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Research Article

Development of Primary Cell Lines from Gill, Kidney, Spleen and Caudal Fin of Common Carp (*Cyprinus carpio*)

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Abstract

Several fish cell lines have been developed for the interests of the fisheries world. This study aimed at developing a primary cell line from gill, kidney, spleen, and caudal fin of a common carp (*Cyprinus carpio*). A healthy common carp weighing 20 g (~1 month) was collected from the Cijeruk Fish Seed Center, Bogor. The development of primary cell lines from the gill, fin, tail, kidney and spleen tissue was performed in cell culture medium Leibovitz's L-15 supplemented with 20% serum fetal bovine, 250 IU Penicillin, 250 µg / ml kanamycin sulfate and 2Mm L-Glutamine, and incubated at 28°C. Primary cell lines of caudal fin and gill began to form a monolayer on day 17 after culture. While the development of cell lines from kidney and spleen, although the initiation of cells and cells spread on the surface into a monolayer, was not perfect; therefore, the passage was unable to be done. Microscopic observations and Giemsa staining showed primary cell lines of caudal fin and gill based on cell morphology consisted of two cell types, fibroblast-like cells and epithelial-like cells. The first passage was done on day 17 when the confluence was more than 50%. The next passage was carried out every 3 weeks when confluence reached 70% -80%. The primary cell culture of gill was successfully passaged as much as 72 and the caudal fin was successfully passed as much as 89 times over 7 years. These new cell lines can be further used to propagate fish viruses and other biotechnology assays.

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1. Introduction

The high stocking density combined with high feed input in modern fish farming has caused severe problems such as deterioration of water quality and the emerge of various fish diseases (Langner, 2011). According to Malole (1990), investigation of disease-causing viruses can be done by using tissue culture, because this method allows the production of more viruses as a source of antigens. Therefore, cell line and model systems are needed, especially for fish that have important economic value (Langner, 2011). Similarly, Langner (2011) stated that cell lines can be used for research in the fields of physiology, virology, pharmacology, toxicology, cancer/carcinogenesis and the modified genetic organisms (GMO) field.

Several cell lines have been developed from several tissues such as ovaries, fins, swimming pouches, heart, spleen, liver, eye muscles, spinal space, brain skin. Among the cell lines, more than 60% of species come from Asia, which contributes to more than 80% of total fish production. According to Lakra et al., (2010), there were currently 283 cell lines which have been produced from finfish worldwide, including freshwater, marine water and brackish water. The cell line technology in fish can be used for fish immunology (Clem et al., 1996; Bois et al., 2001), ecotoxicology (Fent, 2001; Castano et al., 2003; Schirmer, 2006), endocrinology (Bois and Lee, 1991), virology (Wolf, 1988), biomedical research (Hightower and Renfrow, 1988), disease control (Villena, 2003), biotechnology and aquaculture (Bois, 1991).

According to Sumiati *et al.* (2009), primary cell lines of carp's caudal fin has successfully been produced. However, the cell culture cannot be maintained or preserved; thus, many of them finally died. Thus, the present study aimed to develop primary cell lines from caudal fin, kidney, spleen and gill tissues of common carp.

2. Materials and Methods

2.1 Fish sample

Healthy common carp with a bodyweight of 20g was obtained from the Cijeruk Fish Seed Center, Bogor was used in the present study. The fish showed no clinical signs of diseases externally and internally.

2.2 Primary cell culture

The development of primary cell lines was performed according to a protocol developed by Wolf & Quimby (1976) with a slight modification. Firstly, the fish was euthanized by keeping it in cold water for about 15 min and killed by cutting the spinal cord. Thereafter, primary cell cultures were initiated by aseptically collecting caudal fin, kidney, spleen, and gill tissues. The tissues were immersed in a 5.25 % chlorine solution, washed and brought to a cell culture room. Further work was carried out in biosafety cabinet class II.

