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GROWTH CURVE OF BVD VIRAL INFECTIVITY AND ANTIGENS AS MEASURED BY SOME RECENT TECHNIQUES

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INTRODUCTION

Bovine virus diarrhoea virus (BVDV), a common pathogen of cattle was described by Olafson (1946): The virus is not attractive to virologists, its manipulation in culture being beset by problems of frequent advantitious virus contaminations and cultural difficulty (Roeder and Drew, 1984).

Most of the investigations on the behaviour of BVD virus were carried out in primary bovine kidney cell cullure (BK) systems derived from embryonic or new borne calves (Lee and Gillespie 1957; Gillespie et al. 1960 and 1963, and Coggins, 1964), and these studies were restricted to growth kinetics as assayed by infectivity titration (Castrucci et al., 1968; Frey and Liess, 1971; Hafez and Liess, 1972; Parks et al., 1972 Schiff and Storz, 1972; Nuttall, 1980 and Petkova, 1982).

To our knowledge there is no available literature explaining the growth characteristics of Singer strain on primary BK cells, moreover the sequential detection of BVD antigen in primary BK cells by some recent techniques was the aim of the present work.

MATERIALS AND METHODS

Materials:

Virus: The cytopathogenic Singer strain of BVD virus

was used throughout this investigation. The virus was kindly provided by the Ames Iowa laboratories, U.S.A.

Cells: The primary monolayer cultures of bovine kidney (BK) cells) were prepared according to the procedure of Hancock et al. (1959). Eagl's medium containing 10% by volume newly borne calf serum and 5% lactalbumin hydrolysate was used for growth of the cells.

Antiserums:

Bovine anti-BVD serum
Anti bovine Ig-conjugated with peroxidase or with fluorescein isothiocyanate.

N.B: These antisera were kindly supplied by the Ames Iowa Laboratories, U.S.A.

Methods:

Growth curve:

The virus diluted 1/10 was added in a volume of 0.1 ml per tube, to a series of BK monolayer cell cultures and incubated at 37°C. These tubes were removed from the incubator at subsequent intervals of 2,4,8, 12,20,24,48,72,96,120 and 144 hours after inoculation. The fluid phase of each 3 tubes were pooled in screw cap vials and maintained at-70°C until assayed for virus content. The culture tubes containing the cells were resuspended with fresh media to its original volume and exposed to 3 cycles of freezing and thowing, then pooled and clarified at 3000 rpm for 10 minutes. The supernatant representing the cell associated virus were kept at-70°C. Infectivity titration for cell free and cell associated viruses for each time of harvest were assayed and 50% end point was calculated according to the method of Reed and Muench (1938).

Dot ELISA:

According to Hawkes et al. (1982) for the quantitive estimation of viral antigen (S) found in the cell free and cell associated viral portions, irrespective of virus infectivity the Dot-ELISA assay was used to catch the antigenic material of BVD virus. The harvested virus material (cell free and cell associated which was obtained at different intervals of time (see before) were put under screening where double fold serial dilutions were performed and the antigenic material in each dilution was dotted on the nitrocellulose membrane filter and left to interact with reference bovine anti-BVD serum. The positive titers were estimated by reading the development of blue dots on the membrane filter.

Fluorescent antibody technique: (FAT):

To study the development of specific viral proteins of BVD virus in BK cell cultures, the FAT was used in correlation with the microscopical observation for cytopathogenic effect (CPE).

0.1 ml of 1/10 diluted virus was dispensed in cell culture staining chamber system cups containing $5x10^5$ cells in suspension. All cups were then incubated at 37°C in Co_2 chamber. The cups were removed from incubator at intervals 2,4,8,12,16,20,24,48,72,96 and 120 hours postinfection. The cups were treated according to Liess (1966).

Immunoperoxidase technique (IPT):

Sequential development of BVD viral antigen(S) in BK cell culture was studied in a comparative manner between FAT and IPT in correlation with sequential development of CPE. The IPT was applied according to Hyera et al. (1987) with some modification. The sequential detection was started from time hour 20,24,48,72,96 till 120 hours.

