Recent Research in Science and Technology 2009, 1(6): 294–298
ISSN: 2076-5061
www.recent-science.com

ANIMAL SCIENCES

COMPARATIVE SUSCEPTIBILITY OF DIFFERENT CELL CULTURES AND CHICKEN EMBRYO ORGAN CULTURES TO INFECTIOUS BURSAL DISEASE VIRUS OF POULTRY

Shazia Mannan[1*], Muhammad Ibrahim[1], Salman Akbar Malik[2], Khalid Naeem Khwaja[3]
[1]*COMSATS Institute of Information Technology, 520-B Civil Lines, Jail road, Sahiwal, Pakistan*
[2]*Department of Biochemistry Quaid-i-Azam University, Islamabad, Pakistan*
[3]*Animal Health Programme, National Agricultural Research Center, Park Road, 45500, Islamabad, Pakistan*

Abstract
Infectious bursal disease (IBD) is an acute highly contagious viral infection of young chickens often resulting in immunosuppression. Inactivated vaccines play significant role in protection against IBD. Mammalian cell lines could be used for producing such vaccines. In present study twenty-five, local strains of IBD virus were inoculated into chicken embryo bursa cell culture, liver cell culture, kidney cell culture, fibroblast cell culture and *Vero* cell lines for cytopathic effect. Moreover comparative susceptibility of chicken embryo bursa organ, embryo liver organ and embryo kidney organ cultures, to infectious bursal disease virus were studied. Chicken embryo bursa cell line was found to be most susceptible (90%) followed by *Vero* cell lines (70%), fibroblast cell lines (65%), kidney cell lines (50%) and liver cell lines (45%). While chicken embryo bursa organ culture gave maximum cytopathic effect (80%) followed by chicken embryo liver (60%) and kidney organ (45%). From these studies it is concluded that after bursa cell lines, *Vero* cell lines gave maximum cytopathic effect yielding high number of virus particles and are easy to maintain. Thus *Vero* cell lines can be used to produce infectious bursal disease vaccines using local isolates.
Key Words: Infectious bursal disease virus; Chicken embryo cell lines; *Ver*o cell lines; Chicken embryo organ cultures.

Introduction

Infectious bursal disease virus is a member of the genus *Avibirnavirus* in the family *Birnaviridae.* The viral particle is a non-enveloped, icosaherdal capsid with a diameter of 60nm containing two segments of double stranded RNA (Muller *et al.,* 1979).

Infection of 3-6 week old chickens with IBDV causes an acute disease (Gumboro disease), characterized by high morbidity and mortality. Surviving chickens, as well as chickens infected after hatching, develop an immunodeficiency resulting from the depletion of B-lymphocytes. This immunosuppression results in increased susceptibility to opportunistic infections, impaired growth and failure of vaccination. Acute and immunosuppressive forms of the disease have a large economic impact on the poultry industry worldwide (Annett and Hermann *et al.,* 2001).

Lymphoid cells in the bursa of Fabricious are the target cells of IBDV. Lymphoid depletion and the final destruction of bursa of Fabricious result as the predominant feature of the pathogenesis of infectious bursal disease (IBD) (Kaufer and Weiss, 1980). Besides necrosis, marked atrophy of the infected bursa of Fabricious without severe inflammatory response was also reported. This suggests the involvement of apoptotic processes in the pathogenesis of the disease. The induction of apoptosis in IBDV-infected chicken peripheral blood lymphocytes has been reported (Vasconcelos and Lam 1994). Apoptotic cell death was also observed *in Vitro* in IBD-infected *Vero* cells and chicken embryo cells. IBD infection of susceptible chickens resulted in the induction of apoptosis of cells in the bursa as well as in the thymus (Tanimura and Sharma, 1998).

