USE OF PRIMARY QUAIL EMBRYO FIBROBLAST CELLS FOR PROPAGATION AND ASSAY OF AVIAN VIRUSES

MAYADA F. HASOON*, MAJED H. MOHAMMED AND JALADET M.S. JUBRAEL
Scientific Research Center, Faculty of Science, Duhok University,Kurdistan Region, Iraq
* Corresponding author: mayada.hasoon@uod.ac

ABSTRACT. A primary fibroblast cells from embryos of brown quail *Coturnix ypsilophora* has been established and partially characterized. The cells were maintained in Modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum. The cells were able to grow at temperatures between 35°C and 38°C with optimum temperature of 37°C. The growth rate of primary quail fibroblast cells increased as the FBS proportion increased from 5% to 20% at 37°C with optimum growth at the concentrations of 10% or 15% FBS. The cells showed no microbial contamination throughout the period of experiment and the total chromosome number of a diploid cell was 78, according to karyotyping and chromosome analysis.

The susceptibility of quail primary cells for avian viruses was investigated in this study after inoculation with ND and IB viruses. Both viruses showed a satisfactory CPE development and the infectivity were assayed by virus titration ($TCID_{50}$). This suggests that the quail primary cells can be used for isolation of various avian viruses with further steps of infectivity confirmation in the future.

INTRODUCTION

Although embryonating eggs can support the growth of a broad range of avian viruses, many field viruses do not readily grow in eggs. Therefore, obtaining a sufficient number of reliable, high-quality eggs to avoid false negatives (sample mishandling) is a considerable limitation in their use. Many attempts have been made to find suitable alternatives to the use of eggs for avian virus isolation and research purposes. However, all the previously studied cell lines for avian viruses were mammalian in origin, and limitations (including the restricted host specificity of the cell and a possible change in receptor specificity) arise when using a mammalian cell line for avian virus study. Thus, it would be ideal to use a well-established avian-origin cell line for avian virus study.

Infectious bronchitis virus (IBv) infects the respiratory tract, kidneys and oviduct of poultry of all ages, causing delay in growth, mortality, reduced egg production and poor egg shell quality and in many countries the disease remains one of the main problems affecting existing or

developing poultry industries. Until now, there is no cure for the disease (OIE, 1996). Prevention is to import birds from disease-free flocks only or through vaccination. broilers are normally vaccinated at 1 day of age with live attenuated vaccines (Cavanagh & Naqi, 1997). In addition, breeders and egg layers are also vaccinated at approximately 8 week intervals with live attenuated vaccines, and with inactivated vaccines after they start laying eggs (Cook *et al*., 1999).

NDv causes Newcastle disease (ND), which is an acute highly contagious viral disease of domestic poultry as well as other species of birds regardless of age and sex variation (Alexander, 2003). According to the strain variation of NDV, the rate of morbidity and mortality of poultry in a flock due to ND varies from 90-100%, thereby poultry industry all over the world facing serious economic losses every year.

However IBv and NDv infections can also be diagnosed by detection of viral RNA, which make the diagnostic rapid and also dependable (Capua *et al*., 1999; Cavanagh, 2001), but researchers used to produce the virus by growing in embryonated chicken eggs and this takes long time and requires large area for egg incubation. Thus, cell cultures considered are more suitable and less expensive than eggs and also convenient to inspect microscopically for indication of viral proliferation (Dhinakar & Jones, 1997; de Wit *et al*., 1997). There is therefore a serious need to improve IB virus isolation technologies based on quail fibroblast cells (QFC).

In this study, the authors evaluated the characteristics of quail fibroblast cells to investigate their ability to support the growth of avian viruses

MATERIALS AND METHODS

The 8-day-old embryos of quail in this research were provided by chicken breeding farm of faculty of Agricultural, Dept. Animal Production, Duhok Univ. Kurdistan Region, Iraq. MEM (Gibco, USA), special grade fetal bovine serum (Biochrom, German), DMSO (Sigma, USA), Hoechst 33258 (Invitrogen, USA) were used in this study. Reference strains of IB (QX strain) and ND (AG68,1968 *Iraq*. VI. 4a. AF001108) viruses were kindly provided by Al-Kindi Company for *Veterinary* Vaccines & Drugs, Baghdad, Iraq. This study was performed and accomplished at Animal Biotechnology Lab., Scientific Research Center/Faculty of Science/ Duhok Univ.