RESULTS

1. Growth curve infectivity:

From the end titers documented in Table 1, we can notice that the virus infectivity titers began to increase gradually in both cell free and cell associated portions till 8 hours postinfection (PI) with slight difference till 12 hours PI where the titer of the cell free virus position found to give a higher titer $10^{2.5}$, one log more than cell associated virus position $10^{1.5}$. After that the infectivity of the cell free titer gradually increased till 72 hours PI, where the highest titer obtained was $10^{4.6}$, after which the virus infectivity was found to decrease gradually 96,120 and 144 hours PI, with titers of $10^{4.2}$, $10^{3.5}$ and $10^{2.2}$ respectively.

When looking to the cell associated virus part it appears that the virus titer stood at a degree of approximately 1-2 log lower than cell free virus part from 12 till 24 hours PI after which the level of virus titer was found to increase rapidly at 48 hours PI, with a significant titer of 10^{4}, thereafter the virus titer decreased gradually 72,96,120 and 144 hours PI in a parallel manner of the cell free with varying titers of $10^{3.8}$, 10^{3}, $10^{2.5}$ and 10^{2} respectively.

2. Development of viral antigen as assayed by different serological means:

From Table 2 it appears clearly that the antigenic material harvested in cell free part as detected by Dot-ELISA, showed higher titer (1-2 log) more than the cell associated virus part, where the virus titer in both was found to increase gradually till 48 hours PI. At 72 hours PI the cell free portion showed 2^{6} increase in the titer of virus antigenic material compared with the decrease of virus titer of cell associated part 2^{4}. In general the virus titer in both cell associated parts was lower at 96 and 120 hours of

virus harvest, where the cell free titer exceeded that of cell associated in a wide range.

Tracing of viral antigen by FAT revealed that specific viral protein can be demonstrated inside the cells as earlier as 12 hours PI, where no signs of CPE could be observed. The visible changes were found to develop in cell cultures 24 hours PI and increased in intensity till 96 hours PI, where most cells of the monolayer were found detached and suspended in tissue culture supernatant. The highest degree of immunofluorescence was given at 48 hours PI (Table 3). The positive fluorescence appeared to develop mostly intracytoplasmic in a granular form especially 24 and 48 hours PI while control normal BK cells did not show any reaction.

Table 4 documents the comparative tracing of BVD viral antigen(s) with both FAT and IPT in correlation with sequential development of CPE. From the same Table it appears clearly that the IPT was capable of detecting BVD viral antigen(s) inside the infected BK cells as earlier as 20 hours PI, where there was no any signs of CPE. The maximal immunodetection of BVD viral antigen in BK cells could be achieved at 48 hours PI.

Table (1): Growth kinetics of BVD virus (Singer strain) in BK cell culture

Time PI* in hours	Titers of virus infectivity (log 10/0.1 ml)	
	Cell - free	Cell - associated
2	1.2	1.5
4	2.2	1.7
8	1.8	2.6
12	2.5	1.5
16	-	-
20	3.5	2.2
24	3.9	2.2
48	4.4	4.0
72	4.6	3.8
96	4.2	3.0
120	3.5	2.5
144	2.2	2.0

Table (2): Sequential detection of viral proteins for Singer strain in BK cells by using of Dot-Immunobinding assay

Time PI* in hours	Virus titer (log 2)	
	cell-free	cell-associated
2	2	1
4	2	1
8	2	2
12	3	2
16	4	2
20	3	2
24	3	3
48	4	5
72	6	4
96	5	3
120	5	2

* PI : Postinoculation

Table (3): Sequential development of BVD viral antigen (Singer strain) in BK cell cultures studied by Immunofluorescence

Type of assay	2hrs	4hrs	8hrs	12hrs	16hrs	20hrs	24hrs	48hrs	72hrs	96hrs	120hrs
CPE	-	-	-	-	-	±	+	++	+++	++++	0
FAT	-	-	-	+	+	++	+++	++++	++	+	-

hrs: Time in hours postinoculation.
CPE: Cytopathic effect.
FAT: Fluorescent antibody technique.
0 : The sheet is completely detached.
+ and/or- represent the degree of intensity of CPE and immunofluorescence.

