

Establishment and characterization of a new fibroblast-like cell line from the skin of a vertebrate model, zebrafish (*Danio rerio*)

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Abstract

A novel cell line designated as DRS (*Danio rerio* skin) was established and characterized from the skin tissue of wild-type zebrafish, *Danio rerio*, by the explant technique. The cells thrived well in the Leibovitz's -15 medium supplemented with 15% FBS and routinely passaged at regular intervals. The DRS cells mainly feature fibroblast-like morphology. The culture conditions of the cells were determined by incubating the cells at varying concentrations of FBS and temperature; the optimum was 15% FBS and 28°C, respectively. Cells were cryopreserved and revived with 70–75% viability at different passage levels. Two extracellular products from bacterial species *Aeromonas hydrophila* and *Edwardsiella tarda* were tested and found toxic to the DRS cells. Mitochondrial genes, namely COI and 16S rRNA PCR amplification and partial sequencing authenticated the species of origin of cells. The modal diploid (2n) chromosome number of the cells was 50. The cells were transfected with pmaxGFP plasmid tested positive for green fluorescence at 24–48 h post-transfection, confirming the usefulness of the developed cell line for various *in vitro* applications.

1. Introduction

As a vital vertebrate model, zebrafish have advanced in developmental biology, toxicology, and neurobiology [14]. It is mainly because of the fully sequenced, annotated genome, transparent embryos, and the possibility to compare mutants with well-characterized wild-type individuals [3]. It has a high fecundity rate and can develop embryos rapidly ex-utero in less than a week to develop organ systems [8]. The cell lines established from the zebrafish facilitate the correlation of the health-relevant issues of humans. The foremost reason is mainly due to the evolutionary history of conserved gene sequences of zebrafish compared to the human genome to a larger degree, i.e., genetic similarities [17]. Thus, zebrafish form a most promising vertebrate animal model for biomedical applications such as human disease, drug screening, wound healing, and cancer research.

The development of cell lines from zebrafish began in the early 1990s. The majority of the zebrafish cell lines available to the scientific community for *in-vitro* approaches mainly originated from embryonic tissue and featured fibroblast-like morphology. The first report of tissue-specific cell lines (ZPF) and embryonic cell line (ZEM) featuring fibroblast morphology were derived from the fin tissue and mid-blastula, respectively [11]. Most *in vitro* research on zebrafish has been intensively carried out in the liver tissue-specific epithelial cell line ZFL [18]. Over the decades, very few tissue-specific cell lines, viz., ZSSJ (spleen), DrRPE (retinal pigment), DRM (muscle), DrG (Gills), DrF (caudal fin), and DRCF (caudal fin) [54, 32, 25, 33, 31] respectively have been reported from the genetically important model animal. The tissue-specific cell lines from zebrafish find their applications limited to wound healing, ultraviolet radiation biology, contaminants toxicity, and gene expression studies. Although zebrafish is a vital model organism, it lacks other *in vitro* experimental approaches due to the unavailability of suitable cell culture systems [18]. To exploit various *in vitro* research applications, establishing novel tissue-specific cell lines of higher genetic importance is foremost. Hence, we aimed to develop a tissue-specific cell line from the model zebrafish *D. rerio* targeting other *in vitro* research approaches.

Fish skin plays a significant role in the first-line defence system that aids in conferring long-term immunity against the microbes in the aquatic habitat and several other peptide hormones involved in several physiological processes [37]. It serves as a physical barrier by the secretion of enzymes, immunoglobulins, mucins, lectins, and other microbial peptides [5]. The cell lines from the integument system could be employed to understand the mechanisms underlying physiology and wound healing [44]. The susceptibility of zebrafish skin cells to the invading viruses could help study the viral pathogenicity. Moreover, it provides insights into skin antiviral response for elucidating the mechanism of mucosal immunity [28]. The transcriptome profiling of skin cells helps understand the mechanism of the host immune responses against bacterial pathogens, thereby allowing the study of differential gene expression [29].

Further, zebrafish skin also possesses a higher regeneration capacity similar to other organs such as fins, heart, and optic nerve [53]. Therefore, cell lines established from the skin tissue could be applicable in regenerative medicine for producing fish skin grafts [45] and the cytotoxicity assessment of silica nanoparticles [9]. Zebrafish skin also offers a relevant biological model to study pigment research [12]. However, to our knowledge, there is no report on the skin cell line from the wild-type zebrafish. Therefore, we aimed to develop and characterize the cell culture system from the skin tissue of the model organism *D. rerio* by the explant technique and its outcome towards the healthy cell line designated as DRS (*Danio rerio* skin).

2. Materials & Methodology

2.1 Ethical Approval

Institutional Animal Ethics Committee (IAEC) and Board of Studies (BoS), Fish Genetics and Biotechnology Division, ICAR-Central Institute of Fisheries Education, Mumbai, approved all experimental protocols involved in this study. The methodologies involved in the study were carried out following relevant institutional guidelines and regulations.