Cell culture

The quail eggs incubated for 8 days were sterilized using alcohol swabs, and then the embryos were isolated and washed three times with phosphate buffered saline (PBS) supplemented with high level of antibiotic (3% Penicillin-Streptomycin). The embryos were cropped into pieces of 1 mm^3 in size and seeded onto the surface of 25 cm^2 tissue culture flask, and cultured at

Table 1. Media evaluation study. Three types of commercial media were used to evaluate the growth of quail fibroblast cells

serial number	Type of media
1	Basal media of Minimum Essential Medium (MEM Eagle M0268 Sigma®, UK)
2	Roswell Park Memorial Institute (RPMI media-1640 (Sigma-Aldrich®, Germany)
3	DMEM - Dulbecco's Modified Eagle Medium (Gibco® DMEM)

37°C in a humidified atmosphere, 5% CO_2 for 1 to 2 hrs. Modified Eagle's medium (MEM) containing 10% fetal bovine serum (FBS) was added into the flask. The medium was refreshed after 1 to 2 days. The cells were harvested at 80% to 90% confluence using 0.25% trypsin (m/v) solution and were seeded again into culture flasks at the ratio of 1:2 as passage 1 (Guan *et al*., 2005; Zhou *et al*., 2004).

Culture media optimization

Three different media (MEM, RFLP and DMEM) were used to evaluate the growth of quail fibroblast cell (QFC) cultures according to the method of Wolf and Quimby (1976) (Table 1). All media were supplemented with 10% FBS, 1% (100 IU/ml) Antibiotic (Penicillin-Streptomycin), 1% (100 IU/ml) antifungal-Nystatin. A total of 6 wells of tissue culture plate were seeded with new cells at a density of 10^6 cells/ml at initial P1 passage. Duplicate wells of each treatment were treated with different media. 1 ml of old media was removed and replaced with fresh media every next day during the first week of growth period. The cells were trypsinized and the efficiency of each medium was evaluated by the number of viable cells/ml of cell suspension.

Temperature optimization

MEM complete growth media (15% FBS, 1% Penicillin-Streptomycin and 1% Nystatin-antifungal) was used as base medium in this study. Temperature required for growth of quail primary cells was optimized according to the method described by Wolf and Quimby (1976). Cells were cultured in multi-well tissue culture plate with seeding density of 10^6 cells/ml and incubated at 37°C for 24 hours to allow for attachment. Later, each plate with attached cells was incubated in a selected temperature of 35, 37 and 39°C respectively. Every two alternate days, cells at each temperature were counted to determine the best growth. The numbers of viable cells were expressed as cells per ml.

Serum optimization

The optimal fetal bovine serum (FBS) percentage for the growth of primary

quail cells at initial P1 passage was assessed following the technique of Wolf and Quimby (1976). Cells were plated at density of 10^6 cells/ml in multi-well tissue culture plates and incubated at 37°C for 24 hours to allow cell attachment. MEM complete growth media with different percentage of FBS (5%, 10%, 15% and 20%) were selected for cell growth test. Every two alternate days, wells at each FBS percentage were trypsinized and the cells were stained with trypan blue and counted using haemocytometer chamber. The cell concentration was expressed as cells/ml of culture medium.

Microbial detection

Detection of bacteria and fungi

The cells were cultured in complete MEM media free of antibiotics and observed for the presence of bacteria and fungi at 3 days after subculture according to the method described by Doyle *et al.*, 1990).

Detection of viruses

Hay's hemadsorption protocol was used to examine the samples for cytopathogenesis using phase contrast microscopy (Hay *et al.*, 1992; Wu *et al.*, 2008).

Karyotyping and chromosome analysis

Chromosome spreads were prepared, fixed and stained following standard methods (Costa *et al.*, 2005). Cells were harvested when 80 to 90% confluent, and subjected to hypotonic treatment and fixed, then the chromosome numbers were counted from 100 spreads under an oil immersion objective lens upon Giemsa staining. Chromosome distribution was calculated according to the protocol described by Sun *et al.* (2006).

Virus inoculation

The stocks of IB and ND viruses were originally obtained from Al-Kindi com.for vaccines development in Baghdad. Viruses were isolated from the allantoic fluid of embryonated chicken eggs. Initially, 0.3 ml of the virus stock was diluted in 30 ml of 10X PBS giving a dilution of 1:100. The diluted viruses were used to inoculate QFC cells. 85-90% confluent flasks of cells were used for virus passage in an attempt to adapt the virus to replicate in these cells and show CPE.