[*] *Corresponding Author, Email: shazia@ciitsahiwal.edu.pk*

Chickens can be vaccinated with live or inactivated vaccines for protection against IBD. Live vaccines are used in an attempt to control the virulent form of the disease but with only partial success. The level of maternal antibodies in day-old chicks often vary widely and in the face of the persistence of IBD virus on most farms, an effective vaccine regimen is difficult to establish. Live vaccine derived from chicken embryo fibroblast (CEF) cultures or embryonated eggs of SPF chicken is expensive. Vaccines derived from non-SPF chicken may contain extraneous avian viruses or microorganism. Also live vaccine can cause severe immunosuppression, bursal damage and persistence of virus in vaccinated farm. Mammalian continuous cell lines would be suitable alternate for vaccine production. They have several advantages over the use of primary cell cultures. Maintaining a continuous cell line is more cost effective than propagating CEF cultures from SPF chicken embryos (Senthilkumar *et al.,* 2006).

Isolation and serial propagation of IBDV from field cases in cell cultures of chicken embryo origin was found to be very difficult (McFerran *et al.,* 1980). This poor adaptation of IBDV in cell cultures may be due to the strain differences in field viruses. IBDV replicates rapidly in developing chicken embryos but the virus from field cases of the disease does not always grow well in chicken embryos. Recent isolation of wild type virus has relied heavily on the use of susceptible specific pathogen free (SPF) chickens. However the expense inconvenience and risk of this method limit its use to a few research laboratories. Primary cell cultures of chicken embryo origin including chicken embryo kidney cell and chicken embryo fibroblast cells have been successfully employed for the propagation of IBDV isolates from field cases (Lukert and Davis 1974).

Materials and Methods

Isolation of local isolates of IBDV

For isolation of infectious bursal disease virus, 25 samples of bursa and spleen were collected from IBD infected flocks in different areas of Pakistan during the year 2000. Infected bursa and spleen were finely divided in sterile GKN medium (glucose (1g), KCl (0.2g), NaCl (8g), phenol red 1% (2ml), H_2O (900ml)). The suspension was then washed with GKN and minced with sterile scalpel. The homogenate was frozen and thawed three times to release viruses from the cells. The homogenate was then centrifuged at 1500rpm for 15 minutes at 10°C. The supernatant was collected and pellet was discarded. Finally supernatant was filtered and the filtrate was stored at -20°C.

Purification of IBDV

The above filtrate was then purified on a sucrose gradient by ultra-centrifugation at 63,000 x g for five hours at 4°C. The virus band was collected using syringe. The collected material was dialyzed against TNE (Tris. NaCl. EDTA) buffer overnight at 4°C using molecular porous membrane tubing. The dialyzed material was recovered and centrifuged at 50000 x g for 5 hours at 4°C. The pellet was suspended in sterile distilled water and was stored at -20°C.

Chicken embryo kidney, liver and bursa cell culture

Eighteen days old embryonated eggs were taken and after confirming the viability of embryos by candeling they were chilled by placing the eggs in a refrigerator for one hour. The eggs were then placed in egg holder with the pointed end downward and the broader end was wiped thoroughly with 70% ethanol. The eggshell was removed from the broader end closer to the chorioallantoic membrane. The opaque shell membrane was removed with the help of sterile forceps and scissors and the embryo was removed aseptically from the shell into petridishes. The internal organs were removed and placed in petridishes containing GKN with antibiotics. The organs were minced finely with the help of scalpel and washed thrice with GKN. The minced tissue was transferred to a flask to which 1% warm trypsin solution (10 ml/embryo organ) was also added. The flasks were placed on a hot plate for 40 minutes at 37°C. The mixture was gently stirred throughout the incubation using a magnetic stirrer.

The trypsinized tissue was discarded and the pellet was suspended in 5ml of Eagle's Minimum Essential Medium (E-MEM) (Dried medium 4.8g, soda water 380ml, non essential amino acids 5ml, sodium pyruvate, lact albumin hydrolysate 10ml, HEPES (1M) 7.5ml, serum 40ml, penicillin-streptomycin 1ml), with 10% v/v foetal bovines serum (FBS). After counting the cells the concentration of cells in this suspension was adjusted to 10^6 cells per ml by diluting the cells with E-MEM containing 10% (v/v) FBS. The resulting cell suspension was poured in 25ml tissue culture flasks at the rate of 7ml/flask and at the rate 1ml/ well in 24 welled culture plates. The flasks and plates were placed in CO2 incubator at 37°C. They were observed after 24 hours under inverted microscope. The contaminated flasks if any were discarded and the rest were reincubated. The medium was also changed if required in any of the flasks. The monolayer becomes confluent in each flask and plate after 48-60 hours, which was used further for inoculating the virus.