2.2 Experiment animal

A total of 40 healthy wild type fingerlings of *Danio rerio* weighing 2 g and 1.5 cm in length (approximately) were procured from the local aquarium (Mumbai, Maharashtra) and maintained at room temperature in a well-aerated and conditioned water. Donor fish (*D. rerio*) was starved in aerated water for 24 h then sacrificed by euthanization using ice-cold water containing 1000 IU/ml of penicillin, 1000 µg/ml of streptomycin, 25 µg of amphotericin B and dipped in 5% sodium hypochlorite for 5 minutes, followed by surface sterilization with 70% alcohol and operated *in vivo*. Antibiotics were used only during the explant preparation, and the cultures progressed devoid of antibiotics later.

2.3 Initiation of Primary cultures

The method followed to initiate the primary culture was similar to that described for the PHG cell line [40] with some minor modifications. Target skin tissue from three fish was aseptically dissected out by removing the scales, pooled, and washed several times with DPBS (Dulbecco's phosphate-buffered saline) (Hi-media, India) containing 500 IU/ml penicillin, 500 µg/ml streptomycin, and 2.5 µg amphotericin-B (Hi-Media, India). The tissues were aseptically minced into small explants of size 1mm³ seeded into 25 cm² cell culture flasks (Hi-Media, India). Adherence of explants was accomplished by adding 0.2ml of fetal bovine serum (FBS) (Gibco, USA). Then, the flasks were incubated overnight to allow the explant tissues to attach at 28°C based on the acclimatization temperature of the fish [7]. After allowing the tissue to attach, 4 ml of Leibovitz-15 medium (Hi-Media, India) supplemented with 20% FBS was added. One-fourth of the medium was removed and added with fresh medium every 3-4 days. Cell proliferation from the adhered explants and contamination of flasks were observed regularly using an inverted microscope (Nikon, Japan).

2.4 Subculture of monolayers

The primary cultures, upon reaching 80-90% confluency, the cells were harvested using Trypsin-EDTA solution (0.25% trypsin) (HiMedia, India) and 0.2% ethylenediaminetetraacetic acid (EDTA) without dislodging the explants. The detached cells were seeded in a split ratio of 1:2 into 25cm² flasks adding fresh 4 ml of L-15 medium containing 20% FBS. Then the flasks were incubated at 28 °C.

2.5 Cryopreservation

The protocol described by [2] for cryopreservation of cells was followed with minor modifications. Briefly, DRS cells at different passage levels 3,13, 28, and 33 were harvested by trypsinization and followed by centrifugation at 125×g for 5 minutes. The pelleted cells at a 1× 10⁶ /ml density were suspended in L-15 medium containing 10% DMSO and 40% FBS. The cell suspension approximately 1.0 ml was transferred into 2 ml cryovials. Then the cryovials were placed in a freeze control (Mr. Frost) and incubated serially at 4° C for 1 h, -20° C for 2 h, -80° C overnight, and then finally transferred into liquid nitrogen (-196° C). For the revival, cryovials containing DRS cells were thawed in a water bath for 2 mins at 28°C and centrifuged at 125×g for 7 min. After removing the freezing medium, the cells were suspended in L-15 medium containing 10% FBS and were analyzed for cell viability using a TC-20 automated cell counter (Bio-Rad, USA).

2.6 Growth pattern

The optimum temperature for the growth of the DRS cells at passage 17th was determined by incubating the flasks in a BOD incubator (Labtro, India) at varying temperatures ranging from 20, 24, 28, and 32°C over seven days at a seeding concentration of 1x10⁵ cells in 25 cm² culture flasks in triplicates. Then, three flasks from each temperature at which they were incubated were trypsinized, and cell counting was performed using a TC-20 automated cell counter (Bio-Rad, USA) for seven consecutive days

on an alternate day. Similar procedures were performed for the effects of varying concentrations of FBS (10, 15, and 20 %) on cell growth at 28°C over seven days.

2.7 Cell Plating Efficiency

The DRS cells at passage 12th were seeded at 200, 500, and 1000 cells/ml in triplicates to determine the plating efficiency. After incubation for ten days, the spent medium was removed, and the cells were fixed with 5 ml of 1% crystal violet in 25% formalin stain fixative solution for 15 min, rinsed with tap water, and air-dried. Then, the cell colonies were counted under an inverted microscope, and plating efficiency was calculated using the formula described by [16]. Plating efficiency (Y) = $(x/z) \times 100$, Where x= number of colonies counted, z= seeding density of the flask.

2.8 Cell Doubling time

The time interval required for a cell population to double in the middle of the logarithmic phase. The population doubling time of the DRS cell line was calculated at the 11th passage using the formula. Cells population doubling time (DT) = $T \ln (X_e/X_b)$ where T= is the incubation time in any unit, X_e = cell number at the end of the incubation time, X_b = cell number beginning the incubation time [16].