Tissue culture infective dose 50 ($TCID_{50}$)

The infectivity of replicated IBV and ND to QFC cells were determined by calculating 50% end point, as described by Reed & Muench (1938). Ten-fold serial dilution of each virus (NDv and IBv) was prepared in PBS from 10^{-1} to10^{-10}. A 96 multi-well tissue culture microtiteration plate (Titertek, UK) was used to prepare QFC cells monolayers. 100µl of each virus dilution was added in each well of first row leaving last two wells as negative

control. The plate was incubated at 37°C for 1 hour to allow adsorption. Then 100µl of prewarmed maintenance medium (MEM+2%FBS) was added in each well and again incubated at 37°C in 5% CO_2. The plate was observed daily for CPEs. The highest dilution of virus showing 50% CPEs was considered as end point to calculate $TCID_{50}$

RESULTS

Morphological observation of quail fibroblast cells

Cells derived from trypsinized tissue of quail embryo muscles were well attached and started to differentiate after one day of being placed on the bottom of tissue culture flasks (Figure 1A), and then they continued to proliferate and were subcultured when reached 80 to 90% confluent within about 5 days (Figure 1B & C). The cells displayed typical fibrous and fusiform morphology. less than 50% of the cells shown cuboidal epithelial-like appearance, while the fibroblasts grew rapidly and replaced the epithelial cells gradually after 2 passages, and then a relatively purified fibroblast line was obtained (Figure 1). The viability of quail fibroblasts evaluated through trypan blue exclusion tests was 85%.

Optimum media composition

Comparison between three different media of MEM, DMEM and RFLP revealed the best growth for QFC cells maintained in

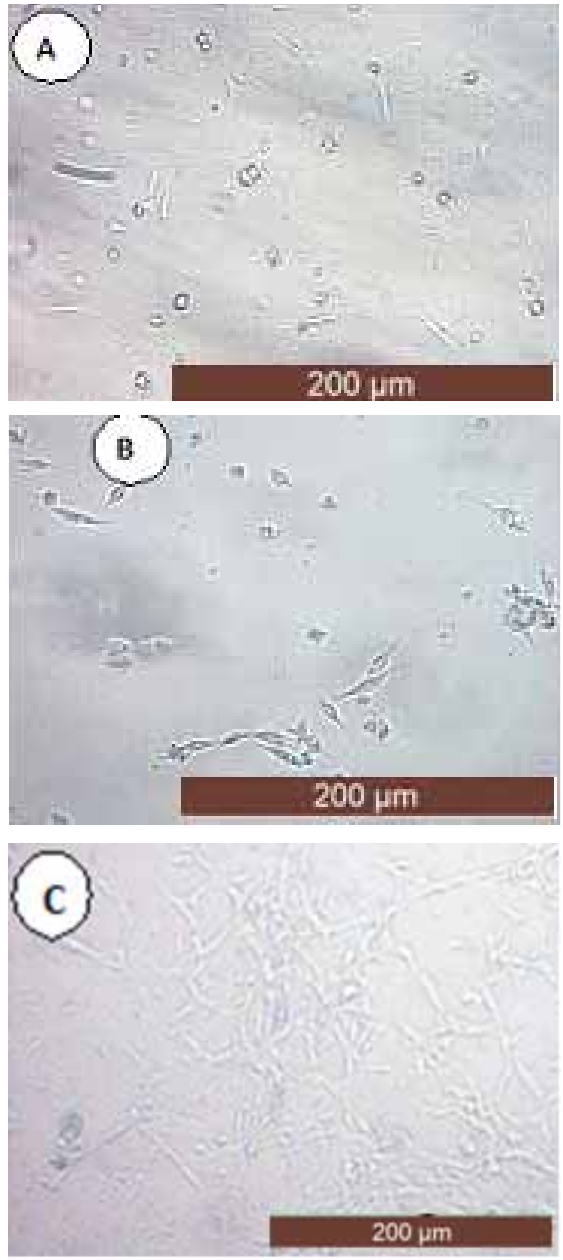

Figure 1. Morphology development of QFC .Primary cells grew and differentiated from the trypsinized quail embryo body muscles; (A) 1 d after initial culturing; (B) 3 ds after culturing; (C) 90% confluent monolayer in about 5 ds after culturing. Unstained cells. 200µm.