The tissues were minced with a sterile blade and scissor and transferred into PBS^{+} solution containing 200 mL PBS (Gibco USA), 10 uL penicillin/streptomycin and 10 uL kanamycin (gibco USA). The tissues were then washed with PBS + solution until the solution appeared clear, followed by incubation at 28°C for 1.5 hours. The PBS++ solution was discarded out and new culture media that containing 50 ml PBS and 50 mL trypsin 0.25% (Gibco USA) was added. The step was repeated twice. The solution was stirred while adding 20 mL of a new PBS ++ solution and incubated for one night at 4° C while stirring at low speed until the solution became murky. Thereafter, 3-5 drops of serum (FBS, Gibco USA)) was added, followed by centrifugation at 3,000 *g* for 5 min at room temperature. Cell pellets were dissolved in 6 ml PBS+ solution and explanted into 25cm^2 tissue culture flasks individually (caudal fin, gill, kidney and spleen) which were previously filled with primary cell culture medium (Leibovitz's L-15 (Gibco.USA) supplemented with 20% Fetal Bovine Serum (FBS), 250 IU penicillin, 250 mg / mL streptomycin, 250 mg / mL kanamycin sulfate and 2 mM L-glutamine. Each flask was inoculated with 15 mL/flask and then incubated at 28°C. Observations were carried out every 24 hours to investigate cell growth using an inverted microscope. When the initiation of cells in the flask was observed, then the media was replenished (Table 1).

2.3 Passage and Cell Culture

The passage was performed when the cell confluence in the flask was more than 50%. One flask in the first passage became two flasks (a split ratio of 1: 2). Cells grew and developed from the nutrients which were available in each media. The next passage was done with a split ratio of 1: 3. The passage was carried out by firstly removing the culture media, followed by rinsing the cells twice with PBS solution. In the new flask, one mL of 0.25% trypsin solution was added and the solution must touch all cell surface areas. Then, culture was incubated for 0.5-1 min until the cell layer appeared white (opaque). The solution was discharged and incubated again for 1-5 minutes. Separation and release of cells from the flask were done by gently tapping the flask. Cells that had been separated from each other were dissolved in 6 mL culture media. When inserting media, the pipette tip should directed the base of the flask where the cell grows to optimize the release of cells at

Table 1. Treatment on primary cell culture of gill, caudal fin, spleen, and kidney.

Day	Treatments			
	Culture of gill cell line	**Culture of caudal fin cell line**	**Culture of kidney cell line**	**Culture of spleen cell line**
1	Cell inoculation in flask	Cell inoculation in flask	Cell inoculation in flask	Cell inoculation in flask
4	Observation of cell attachment on a flask	Observation of cell attachment on a flask	Observation of cell attachment on a flask	Observation of cell attachment on a flask
12	Replenishing 50% of cultured media with new culture media	Replenishing 50% of cultured media with new culture media	Replenishing 50% of cultured media with new culture media	Replenishing 50% of cultured media with new culture media
17	Passage 1	Passage 1	Passage 1	Passage 1

the base of the flask. Then the cell suspension was divided into 3 flasks (containing 2 mL/flask). The flasks were labeled (Cell Name, Passage Number, Passage Date) and incubated at 28°C in an incubator. Growth was observed every day.

2.4 Staining procedures

The cell staining procedure was carried out according to the protocol of Freshney (2005). In brief, the culture media were removed, and cells were rinsed with 5 mL PBS. PBS: Methanol (1: 1) solution was added to the flask as much as 5 mL, allowed to stand for about 2 min then part of the solution was replaced with 5 mL ethanol and incubated for 10 minutes. The step was carried out 2 times. At this stage, cells could be stored or immediately stained. If stored, it must be rinsed with methanol before staining. For the next step, two mL of Giemsa coloring put into a flask and incubated for 2 min then added 8 mL Aquadest and incubated again for 2 min. The solution was discarded and rinsed with water until the excess color was disappearing. Cells were rinsed with distilled water and observed under an inverted microscope.

3. Results and Discussion

The results showed that the cell lines of gill, caudal fin, kidney and spleen tissue of carp grew well in Leibovitz's L-15 media supplemented with 20% Fetal Bovine Serum, Penicillin 250 IU, Streptomycin 250 µg / mL, kanamycin sulfate 250 µg / mL and L-glutamine 2 mM and incubated at 28°C. After 4 days, the primary cell of gill and the caudal fin was begun to be observed in the flask (Figure 1), indicated by the attachment of those cells on the flask. Meanwhile, the primary cell of the kidney and spleen were unable to grow indicated by fewer cells which were attached to the flask, and many-of them were floating.