2.9 Species authentication by PCR amplification

Template DNA from the DRS cell line at the 10th passage and the skin tissue of *D. rerio* were isolated following [39] with minor modifications. The origin of the DRS cell line was authenticated by amplification of mitochondrial gene regions such as COI (Cytochrome C oxidase subunit I) and 16S rRNA of *D. rerio* using universal pair of primers, FishF1 and FishR1 [50] and 16sf1F140 and 16sf1R1524 [55] respectively. The primers sequences, PCR master mix composition, and the thermal regime for the COI and 16S rRNA gene amplification are mentioned in Table.1,2,3,4, respectively. The amplified products were analyzed using 1.0 % agarose gel containing ethidium bromide and visualized using gel documentation (BioRad). The amplified products were purified and subjected to sequencing by the external sequencing facility (Xcelris, Pvt. Ltd). The resultant sequences were aligned and compared to the known sequence of the species in the NCBI GenBank reference database (standard database 'nr/nt') using the Basic Local Alignment Search Tool (BLAST).

2.10 Chromosome analysis

Cells with 80% confluency at passages 16 and 21 were used for chromosome preparations following [22] with some modifications. Briefly, the cells were incubated at 28°C in a complete growth medium containing 0.04 % of colchicine for 4 hrs. The cells, after incubation, were trypsinized and pelleted by centrifugation at 367g for 10 mins. The supernatant was removed, and cells were treated with 5ml of hypotonic solution KCl 0.07M for 25-30 mins. Then the cells were prefixed with 2ml of freshly prepared Carnoy's fixative (3:1) (methanol: glacial acetic acid) for 5 mins. Again, the cell suspension was centrifuged at 367g for 10 mins, followed by washing the pellets with ice-cold Carnoy's fixative twice. The

pellets were resuspended in 0.5ml of fixative solution and dropped onto a clean glass slide using the conventional drop splash technique. Finally, glass slides were air-dried, stained with 5% Giemsa for 20-30 mins, and observed under light microscope (Nikon, USA) at 100X magnification. A total of 100 metaphase spreads were counted for chromosome analysis.

2.11 Transgene Expression

DRS cells at the 17th passage were seeded into a six-well plate at a density of 1×10^5 cells/well and incubated overnight at 28°C. Transfection was carried out using pmaxGFP plasmid DNA concentration of 400ng and reagent Lipofectamine 3000 (Invitrogen, USA) following the manufacturer's protocol. The transfected cells were incubated overnight at 28°C in the BOD incubator. Then the spent medium was replaced with a fresh medium after 4-6 hours and tested for transgene expression. The number of cells positive for the green fluorescence was counted using ImageJ software (<https://wsr.imagej.net/distros/win/ij153-win-java8.zip>). The transfection efficiency was calculated based on the ratio of cells positive to the fluorescence and the total number of cells in 20 independent light fields [52].

2.12 Bacterial extracellular products (ECP's) testing

For bacterial extracellular products (ECP's) testing, two bacterial species, *Aeromonas hydrophila* and *Edwardsiella tarda*, were isolated from the diseased animal. The extracellular products were obtained by following the protocol described by [6] with some minor modifications. Briefly, the bacterial cells were incubated at 28°C for 48 h after spreading onto a cellophane sheet overlaying an agar plate. The cells were harvested by centrifugation at $13,000 \times g$ for 20 min at 4°C, and the supernatant was filtered through a 0.22- μm filter to get bacterial ECP's as well as heat-killed bacteria and stored at -80° C until further use. The protein concentration of the isolated ECP's was estimated by the BCA™ (Bicinchoninic acid) protein assay using Nanodrop™ (USA). The DRS monolayer was inoculated with 0.1ml of 10-fold serial dilutions of bacterial ECP's and heat-killed bacteria. Wells inoculated with saline solution (0.85%) were treated as negative controls. The plates were incubated at 28°C, and the effect of ECP's and heat-killed bacteria on the monolayers was observed after 24 and 48 h.

2.13 Mycoplasma detection

Mycoplasma presence in the DRS live cells and culture medium was tested at the 15th passage using MycoFluor™ Mycoplasma Detection Kit (Invitrogen, USA), following the manufacturer's instructions with minor modifications. Briefly, 1 volume of 20X concentrated MycoFluor reagent was added to 9 volumes of DRS cell suspension (1×10^5 cells/ml) incubated at RT for 10 min and observed using a near-ultraviolet fluorescence filter (excitation at 365 nm) (Nikon, USA).

3. Results

3.1 Explant technique and subcultures

The explant primary culture technique initiated the monolayer of cells from the skin tissue of zebrafish, and routine subculture led to the development of the cell line designated as DRS (*Danio rerio* Skin). The cells started to proliferate from the explant tissue after five days of explant preparation, and a confluent monolayer was noticed on the 20th day. The cells were initially grown in L-15 medium supplemented with 20% FBS, then reduced to 15% after the 12th passage level. Initial subcultures were slow-growing and started to proliferate rapidly after the fifth passage. Thereon, the cells were routinely maintained and passaged at a ratio of 1:2 at 3-4 days intervals. The morphology of the primary cells was composed of epithelioid and fibroblastic during the initial cultures (Fig.1). Later, the fibroblastic morphology predominated after the 18th passage. The cell line has been passaged for over 36 subcultures since its initiation in March 2022.