Table 2: Culture media used to evaluate the growth potential of primary QFC cells

Medium	Cell viability and confluency	No. of days to reach confluent
MEM	+++	7
DMEM	+	14
RPMI	+/-	-

(+++): Excellent growth, <10^6 viable cells, <80% confluence
(+): Medium growth, 10^3 viable cell, 20-40% confluence
(+/-): Little growth, 10-10^2 viable cells
(-): No growth, no confluence

MEM media. QFC cells seeded at initial density of 10^6 cell /ml and maintained with complete growth MEM media exhibited a high yield of viable cells (7x10^6 cell / ml) and reached confluence within 7 days (Table 2). Cells that were maintained with DMEM complete growth media comprised cell viability of 10^3 cell /ml and reached confluence after 14 days. Cells that grew in RPMI complete growth media showed low viability of 10^2 cells /ml and no confluence was achieved.

Optimum temperature

QFC cells maintained in MEM complete growth media and seeded at initial density of 10^6 cell /ml exhibited different growth levels at different temperatures of 35°C, 37°C, and 39°C . The cells were able to grow at temperature range from 35 to 39°C. However, maximum growth with high viable cells (6.5 x10^6 cell /ml) was obtained at 37°C after 7 days post- culture (Figure 2).

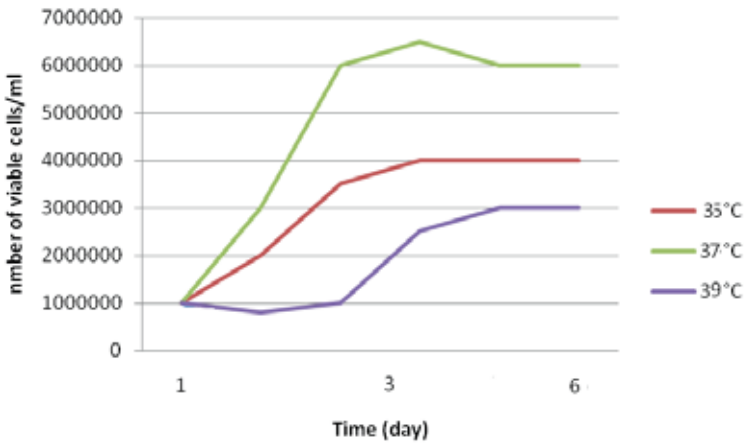

Figure 2. Various incubation temperatures used to evaluate potential growth of QFC cells. Optimum growth was achieved at 37°C while at 35°C, and 39°C the cells showed less growth.

Optimum serum concentration

QFC cells seeded at initial density of 10^6 cell /ml and maintained in MEM media supplemented with different levels of serum (5, 10, 15 and 20%), were exhibited viable cell yield of $9x10^6$ cell /ml on day 6^{th} post- seeding at level 20% serum. Cells maintained in media supplemented with 15 and 10% serum showed less in number of viable cells yield ($6 x10^6$ and $5.5 x10^6$ cell /ml respectively) at day 6^{th} post-seeding. Only $0.8 x10^5$ cell /ml viable cells obtained when cells maintained in media supplemented with 5% FBS.

Microorganism detection

The medium was clear all the time and no abnormalities were observed under the microscope. As is shown by the cytopathogenic evidence for virus contamination was also negative. The results indicated that the QFC were free of bacterial, fungal and viral contamination.

Chromosome number

In this experiment 100 representative spreads at metaphase of passage 1 to 3 were observed under the microscope to count the chromosome number of diploid QFC, including the mean proportion of diploid cells which was 78±1% (Figure 3).

Cytopathic effect (CPE)

Infected cells monolayer of QFC was steadily became rounded and broken as the virus proliferates to occupy extra cells in culture. In the first and second passage, the infectivity of both viruses (IB & ND) were sluggish and not very clear as the virus was just begin to adapt on cells. Through the third passage, CPE was rapid and changes have been recognized within