Chicken Embryo Fibroblast Cultures

Chicken embryo fibroblast culture was prepared by using 9-10 days old embryonated eggs. Briefly, in this procedure after taking out the embryo from the egg, head, limbs and viscera were removed and remaining mass was processed similarly as above.

Vero cell line culture

Precultured *Vero* cell lines at late log phase were obtained in the form of a monolayer and in order to store the seed stock the cell lines were trypsinized with 1% trypsin for 30 minutes. Then centrifuged and resuspended at approximately 10^7 cells /ml in culture media containing serum and preservative dimethyl sulphoxide (DMSO) 10%. Cell suspension was dispended into 5 ml prelabeled plastic tubes and were capped. The tubes were kept in a freezer at –20°C and then stored in liquid nitrogen container at–70°C.

In order to recover the frozen cells, the tubes were retrieved from the freezer and placed in water at 37°C. When thawed the tubes were swabbed with 70% ethanol and 10ml medium was added slowly to them, diluting the cells and preservative. After that it was centrifuged for 10 minutes at 100 x g. The supernatant medium with preservative was discarded and cells were resuspended in fresh medium for culture.

Chicken embryo kidney, liver and bursa organ culture

For preparing organ culture 16 days old embryos were used. Here the respective organs were removed and chopped into $1mm^3$ pieces. These were washed three times with GKN and finally with the help of a scalpel blade the clumps of organs were placed in the tissue culture flasks. About 1ml of E-MEM with 20% v/ v foetal bovine serum was added in each flask and incubated at 37°C for 24 hours. After that 5ml E-MEM with 10% foetal bovine serum was added to the flasks and they were further incubated at 37°C till a monolayer became apparent in the flasks, which was used for further propagation of virus.

Virus inoculation in tissue culture and organ culture and Vero cell line culture

Infectious bursal disease viral isolates were inoculated in cultured cells, in a biosafety cabinet with vertical airflow. For this media was removed from the cultured flask. Under sterile conditions near a burner flame 5ml of GKN was gently poured into the flask at the opposite side of the monolayer. After recapping the flask the GKN solution was gently swirled on the monolayer by rotating the flask and then the solution was carefully decanted. 0.5 ml of the virus preparation was poured on to the cells in the flask, which was then incubated for 45 minutes at 37°C. To the flask 5ml of E-MEM containing 2% (v/v) FBS was added and reincubated at 37°C. The flask was daily examined microscopically for the required cytopathic effects. When more than 75% CPE was observed the contents of the flask were frozen and thawed 3 times to release virus particles from the cells. The material was configured at 1500 rpm for 10 minutes at 10°C. The pellet was discarded and the supernatant was filtered using 0.2μ syringe filter. Many aliquots of the filtrate were prepared and stored at -20°C. A sample of this filtrate was tested by agar gel precipitation test for confirming the presence of virus. This was referred as passage-1 (P-1) of the virus in tissue culture.

Results and Discussion

For *in vitro* inoculation of samples, 5 types of primary cell lines were used. These were chicken embryo fibroblast, Chicken embryo kidney, chicken embryo liver, chicken embryo bursa and *Vero* cells. From the results after third passage it is clear that overall chicken embryo bursa cell lines gave maximum, cytopathic effect, followed by *Vero* and chicken embryo fibroblast. A minimum cytopathic effect was seen in chicken embryo kidney and chicken embryo liver cells. This reveals that IBD virus isolates are more susceptible to chicken embryo bursa followed by *Vero* and chicken embryo fibroblast cells. This is supported by the previous report that generally IBD viruses propagated in bursa cell lines had all structural proteins in regular proportions in contrast to chicken embryo fibroblast which tend to produce incomplete particles (Muller *et al.,* 1986).