3.2 Cryopreservation

DRS skin cells at different passage levels 3,13, 18, 28, and 33 were stored in liquid nitrogen (-196°C) for over two months. The skin cells were recovered by thawing and seeded to form a confluent monolayer about 7 days after plating without showing any changes in the morphology. The average viability of the DRS was estimated to be 70-75% approximately.

3.3 Growth pattern

DRS cells could grow well in optimum conditions of 24 to 32°C. However, the highest growth rate was observed at 28°C (Fig.2A). i.e., the temperature required for the acclimatization condition of the fish. The growth of the cells increased from 10% to 20% FBS concentration and significantly better growth was observed at 15%, but the highest growth was observed at 20%. (Fig.2B)

3.4 Cell plating efficiency

The cells were seeded at a density of 200, 500, and 1000 cells/ flasks calculated plating efficiency of the DRS at the 12th passage level was 17, 25, and 41%, respectively, with no significant differences between replicates.

3.5 Cell doubling time

The calculated doubling time of the DRS cells was found to be 31h at passage level 11th.

3.6 Species authentication by mitochondrial gene analysis

Mitochondrial gene analysis, namely COI and 16S rRNA, revealed the species of origin of the DRS cells was from zebrafish *D. rerio*. The PCR amplification of COI and 16S rRNA genes yielded the expected PCR products of size 655 bp and 1380 bp, respectively (Fig.3 & Fig.4). The subsequent comparative analysis

of the aligned sequences with the known voucher specimen from *D. rerio* demonstrated 96-97% sequence similarity. The sequences have been submitted to the NCBI database for their accession number.

3.7 Chromosome analysis

The cells at passage levels 16 and 21 were arrested at metaphase using colchicine at a final concentration of 0.04% (Fig.5A). The result of counting a total of 100 metaphase spreads revealed that the diploid chromosome ($2n$) number ranged from 36 to 63 (Fig.5B). However, most cells were observed with a diploid chromosome number ($2n=50$).

3.8 Transgene expression

The DRS cell line at the 17th passage was successfully transfected with the pmaxGFP vector under the control of the CMV promoter, resulting in stable transfection with a green fluorescence signal as early as 24 hpt (hours post-transfection) (Fig.6). The transfection efficiency of the DRS cells calculated was 7% approximately. The result suggests that the developed cell line has the potential to be applied in transgenic studies.

3.9 Bacterial Extracellular Products testing

Two bacterial strains, *A. hydrophila* and *E. tarda*, proved cytotoxic to the DRS cells at a concentration of 1/10 dilutions of bacterial ECP's. No cytotoxicity was observed in cells exposed to heat-killed bacteria. The morphological changes of the cells after exposure to the bacterial ECP's observed after 12h were rounding, shrinking. The partial detachment of cells was noticed after 24h of inoculation (Fig.7). The destruction of the confluent monolayer was observed after 48 h, and no significant changes were observed compared to the PBS-treated control group.

3.10 Mycoplasma detection

The developed cell line was free from mycoplasma contamination as no stained nuclei associated with the DRS cells were observed.

4. Discussion

Most of the zebrafish cell lines have been established either from the embryonic stages or mutant strains such as AB and SJD types [22]. The cell lines reported from the wild-type zebrafish are very much limited that include DRCF [31]; DrG (*Danio rerio* Gill), DrRPE (*Danio rerio* Retinal Pigmented Epithelium) [32,33]; DRM (*Danio rerio* muscle) [25] and DrF (*Danio rerio* Fin) [22]. To our knowledge, this is the first report on the skin cell line from the wild-type zebrafish.

The explant method of primary culture offers the advantage of aiding the tissues to proliferate into a large number of cell types and reducing stress in culture conditions [47]. It also has the potential to aid the cell's survival, attachment, and differentiation into another type by releasing the necessary growth

factors [21]. The most attractive characteristic of the explant technique is that it does not affect the biological characteristics of the cells [27], unlike the enzymatic digestion method. As mentioned earlier, the previous reports of cell lines from the zebrafish, namely DrF, DrG, and DRCF, were successfully developed by the explant technique. Therefore, the explant technique is a suitable method to develop cell lines from zebrafish. The cell line established in this study is also in accordance with previous reports of zebrafish cell lines.

The developed cell line was grown in L-15 medium supplemented with 15% FBS. The morphology of the DRS cells was observed with a heterogeneous population of cells during the initial passages. Such co-existence of heterogeneous cell types during the initial phases was observed with morphological change into a single type, i.e., fibroblast, as the culture progressed after passage 18th. Such morphological change of one cell type over the other has been reported commonly in many established cell lines [56,10, 43].