In the primary culture of the kidneys and spleen on the 12th day, media replacement was performed. Although cell initiation has taken place and cells began to spread on the surface of the flask, its development was not perfect. According to Sumiati *et al.*, (2009), medium replacement which was too soon in which the cells had not completely attached to the flask, was thought to be the cause of the cells not growing properly and eventually dying.

The first passage was performed on the 17th day for gill and caudal fins when confluence exceeded 85% in gill flasks and 70% in the flask of caudal fins with a split ratio of 1: 2 (Figure 2). While the passage could not be performed in the primary culture of the kidneys and spleen due to the incomplete growth even on day 17 after culture. The next passage was carried out every three weeks with confluence reaching 70% -80% with a split ratio of 1: 3. In the next passage, the concentration of Fetal Bovine Serum media reduced from 20% to 10%

To keep the primary cell alive, a passage was performed by replacing a half or a 100% old growth medium with a new medium (Paul, 1972; Malole 1990). The culture of cells by in vitro requires several media for their growth. The first passage was carried out on day 17. Confluence was reached on day three on the first passage of the primary culture of gill and caudal fin, which reached 80%. Cell growth is a process of cell division where after one cell is divided into two. Each cell will experience cell growth and then become mature and ready to multiply again (Juwono, 2000).

The culture of primary cells of gills, caudal fin, spleen, and kidney occurred initially on day 2 (Table 2). The growth was seen from the confluence and continue to increase so that it can be passage. As for the culture of kidney and spleen cells, even though cell initiation