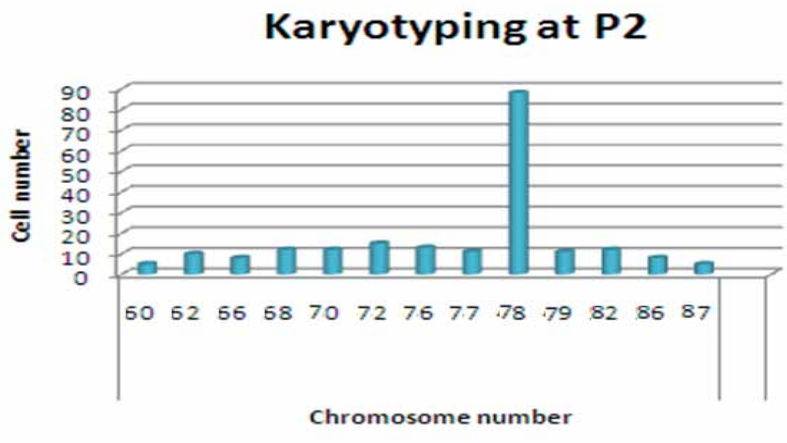

Figure 3. Chromosome analysis of primary quail fibroblast cells showing the chromosome number distribution at passage P2 with model peak at 78

Table 3: Virus titer determined by TCID50

Virus category and passage level	Virus titer TCID50/ml	
	IBv	NDv
1	4.5	2.5
2	6.5	3.5
3	7.4	4.7

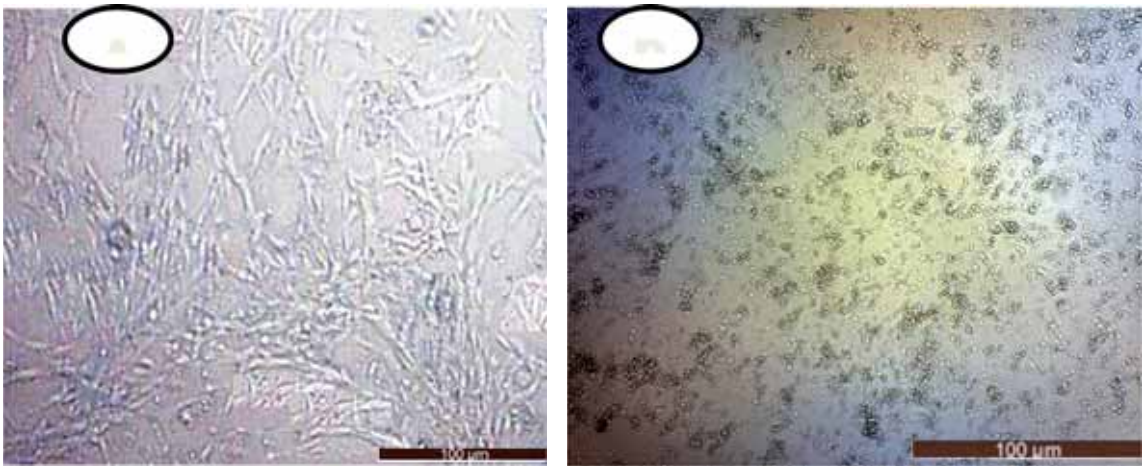

Figure 4: Cytopathology produced by IBv. infection in quail fibroblast cells 48hrs postinoculation.100µm.

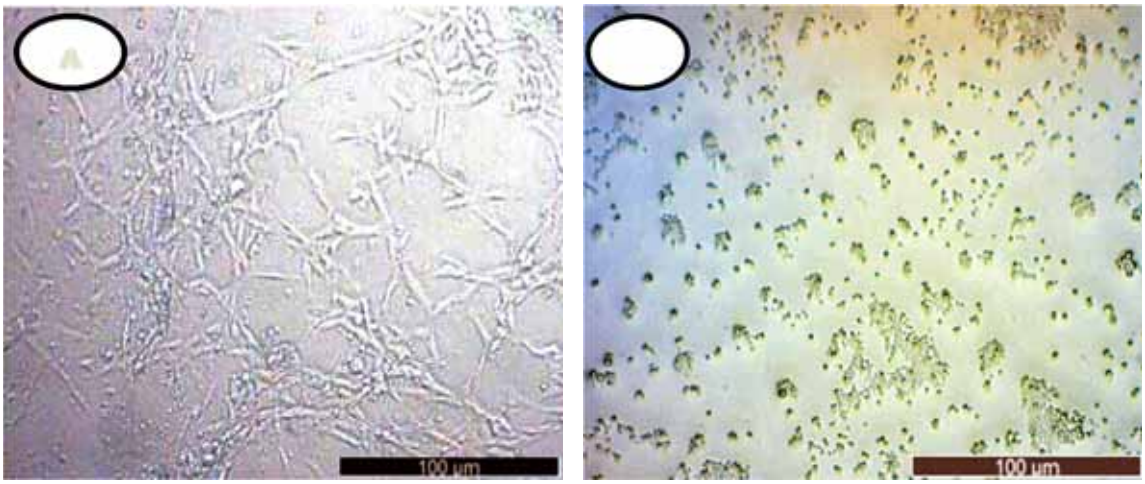

Figure 5: Cytopathology produced by NDv. infection in quail fibroblast cells 72hrs postinoculation. x 100µm.

two days (in compare with the control). It was characterized by rounding of cells, failure of adhesion, vacuolization in cells, clustering of infected cells (Figures 4 & 5).