To avoid loss of cells by contamination or to reduce the genetic or phenotypic changes during the routine subcultures using a high volume of trypsin [43], cryopreservation of cultured cells is the best and also suitable method for the long-term preservation of cells [27]. Cryopreservation at different passage levels followed by the revival after two months of cryo-storage, the DRS cells were successfully recovered with a viability of 70-75% approximately, forming a confluent monolayer of cells after 7 days of plating, which indicates that the cryo-storage is not detrimental to the cells. No changes in the indicators of the functionality of cells, including morphology, attachment, and growth [30], were observed after recovery, which is in agreement with the earlier reports [32,45].

The precise *in vitro* culture conditions such as temperature, growth medium, nutritional supplements including FBS, and growth factors are vital for normal cellular activities. The growth condition of the cell line was studied by incubating the cells at varied temperatures and FBS serum levels. The outset growth temperature for the DRS cells was 28°C, where the cells thrived well based on the acclimatization temperature of the fish. The cells proliferated in large numbers in L-15 medium supplemented with 20% FBS during the initial cultures. But, considering the cost-effectiveness of the culture period, the cell line was maintained optimally at 15% FBS, which is also in conformity with other established cell lines from the zebrafish DRM [25] and DrF [22].

DRS at passage level 12th was seeded with a cell density of 1×10^3 cells/ml, resulting in the highest plating efficiency of 41%, and there was no significant difference between the replicates. In contrast, the cells seeded at low density resulted in relatively lower efficiency. Similar findings have been reported in various cell lines, such as HCF cells [1] and PHG cells [40]. Such reduced plating efficiency of cells seeded at lower density is mainly due to the non-linear correlation between the seeding density and the plating efficiency [35]. However, higher seeding density could enhance the plating efficiency [42] or by using other conditioning medium [24].

The population doubling time of the developed DRS cells estimated was 31h at the 11th passage. The correlation between the cell doubling time and FBS concentration at which the cells were grown resulted

in increased population doubling, i.e., the higher the optimum level of FBS concentration, the higher the replicative lifespan of cells [56, 15].

Misidentification or cross-contamination of the cell lines is the major hindrance in cell culture systems intended for various *in vitro* applications. Hence, characterization of the cell line for its origin or any inter or intra-species cross-contamination using molecular approaches helps in studying the integrity of the cell line developed and also facilitates research accuracy [19]. Among the number of techniques for cell line authentication, PCR-based amplification of COI gene using sequence-specific primers was employed to identify the species origin of developed cell lines [34]. This study confirmed the origin of the DRS cells by COI and 16S rRNA gene amplification using universal pair of primers. Similarly, many cell lines have been characterized for their species identity by COI and 16S rRNA gene amplification routinely [1,13,43].

The distribution of the chromosomal numbers per cell is the crucial property of a cell population for both the performance and timeline of the cell line [49]. The commonly employed technique for measuring abnormalities in the chromosome number and the quality of the cell line is chromosome analysis [4]. The established cell line DRS cells exhibited a diploid (2n) chromosome number of 50 in the majority of the cells, which agrees with earlier reports from the zebrafish [22,31].

The ability of the cells to express the foreign genes determines the feasibility of the cell line in exogenous gene expression studies. In our study, the cell line was stably transfected with the plasmid DNA encoding green fluorescence protein with 7% transfection efficiency. Generally, higher transfection efficiency is needed only for the research targeting applications in biochemical studies or siRNA transfection [41]; however, the resulted transfection efficiency from this study is sufficient to study *in vitro* gene expression of various recombinant constructs.

Bacterial ECP's *A. hydrophila* and *E. tarda* were toxic to the DRS cells. The primary role of the ECP's secreted by bacterial pathogens is to counteract the host defence mechanism by increasing the virulence of the pathogen [36]. Previously, *A. hydrophila* and *E. tarda* have been reported to cause cytotoxic effects and invasion into the ZF4 cells, respectively [38, 57]. The *in vitro* bacterial ECP's cytotoxicity of the DRS cells from this study is similar to the observations of established cell line PCF [20] and could be correlated with the *in vivo* acute toxicity of bacterial pathogens. Hence, *in vitro* approach of bacterial ECP testing could be an alternative tool for *in vivo* assays.

Mycoplasma prevalence has been estimated at more than 20% of *in vitro* cultures, possibly through contaminated culture reagents or poor aseptic techniques [48]. It usually evades visual inspection and persists in the culture for a prolonged period without severe characteristic changes in the cell. However, its presence significantly affects the culture by altering cellular parameters like cell growth, metabolism, chromosomal aberrations [51] and cell proliferation [23]. In this study, the fluorescent MycoFluor™ Mycoplasma detection kit was used to test the presence of mycoplasma. However, the fluorochrome did not stain the nuclei of the DRS cells associated with mycoplasma. Therefore, the cultures are considered to be free from mycoplasma contamination.