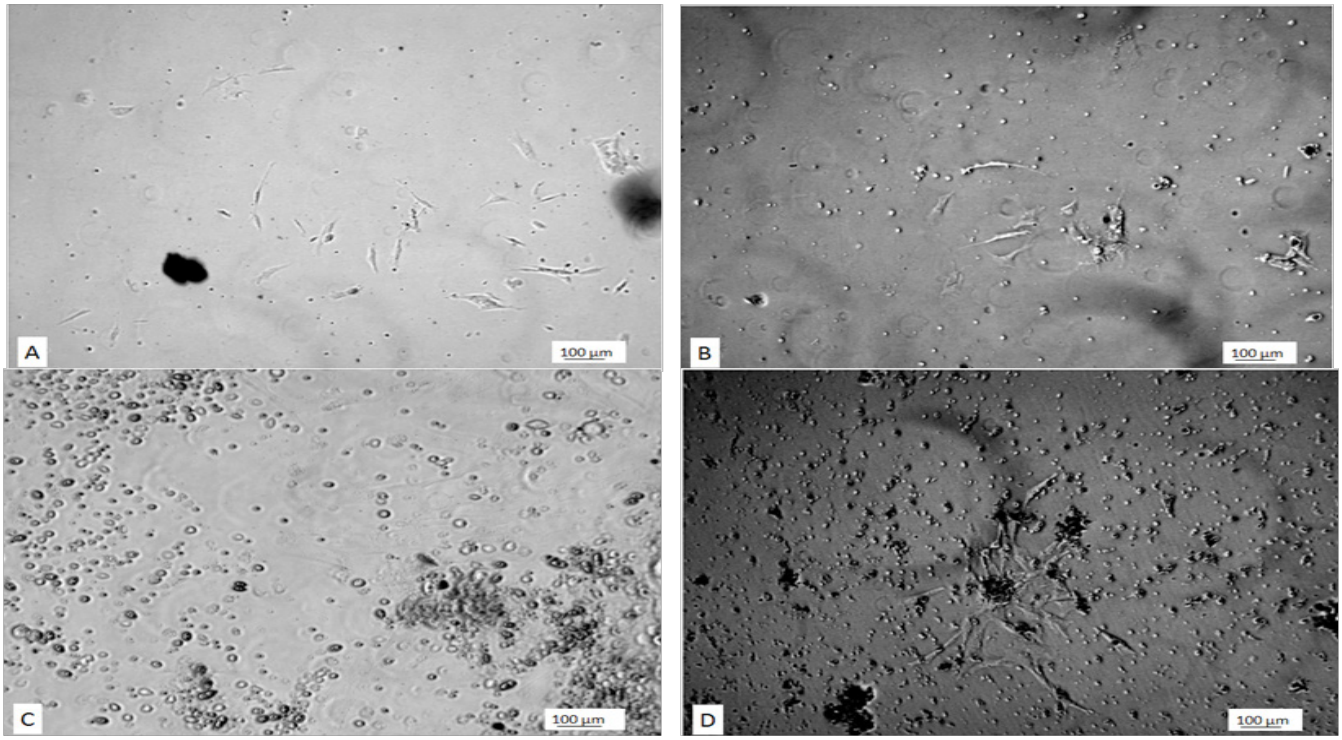

Figure 1. Cell growth on day 4. (A) primary cells of gill; (B) primary cells of caudal fin; (C) primary cells of the kidney; and (D) primary cells of spleen growth on Leibovitz's L-15.

