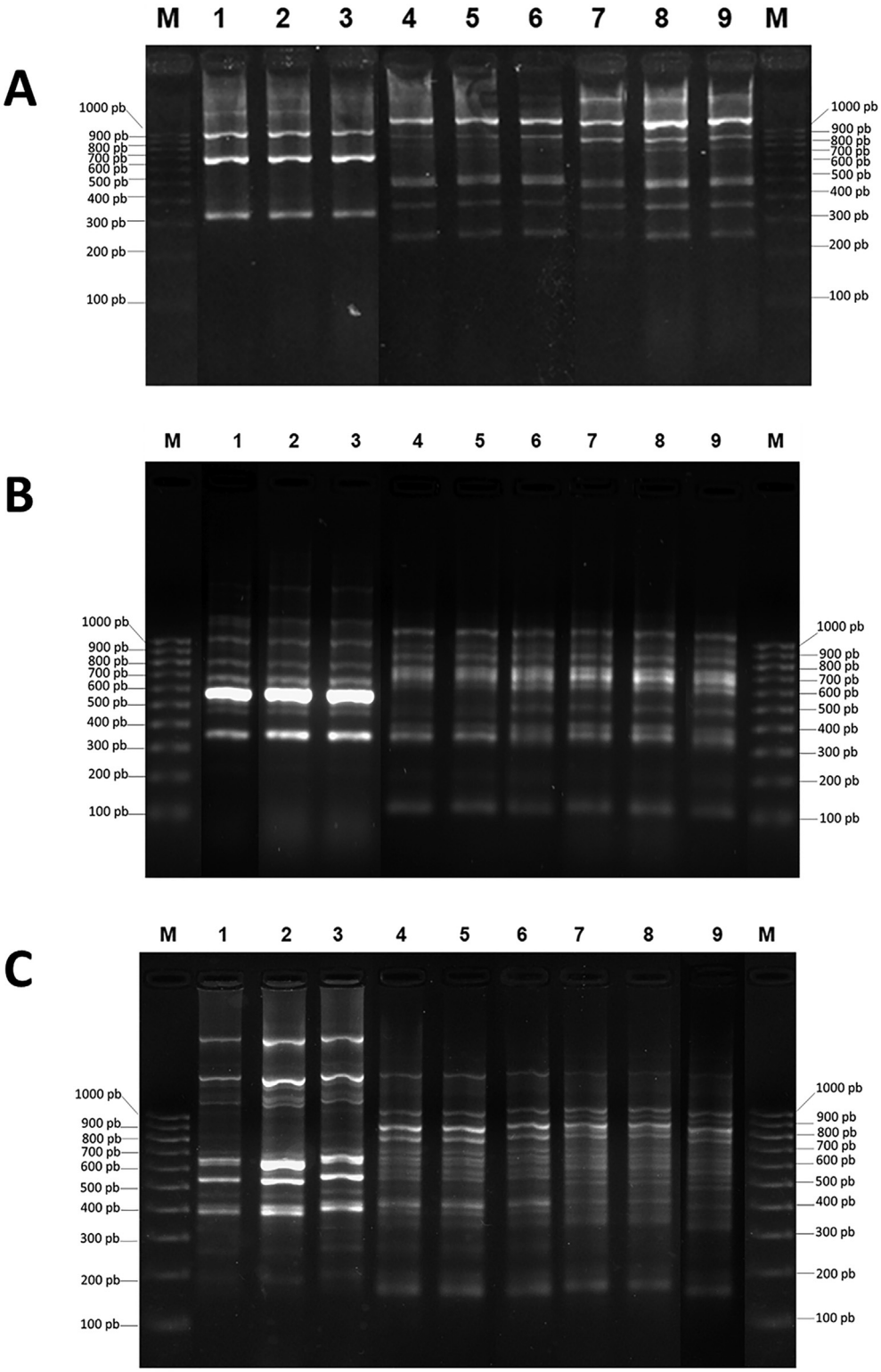

Figure 6. *Calliphora vicina* genomic DNA RAPD-PCR profile. A. RAPD-PCR profile obtained with primer A2. B. RAPD-PCR profile obtained with primer A10. C. RAPD-PCR profile obtained with primer A20. M: 100 bp molecular weight marker. Lanes 1–3 show Lulo cell control line. Lanes 4–6 show the *Calliphora vicina* CV-062020-PPB cell line. Lanes 7–9 show *C. vicina* fly adult tissue.