5. Conclusion

The DRS cell line was successfully developed from the skin of *D. rerio*. The cell line was further characterized by its growth parameters like temperature and FBS concentration. The other routine cellular parameters like cryopreservation, plating efficiency, and doubling time were calculated. The cell line was authenticated for its species of origin using mitochondrial genes such as COI, 16S rRNA, and chromosome analysis. The exogenous gene expression was confirmed by cells showing a positive signal to the green fluorescence. Testing the cell line using a mycoplasma detection kit revealed that the cell line is devoid of contamination. Two bacterial extracellular products (ECP's) were tested and found toxic to the DRS cells. This is the first report on the skin cell line from the wild-type zebrafish that would contribute to the research community as a valuable *in vitro* tool.

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Declarations

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Competing Interests

The authors have no competing interests to disclose.

Authors Contributions

Benchmark, data collection, and analysis, first draft writing - **Arjunan Sathiyarayanan**

Maintenance of cell line - **Yashwanth BS**

Assistance in bacterial ECP testing- **Nevil Pinto**

Guidance and methodology in transfection studies - **Dimpal Thakuria**

Manuscript curation and validation- **Aparna Chaudhari**

Guidance in characterization Techniques - **Gireesh Babu P**

Ethics Approval

All applicable institutional guidelines for the care and use of animals were followed and approved by Institutional Animal Ethics Committee (IAEC), ICAR- Central Institute of Fisheries Education, Mumbai, Maharashtra, India.

Consent to Participate/ Publish

All the authors have read, approved the final manuscript, and given their consent to participate in this report and submit it to the journal.

Tables

Mitochondrial gene region	Primer Name	Primer sequence (5'-3')	Length (bp)	References
COI	Fish F1	TCAACCAACCACAAAGACATTGGCAC	26	Ward et al. (2005)
	FishR1	TAGACTTCTGGGTGGCCAAAGAATCA	26	
16S rRNA	16sf1F140	CGYAAGGGAAHGCTGAAA	18	Zhang and Hanner (2012)
	16sf1R1524	CCGGTCTGAACTCAGATCACGTAG	24	

Table 1. List of primers used for the amplification of mitochondrial genes COI and 16S rRNA.

Components	Volume/reaction (µL)
Template (200 ng/µL)	0.5
Buffer (10 ×)	1.25
Forward primer	0.5
Reverse primer	0.5
dNTPs (10 mM)	0.25
Taq polymerase (5 units/µL)	0.15
D/W	9.35
Total volume - 12.5 µL	

Table 2. Composition of PCR master mix for COI and 16S rRNA.

Steps	Condition		Cycles
	Temperature (°C)	Time	
Initial denaturation	94	5 min	1 cycle
Denaturation	94	30 s	35 cycles
Annealing	54	30 s	
Extension	72	45 s	
Final extension	72	7 min	1 cycle
Soak	4	Forever	-

Table 3. Thermal regime for cytochrome C oxidase I (COI) gene.

Steps	Condition		Cycles
	Temperature (°C)	Time	
Initial denaturation	95	5 min	1 cycle
Denaturation	95	40 s	35 cycles
Annealing	53	60 s	
Extension	72	45 s	
Final extension	72	7 min	1 cycle
Soak	4	Forever	-

Table 4. Thermal regime for 16S rRNA gene.