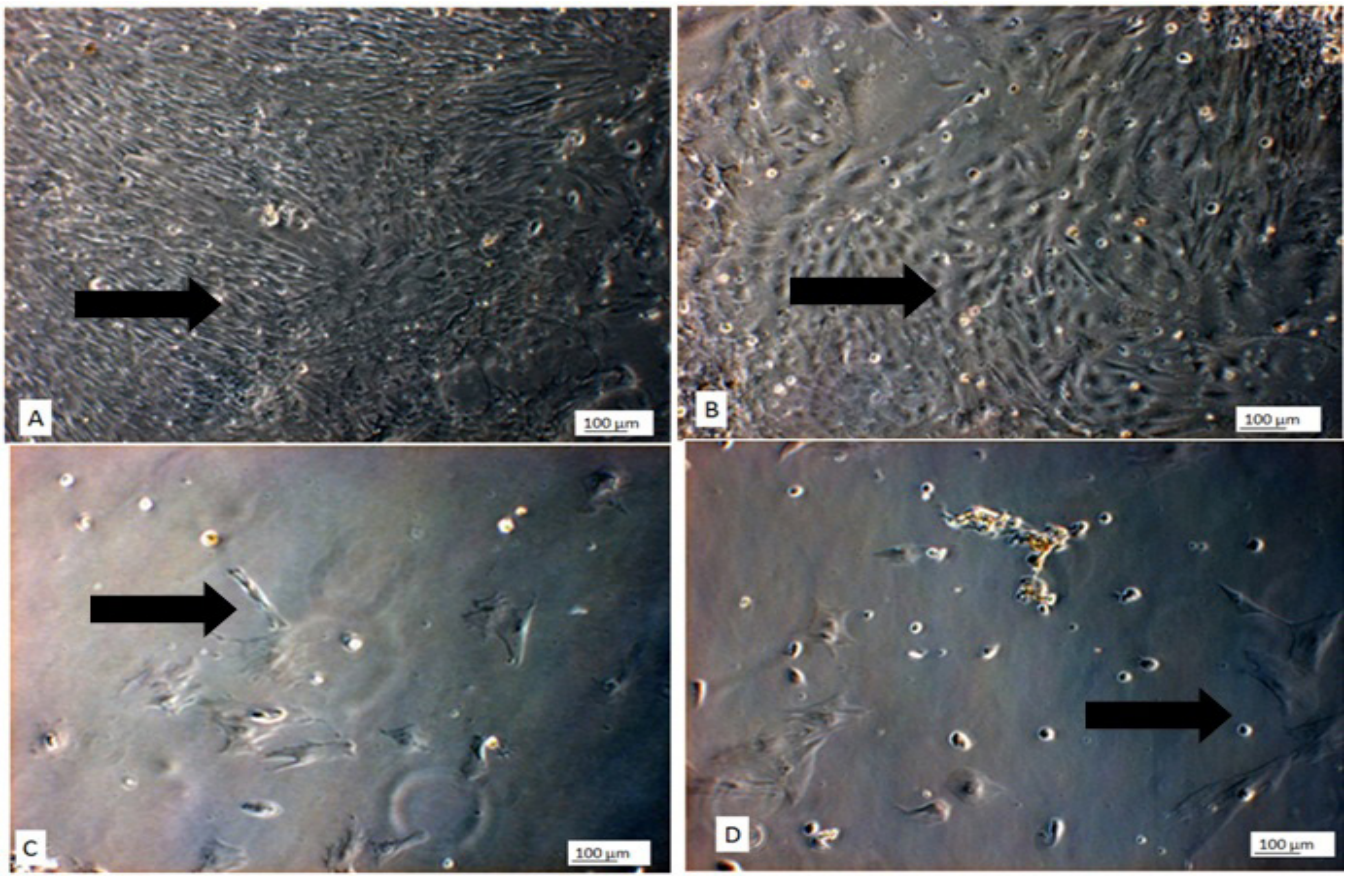

Figure 2. the first passage on day 17. (A) A monolayer of primary cell culture of gill; (B) monolayer of primary cell culture of caudal fin; (C) growth of primary cell culture of the kidney; (D) growth of primary cell culture of the spleen.

has occurred and the cells have spread to the surface of the flask, the development is not optimal to be a monolayer and eventually died. Culture media in the flask culture of gill and a caudal fin which had been changed and washed with PBS, showed cell development. In the culture of kidney and spleen cells that were attached to form a single cell. This results in the cell not growing completely and eventually dying prematurely. Cells that originated directly from organs, namely primary cells, can only grow if they are attached to the surface of the flask (Malole, 1990).

The main factor affecting the growth of cells is culture media (Phelan, 1998). The are several growth media which are required for culturing cells by in vitro (Sugiri, 1992). Media is the living place of cultured cells, therefore the composition of the media influences cell growth. Media that are needed in culture generally requires a composition suitable for a particular cell (Suprapto and Nunik, 2010).

There was a time difference in the two cultures in achieving a minimum density for a passage. Gill cell culture on day 16 reached 75% confluence. Whereas in the caudal fin primary cell culture on the 16th day reached 50% confluence. The confluence of gill primary cell culture was faster than caudal fin primary cell culture. This occurred because the cell types attached to the gill primary cell culture were more numerous than the caudal fin primary cell culture

The growth of primary cell cultures that make up the monolayer occurred in gill and fin cell culture. The confluence of gill primary cell culture and caudal fin primary cell culture can reach more than 50% at the 17th passage so that it can be done passage. According to Sumiati et al. (2009) passage was only done after reaching a growth rate (confluence) of more than 50%.

Culturing of gill and caudal fin cells was successfully performed until 72 passages with a confidence level of 80% -100% (Figure 3). Gill cells can grow and multiply properly. However, gill cell culture after the 60th passage showed a slowdown in growth. After 72 passages, the primary gill culture does not develop again and eventually died. While in the caudal fin primary cell culture, the passage has been done 89 times. Furthermore, the caudal fin cell was stored at 10% DMSO in liquid nitrogen.

Identification of cell types was done using a microscopic observation and Giemsa staining, according to Freshney, (2005). The result showed that the primary caudal fin cell culture and primary gill cell culture consisting of two cell types: fibroblast-like and epithelial-like cells (Figure 4). At the beginning of the growth, cell types appeared fibroblast-like cells. Then the passage was performed and it was also found the epithelial-like cell types. The dominant growth of fibroblast-like cells occurred at the beginning of growth, while epithelial-like cells at the beginning were not observed.