Lukert and Davis, 1974, have also found IBD virus to be more adapted to chicken embryo bursa than chicken embryo fibroblast. *Vero* cell lines have also been reported susceptible to IBDV.

Kibenge *et al.,* (1988), studied the growth of five strains of IBDV, three strains of serotype 1 (SAL, D-78, 2512), one of serotype 2 (OH) and one variant (variant-A) in *Vero* and Chicken embryo Fibroblast (CEF) in order to characterize the replication of different strains of IBDV. For all five strains, the latent period in *Vero* cells ranged from 12-18 hours, which was longer than 1-6 hours, the latent period observed in CEF cultures for strains SAL, D-78 and OH. Virus strains SAL, D-78 and OH which was examined in both Vero cells and CEF cultures also had a more extensive maturation phase and higher yields of virus than in CEF cultures. The 2512 and variant-A strains did not replicate on CEF.

Table 1: Site of Sample Collection, Flock ID and Infectious Bursal Disease Strains isolated from the samples

S.No.	Name of Poultry Farm	Flock-ID	Strain-ID
1	Poultry Research Institute, Rawalpindi	F-1	IBDV-1
2	Amjad farm, Chakri road	F-2	IBDV-2
3	Ejaz farm, Lehtrar	F-3	IBDV-3
4	FB farm, Faisalabad	F-4	IBDV-4
5	Gulnawaz farm, Faisalabad	F-5	IBDV-5
6	Marzipura farm, Faisalabad	F-6	IBDV-6
7	Irum farm, Faisalabad	F-7	IBDV-7
8	Irum farm, Faisalabad	F-8	IBDV-8
9	Irum farm, Faisalabad	F-9	IBDV-9
10	Irum farm, Faisalabad	F-10	IBDV-10
11	Poultry Research Institute, Rawalpindi	F-11	IBDV-11
12	Poultry Research Institute, Rawalpindi	F-12	IBDV-12
13	Poultry Research Institute, Rawalpindi	F-13	IBDV-13
14	Poultry Research Institute, Rawalpindi	F-14	IBDV-14
15	Poultry Research Institute, Rawalpindi	F-15	IBDV-15
16	Poultry Research Institute, Rawalpindi	F-16	IBDV-16
17	Poultry Research Institute, Rawalpindi	F-17	IBDV-17
18	Qadeer farm, Begewal	F-18	IBDV-18
19	Ramazan farm, Begewal	F-19	IBDV-19
20	Shabir farm, Abbotabad	F-20	IBDV-20
21	Saleem farm, Abbotabad	F-21	IBDV-21
22	Saghir farm, Abbotabad	F-22	IBDV-22
23	Tahir farm, Abbotabad	F-23	IBDV-23
24	Yaqoob farm, Abbotabad	F-24	IBDV-24
25	Akram farm, Abbotabad	F-25	IBDV-25

Lam (1988), reported that IBDV types 1 and 2 were able to induce direct lysis of chicken bursal cells, thymus cells and peripheral blood lymphocytes in chromium release assays. These two viruses were unable to lyse two established lymphoblastoid cell lines, although IBDV-1 was capable of multiplying in MSB-1 cells.

Kibenge and Mekanna (1992) examined 26 strains and isolates of IBDV by virus propagation on ovine kidney (OK) cell line, *Vero* cell line and chicken embryo fibroblast (CEF) cultures. Virus was isolated from 3 of 26 samples on OK, *Vero* and CEF cultures respectively. However in contrast to IBDV replication in *Vero* and CEF cultures, isolated virus was unable to induce serially sustained cytopathic effects (CPE) during successive passages in OK cell line unless cell lysates were treated with chloroform between alternate passages. The cytopathogenicity of the untreated virus passages in OK cells was revived and maintained upon passage in *Vero* cells. An initial single passage of laboratory or field material in OK cells followed by further passages in *Vero* cells resulted in virus isolation from 6 to 26 samples, which was better virus recovery than when either cell line was used alone or when CEF cultures were used.