Figures

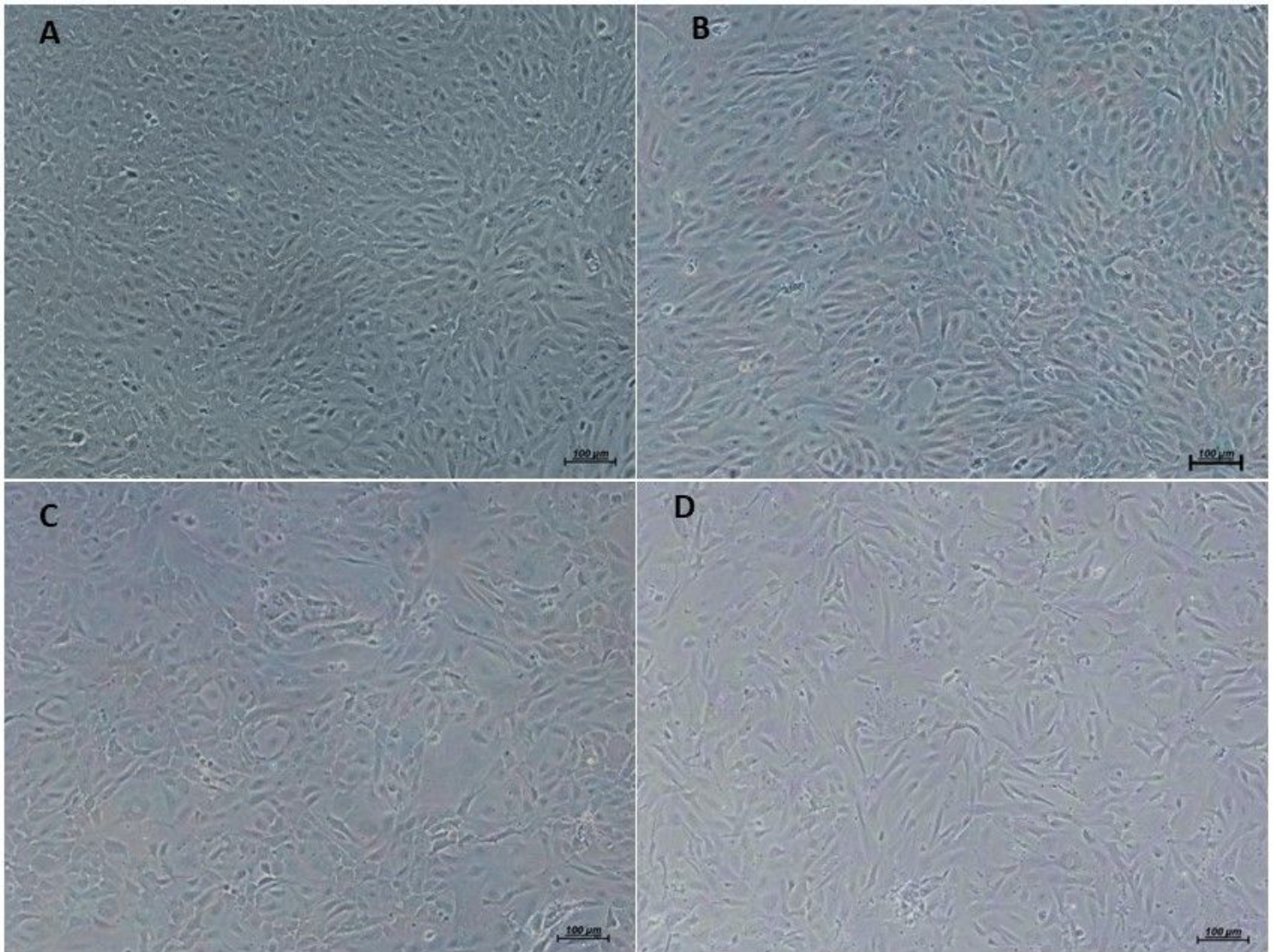


Figure 1

A. Phase-contrast photomicrographs of DRS cells confluent monolayer after 20 days of explant preparation (X 100).

B. Phase-contrast photomicrographs of DRS cells confluent monolayer after two days of passage 2nd (X100).

C. Phase-contrast photomicrographs of sparsely proliferated DRS cells after two days of passage 4th (X100).

D. Phase-contrast photomicrographs of sparsely proliferated DRS cells after two days of passage 14th (X100) (Scale Bar =100 μ m)

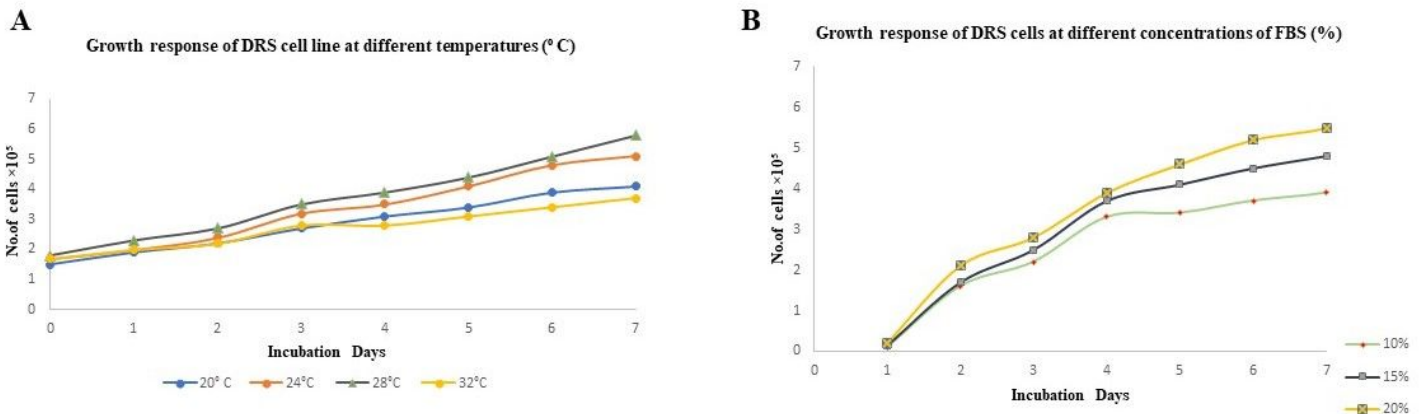


Figure 2

Growth response of DRS cell line at passage 17th **(A)** Different incubation temperatures ($^{\circ}$ C) **(B)** Different concentrations of FBS (%) (Values are means \pm SD) (n=3).