$TCID_{50}$

The infectivity titre of both viruses was found to increase gradually from the 1st to the 3rd passage. The $TCID_{50}$ titer was 7.4 for IBv and 4.7 for NDv after 3 passages (Table 3)

DISCUSSION

Quail embryonic fibroblasts (QFC) were selected in this experiment as they offer many advantages, such as high vitality, easy access, and low probability of bacterial and other contamination. QFCs were successfully established from 40 embryo samples by adherent culture. The biological and morphological characteristics may be altered and cells may stop dividing by *in vitro* culture after many passages, so a minimal number of passages are recommended to protect the cells against degeneration and death (Bai *et al*., 2011). Morphological observation indicated that the cells developing a fibroblast-like appearance during the primary and the first several passages of trypsinized tissues. Due to their different tolerance to trypsinization, the fibroblasts-like cells detached from the flasks earlier when digested with trypsin and adhered again quickly after passage, (Rosenberg etal., 1991). For this reason, a purified fibroblast line could be obtained after 2-3 passages.

Infectious bronchitis (IB) and ND virus, early described in 1930 (Schalk & Hawn, 1931) continues to be main causes of disease in poultry of all ages and types in all parts of the world (Anon, 1991). The disease is occurring in all countries with a concentrated poultry industry, with the occurrence of infection up to 100% in most locations (Ignjatovic & Sapats, 2000).

Among the various media tested were DMEM - Dulbecco's Modified Eagle Medium, Eagle's minimum essential medium (Eagle's MEM), and Roswell Park Memorial Institute (RPMI) medium. MEM media was found to be the most suitable for the attachment and proliferation of QFCs cells. Several researchers have reported the successful used of MEM to support the growth of poultry cells (Lakra *et al.*, 2006; Hameed *et al*., 2006). However, some primary cell lines required specific culture medium designed specifically to optimize growth during development of the primary culture (Wang *et al.*, 1995), more than 80% of the cell lines established after 1994 used MEM media.

The growth temperature range used for QFC cells in this study was 30-39°C with optimum growth at 37°C. These results were consistent with other results reported previously (Nicholson *et al*., 1987; Tong *et al*., 1997, Kang *et al*., 2003; Hameed *et al*., 2006). One of the advantages of cell lines that grow over a wide temperature range is their potential suitability for isolating wide

range of pathogenic viruses (Nicholson *et al*., 1987).

The growth rate of QFC cells increases as the FBS concentration increased from 5% to 20%. However, a 10% concentration of FBS also provided relatively good growth and this is an advantage to be recommended to maintain those cells of QFC at low cost (Ye *et al*., 2006). In addition, eliminating serum proteins, often improves product quality and/or cell growth. As Barnes and Sato (1980) suggested in their strategies for optimizing cell growth; the goal is sometimes not to maximize cell growth rate, but rather to optimize viability and growth condition, whereas serum reduction is, almost useful in meeting this goal.

The newly developed QFC primary cell line was free from contaminating microbes as detected by tests on special media as described earlier by Hopert *et al*., (1993).

The proportion of 2n = 78 cells were 98% as detected in 100 cells. Most poultry chromosomes were very small ones, which were easily lost in the preparation process and by the interference of dye, rendering the difficulty to count chromosome number and to observe the morphology. Therefore, time point and duration of colchicine administration and low-osmotic duration should be precisely controlled in experiments and must be tightly controlled within appropriate time (Ashraful, 2012).

The cytopathic effects (CPEs) appeared after 72 hours of infection in third passage and this observation was also noticed by (Hopkins, 1974), but slightly varied from findings of Mahgoub *et al.* (2010) where the IBV and ND were adapted to Vero cell line after the third passage. The difference might be due to the cell culture passage level of the virus strains used or variation in sensitivity of cell culture to different strains. The total infectious titer at passage 3 was found to be $10^{7.4}$ and $10^{4.7}$ $TCID_{50}$/ml for IBv and NDv respectively. These finding supports the previous report by (Otsuki *et al*., 1979).

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