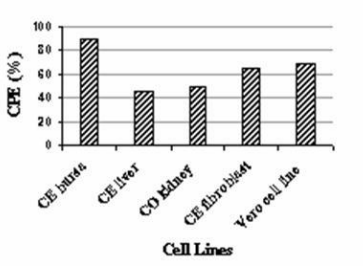
Table 2: Percentage of Cytopathic Effect on day-5/passage-3 on CEF, CEK, CEL, CEB, and *Vero* Cell lines

S.No.	Flock-ID	Strain-ID	Cell lines and %ge of CPE on day-5/passage-3				
			CEF (%)	CEK (%)	CEL (%)	CEB (%)	Vero (%)
1	F-1	IBDV-1	70	65	35	85	65
2	F-2	IBDV-2	75	50	40	80	60
3	F-3	IBDV-3	65	60	50	75	70
4	F-4	IBDV-4	70	55	40	95	55
5	F-5	IBDV-5	60	60	45	85	70
6	F-6	IBDV-6	60	50	35	80	70
7	F-7	IBDV-7	70	45	40	85	75
8	F-8	IBDV-8	65	55	50	80	75
9	F-9	IBDV-9	75	50	45	80	70
10	F-10	IBDV-10	70	65	45	90	75
11	F-11	IBDV-11	60	55	50	80	75
12	F-12	IBDV-12	65	60	45	75	70
13	F-13	IBDV-13	70	50	50	80	70
14	F-14	IBDV-14	65	65	40	85	65
15	F-15	IBDV-15	55	55	55	80	65
16	F-16	IBDV-16	65	50	40	95	70
17	F-17	IBDV-17	65	55	40	95	75
18	F-18	IBDV-18	60	50	50	85	70
19	F-19	IBDV-19	65	55	55	80	70
20	F-20	IBDV-20	70	45	35	80	65
21	F-21	IBDV-21	70	60	50	90	65
22	F-22	IBDV-22	75	50	40	95	70
23	F-23	IBDV-23	60	45	45	90	65
24	F-24	IBDV-24	65	40	45	95	65
25	F-25	IBDV-25	65	65	50	80	60

Table 3: Percentage of Cytopathic Effect on day-5/passage-3 on CE Bursa, CE Kidney and CE Liver organ cultures

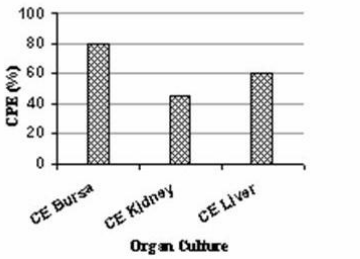
S.No.	Flock-ID	Strain-ID	%age CPE on organ cultures		
			CE bursa (%)	CE kidney (%)	CE liver (%)
1	F-1	IBDV-1	80	35	65
2	F-2	IBDV-2	70	40	70
3	F-3	IBDV-3	65	50	60
4	F-4	IBDV-4	85	40	65
5	F-5	IBDV-5	75	45	55
6	F-6	IBDV-6	70	35	60
7	F-7	IBDV-7	75	40	65
8	F-8	IBDV-8	70	50	60
9	F-9	IBDV-9	70	45	65
10	F-10	IBDV-10	80	45	55
11	F-11	IBDV-11	70	50	60
12	F-12	IBDV-12	65	45	60
13	F-13	IBDV-13	70	50	65
14	F-14	IBDV-14	75	40	60
15	F-15	IBDV-15	70	55	55
16	F-16	IBDV-16	85	40	60
17	F-17	IBDV-17	85	40	65
18	F-18	IBDV-18	75	50	60
19	F-19	IBDV-19	70	55	65
20	F-20	IBDV-20	70	35	70
21	F-21	IBDV-21	80	50	70
22	F-22	IBDV-22	85	40	75
23	F-23	IBDV-23	80	50	60
24	F-24	IBDV-24	85	45	65
25	F-25	IBDV-25	70	45	60

Figure 1: Comparative cytopathic effect of local isolates of infectious bursal disease on different embryo cell lines.