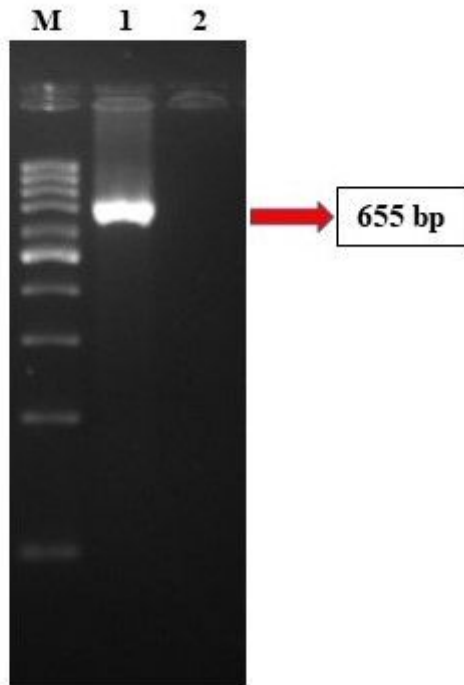


Figure 3

PCR Amplification of 655 bp fragment of *D. rerio* genome using oligonucleotide primers from the conserved portions of COI (Lane M- 100 bp DNA marker ladder (Fermentas), Lane 1- DRS cells, Lane 2- Negative control (Negative control - without DNA template)).

Figure 4

PCR Amplification of 1380 bp fragment of *D. rerio* genome using oligonucleotide primers from the conserved portions of 16S rRNA Lane M- 100 bp DNA ladder (Fermentas), Lane 1- DRS cells, Lane 2- Negative control (Negative control- without DNA template, Lane 3- Positive control (skin tissue from zebrafish))

Figure 5

Chromosome analysis of DRS cells at passage 16th (**A**). Metaphase spread of DRS cells (**B**). Distribution of Chromosome number among the metaphase spread (scale bar= 20µm)

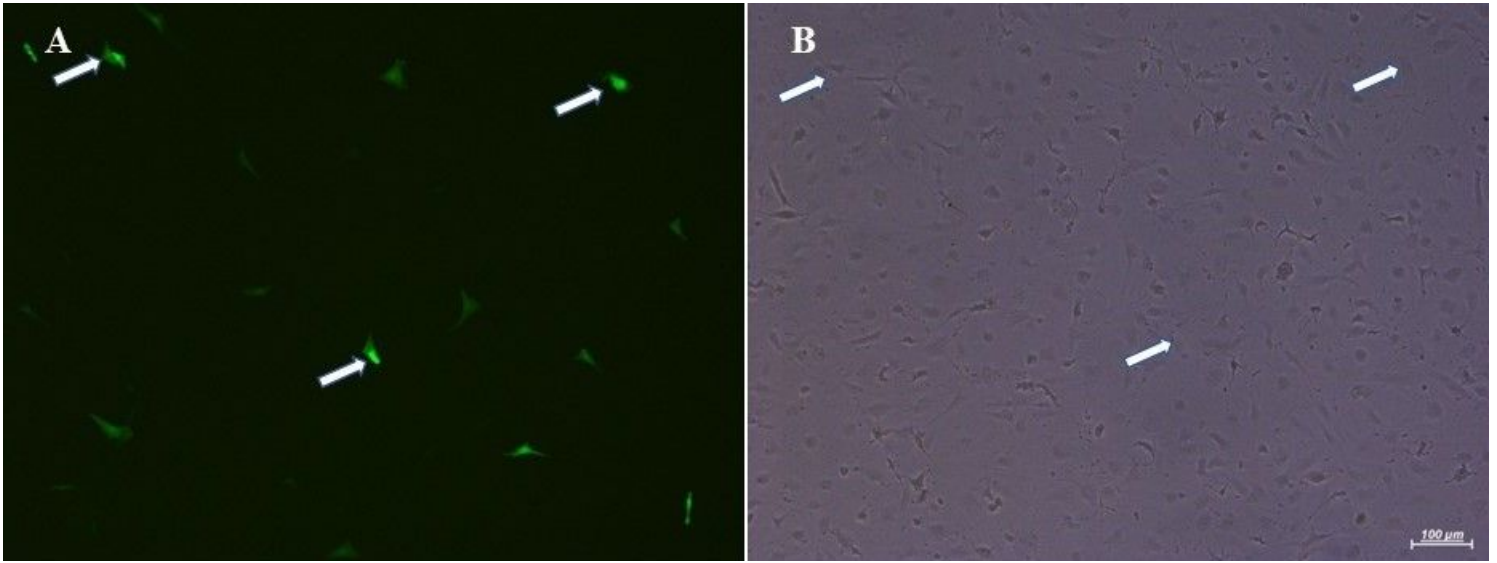


Figure 6

Transfection of DRS cells with pMaxGFP plasmid vector (A) Fluorescent microscopic image of DRS cells (B) Non-fluorescent phase-contrast microscopic image (Arrow indicates- cells positive to the GFP signal) (Scale bar=100μm)

Figure 7

Cytotoxic effect of bacterial extracellular products in the DRS cell line (A) *A. hydrophila* (B) *E. tarda* (C) Control (Scale bar=100μm)