Table 2. Development of primary cells in culture flasks

Day	**Treatment**			
	Culture of primary gill cells	**Culture of primary caudal fin cells**	**Culture of primary kidney cells**	**Culture of primary spleen cells**
2	Cell initiation division	Cell initiation division	Cell initiation division	Cell initiation division
4	Cell began to develop in several places	Cell began to develop in several places	Cell began to develop in several places	Cell began to develop in several places
5	Monolayer development reached confluence at 45%	Monolayer development reached confluence at 25 %	Monolayer was not developing	Monolayer was not developing
14	Monolayer development reached confluence at 65%	Monolayer development reached confluence at 40%	Monolayer was not developing	Monolayer was not developing
16	Monolayer development reached confluence at 75%	Monolayer development reached confluence at 50%	Cells died	Cells died
17	The fist passage after confluence reached 85% until passage-72	The first passage after confluence 70% and kept developed until passage-89		

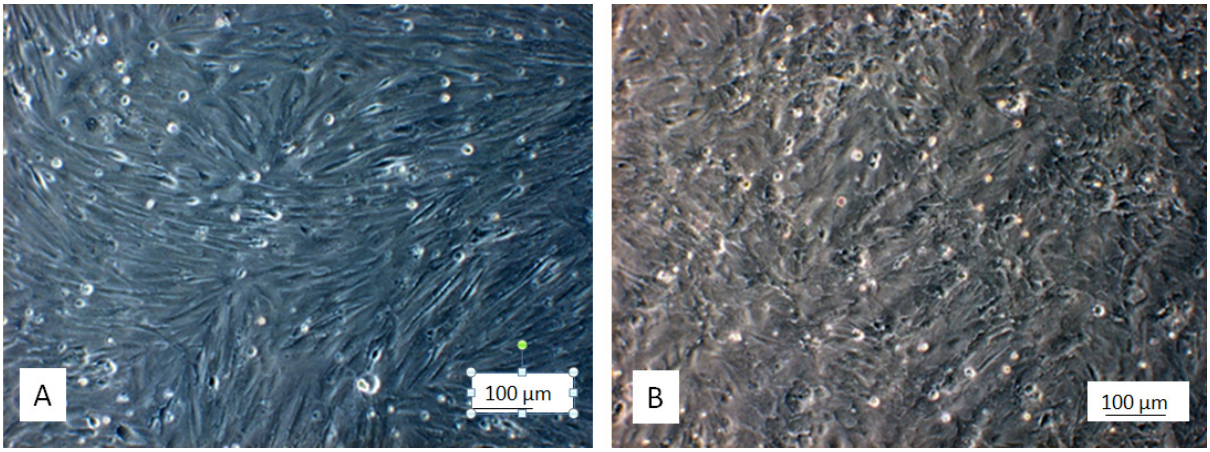

Figure 3. (A) primary gill cell culture reaching cell growth 80% forming monolayer; (B) primary caudal fin cell culture with confluence of 100 %

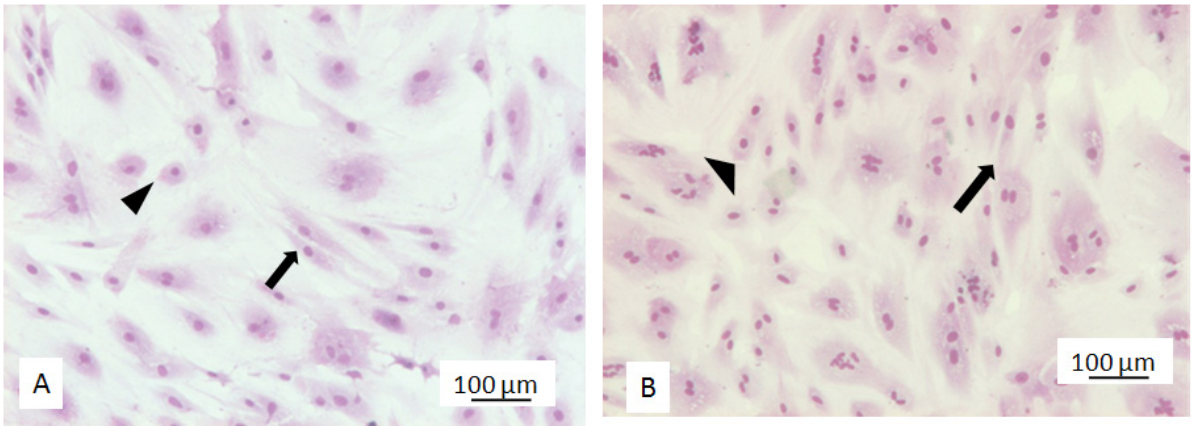

Figure 4. Morphology of cell after staining with Giemsa viewed under an inverted microscope. Arrows (▲) are *fibroblast- like*, small arrows (⬆) are *epithelial- like*).(A) primary gill cell culture ; (B) primary caudal fin cell culture.