Ture and Saif (1992) analyzed and compared structural polypeptides of six tissue culture origin (BGM-70 continuous cell line) infectious bursal disease viruses representing classic and variant strains of serotype 1 and one serotype 2 strain by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Additionally, they propagated two of the variant strains *in vivo* in bursa of Fabricious and compared with those grown in cell culture. They noted that differences among the structural proteins of serotype 1 viruses were minor and probably of no value in differentiating these viruses. However, distinct differences were observed between serotype 1 and 2 viruses. The bursa derived viruses were different from those propagated in cell culture in molecular weights and in proportions of the proteins. The bursa derived strains had protein migration patterns similar to those of tissue culture incomplete virus particles.

Figure 2: Comparative cytopathic effect of local isolates of infectious bursal disease on different embryo organ cultures.



Thus mammalian continuous cell lines would be suitable alternates for vaccine production. They are preferred over the use of primary cell cultures. Maintaining a continuous cell line is commercially more beneficial than propagating CEF cultures from SPF chicken embryos.

The most common culture system used for the growth of IBDV is CEF However; the titers of virus obtained in CEF are not as high as that in bursa derived vaccine. Thus the preparation of inactivated vaccines using cell culture requires bulk culture to be done to achieve the antigenic content required to induce adequate immunity.

References

Annett, J., Herma, N., and Hermann, M., 2001. Apoptosis is induced by infectious bursal disease virus replication in productively infected cells as well as in antigen-negative cells in their vicinity. *Journal of general Virology*, **82**, 1107-1115.

Kaufer, I., and Weiss, E., 1980. Significance of bursa of Fabricious as target organ in infectious bursal disease of chickens. *Infection and Immunity*, **27**, 364-367.

Kibenge, F. S. B. and Mekanna, P. K. 1992. Isolation and propagation of infectious bursal disease virus using the ovine kidney continuous cell line. *Avian Diseases***.** **36**(2); 256-264.

Kibenge, F. S. B., Dhillon, A. S. and Russell, R. G 1988. Growth of serotypes I and II and variant strains of infectious bursal disease virus in *Vero* cells. *Avian Diseases*, **32**: 298-303.

Lam, K. M. 1988. Lysis of chicken lymphocytes by infectious bursal disease viruses. *Avian Diseases*, **32**: 818-821.

Lukert, P. D. and Davis, R. B. 1974. Infectious bursal disease virus: growth and characterization in cell cultures. *Avian Diseases*, **18**: 243-250.

Mc Ferran, J. B., Mc Nulty, M. S., Mc Killop, E. R., Conner, T. J., Mc Cracken, R. C., Collins, D. S. AND Allan, G. M. 1980. Avian Pathology, **9**: 395-404.

Muller, H., Lang, H., and Becht, H. 1986. Formation, characterization and interfering capacity of a small plaque mutant and of incomplete virus particles of infectious bursal disease virus. *Virus Res*. **4**: 297-309.

Muller, R., Kaufer, I., Reinacher, M. and Weiss, E. 1979. Immunofluorescent studies of early virus propagation after oral infection with infectious bursal disease virus. *J. Virol.* **41**: 333-337.

Senthikumar, P., Thangavelu, A., Raj Dhinakar, G., and Koteeswaran, A. 2006. Antigenic content of *Vero* cell adapted infectious bursal disease virus produced by different culture methods. *Tamilnadu J. Veterinary and Animal Sciences*, **2**(3) 102-108.

Tanimura, N., and Sharma, J. M. 1998. *In-situ* apoptosis in chicken infected with infectious bursal disease virus. *Journal of Comparative Pathology*, **118**, 15-27.

Ture, O. and Saif, Y. M. 1992. Structural proteins of classical and variant strains of infectious bursal disease viruses. *Avian Diseases*, **36** (4): 829-836.

Vasconcelos, A. C., and Lam, K. M. 1994. Apoptosis induced by infectious bursal disease virus. *Journal of General Virology*, **75,** 1803-1806.