Cultured insect cell karyotypes show increased resolution, homologous chromosome separation and facilitate chromosomal structure measurements (Bello et al., 1995). The *C. vicina* embryo cell diploid chromosomal configuration in the present work was 2n = 12; this has also been reported in different Calliphoridae species (Ullerich and Schöttke 2006), including *L. sericata* (Chirino et al., 2015; El-Bassiony 2006), *Chrysomya megacephala* and *Ch. putoria* (Parise-Maltempi and Avancini 2001), *Triceratopyga calliphoroides*, *L. porphyrin*, *Ch. pinguis*, *Xenocalliphora hortona* (Agrawal et al., 2010) and *L. cluvia* (Chirino et al., 2015). The 2n = 12 diploid chromosome configuration found in *C. vicina* karyotypes has also

Table 3. RAPD-PCR band similarity coefficients between *Calliphora vicina*, the CV-062020-PPB embryonic cell line and adult *C. vicina* fly cells.

Primers	*C. vicina* CV-062020-PPB cell line vs. Adult *C. vicina* tissues	*C. vicina* CV-062020-PPB cell line vs. LULO cell line
A2	0.93	0.37
A10	0.96	0.48
A20	0.88	0.66

been reported in some Muscidae and Sarcophagidae species (Parise-Maltempi and Avancini 2000, 2007).

C. vicina chromosome length differed from that reported in a previous study for this species conducted in north-western Egypt (El-Bassiony 2006). Differences might be explained by evolutionary changes due to the flies' adaptations to geographical areas or scenarios where the specimens had been collected, probably due to particular differences in the tissues used to produce the karyotypes or the equipment used to produce morphometric data. Similarity would thus only be maintained regarding X and Y chromosome classification (El-Bassiony 2006). Size variations have also been reported in Calliphoridae family species *Chrysomya albiceps* and *Ch. rufifaciens* (Parise-Maltempi and Avancini 2001).

Molecular typing methods are very useful for discriminating between species and members of different species. *C. vicina* embryo cell culture molecular pattern based on RAPD-PCR markers revealed similarity coefficients that correlated very well with those reported in dipteran species such as *L. sericata* (Acuña Morera et al., 2011), *S. magellanica* (Cruz and Bello 2012), *Aedes aegypti* (Ardila et al., 2005), coleopteran species such as *Leptinotarsa decemlimeata* (Long et al., 2002) and *T. castaneum* (Mahmoud and Kamel 2019) and in Lepidoptera such as *S. exigua* (Chaeychomsri et al., 2016). The similarity coefficient confirmed evaluated cell line identity and shared relationship with different samples from the same insect species in each of the aforementioned studies.

This study has reported a new *C. vicina* embryo tissue-derived cell line (CV-062020-PPB) which was morphological, cytogenetically and molecularly characterised. This cell line could be useful for isolating AMPs and other molecules involved in healing difficult-to-heal chronic wounds, as has been reported *in vivo* using larval therapy methodology involving Calliphoridae family species. It will be available for the multiple biomedical and biotechnological applications described for insect-derived cell lines.

Declarations

Author contribution statement

Ingred Pinillos and Cindy Pérez: Performed the experiments; contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data; Wrote the paper.

Orlando Torres and Manuel A. Patarroyo: Contributed reagents, materials, analysis, tools or data; Wrote the paper.

Felio Bello: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

Dr FELIO Bello was supported by Ministry of Science and Technology [Project code 124380864546, grant number FP 80740–152-2019]].

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

The authors would like to thank Yuly Eilen Bernal MSc for her support regarding the bacteriological and molecular techniques used in *C. vicina* embryonic cell characterisation. We would also like to thank Jason Garry for carefully reviewing the manuscript.

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