These results are in accordance with Gibco Cell Culture Basics (2010), which stated that cultured cells could be divided into three categories based on their shape and profile, fibroblastic-like cells with bipolar or multipolar characteristics, elongated shape and can grow attached to the substrate. *Epithelial-like cell* has a polygonal shape with regular dimensions and can grow attached to the substrate. *Lymphoblast-like cells* were round in shape and commonly grow in suspension without sticking to the surface of the substrate. Primary cell culture consists of various cells originating from their original organs (Paul, 1972; Malole, 1990; Freshney 2005).

The gill and caudal fin cells, which have been maintained for seven years, can still be developed and passaged for 72 and 89 times with a confluence of about 80% in the first passage. From the first passage to the 5th passage, gill and caudal fin cells required 3 weeks for each passage. The media used in the primary culture as quite similar to media for maintenance, in which only the serum level used was reduced to 10%. According to Sumiati et al., (2009), growth media for producing primary cell culture should contain 20% serum levels to stimulate the growth of cells until it developed into a monolayer. Moreover, for cell maintenance, the serum can be as much as 10%.

A passage is required to prevent cell death due to running out of nutrients. The more cell culture is passaged, the shorter the time interval required to grow and the development of the cells. This fact is very similar to what had been observed in the present study in which the growth of cells (gill and caudal fin) was much faster at passage 5 compared to previous passages. From passage 6 to 72, cell of gill and caudal fin required one week for every passage. The culture of caudal fin cells could be passaged at 89 times.

According to Suprapto and Nunik (2010), cells of fish culture can grow in several media, including

L-15, MEM (Minimum Essential Medium) and TCM (Tissue Culture Medium). The fastest and best growth of fish cell culture was reported in L-15 media with 20% Fetal Bovine Serum (FBS) (Kalamendra 2010; Lakra et al., 2006). Many researchers also reported on the suitability of L-15 for cell culture compared to other media (Fernandez et al., 1993 Lai et al., 2000; Kumar et al., 2001; Lai et al., 2003; Lakra et al., 2006; Hameed et al., 2006; Ye et al., 2006; Qin et al., 2006; Suprapto and Nunik, 2010), because L-15 is designed to maintain pH in physiological ranges under normal atmospheric conditions without adding CO_2. Similarly, the present study also showed that cell cultured in L-15 medium at pH 7.4 had faster growth and multiplication. The optimal growth temperature was found at 28 ° C. This result was the same as previous studies using fish cells (Tong et al., 1997; Lakra et al., 2006b)

4. Conclusion

Primary cell lines have been established from gill and caudal fins of a common carp. Primary cell lines of caudal fin and gill which grew after growth medium changing on day 12. While the primary cell of kidney and spleen cells could not be developed and eventually died. The gill and caudal fin cell lines were successfully produced using a specific tissue culture medium which was Leibovitz's L-15 (Gibco.USA) supplemented with 20% serum, 250 IU penicillin, 250 µg / mL streptomycin, 250 µg kanamycin sulfate, and 250 mg / mL L-glutamine. Based on the cell morphology, the primary cell line of gill and caudal fin consisted of two cell types: fibroblast-like cells and epithelial-like cells. The primary gills were successfully passaged until 72 times. While the primary fin cells were successfully passaged for 89 times. The primary cell line of caudal fin had been successfully produced and maintained which later can be used for viruse propagations and other biotechnology assays.

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Authors' Contributions

All authors have discussed the results and contributed from the start to the final manuscript; Insariani, Trisniaty and Firma: responsible for the overall data collection and writing the initial until final draft of this paper. Freddy Riatmono: responsible for some data collection, particularly secondary data, and finding discussions. Insariani, Trisniaty, Firma, Freddy Riatmono, Abdul Ghani Amri Siregar: besides being involved in a few field works, they are also involved in the discussion of this paper.

Conflict of Interest

The authors declare that they have no competing interests.

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