Table (4): Comparative sequential tracing of BVD (Singer strain) viral antigen(s) in BK cell cultures with the immunofluorescence and immunoperoxidase techniques in correlation with CPE development.

Test	Time PI in hours	20	24	48	72	96	120
FAT		+	++	+++	++	+	-
IPT		+	++	+++	++	+	-
CPE		±	+	++	+++	++++	-

PI : Postinoculation
FAT : Fluorescent antibody technique
IPT : Immunoperoxidase technique.
+ and or: Marks showing the degree and intensity of immune reaction as well as CPE given by infected BK cells.

DISCUSSION

In this work the growth characteristics of BVD virus (Singer strain) in BK cells, the sequence of production of cell free and cell associated virus particles as well as the development of specific BVD viral antigens inside the cells have been investigated.

When looking to the data documented in Table 1 we can conclude that the maximal viral infectivity could be harvested from the cell free portion of the infected BK cells 72 hours PI with a titer of $10^{4.6}$. Concerning the cell associated virus portion the maximal harvest could be obtained 48 hours PI (10^{4}). The variation concerning the timing at which maximal titer of BVD virus could be achieved has been discused by many authors working with other strains of BVD virus, where the maximal titers were obtained between 48 and 96 hours PI as reported by Gillespie et al. (1963); Castrucci et al. (1968); Frey and Liess (1971); Schiff and Storz (1972) and Nuttall (1980), where it has been also stated that the

estimated titer of the different strains of BVD virus differ greatly with all tested strains in spite of equal input of virus multiplicity. The observed decline in virus titer of the cell free part after reaching its peak can be attributed to the influence of sustained elevation of temperature during incubation, where large number of free virus particles became inactivated as reported by Taylor et al. (1963); Hafez and Liess (1972); Potter et al. (1984) and Pagnini et al. (1984).

The interaction of the sensitive technique, namely Dot-ELISA in the present work has helped greatly in detection and tracing of BVD viral protein in BK cells where the Sequential development of proteins of Singer strain could be followed in either cell free and cell associated virus parts at different times PI. The virus antigenicity was found to reach its maximal titer (2^6) 72 hours PI (Table 2) in the cell free portion opposed with (2^5) for cell associated virus 48 hours PI. The results presented in this work support us with the evidence that the Dot-ELISA could be successfully used as a sensitive technique for tracing of viral antigens as reported for IBR virus (El-Shahedy 1988).

The successful detection of BVD viral antigen in infected BK cells with the immunofluorescence as early as 12 hours PI with the appearance of intracytoplasmic positive fluorescence in most cases. The failure to detect specific viral antigen earlier than 12 hours PI may lie on the type anti-BVD serum used in this work, where it is not well enshured against strain of BVD virus is directed. Roizman and Schluedberg (1961) stressed on the difference in the type of specific fluorescence observed in BVD infected cells and stated that the cells found in different stages of mitotic cycles are not uniformly susceptible to infection with different viruses or even with the same virus. Table 3 supplies us with the evidence about the best time at wchich the BVD viral protein antigen could be maximally detected, where 48 hours PI was

found to be the best time. The same Table reveals the advantage of FAT in tracing virus infection in BK cells other than following the CPE which have been found to develop and became microscopically observable only 24 hours. PI.

Immunoperoxidase technique (IPT) has been used for diagnosis of BVD virus (Holm Jenson, 1981; Bauli et al. 1984 and Hyera et al. 1987). In our work we used IPT for detection and tracing of BVD antigen where the optimal reactions obtained was achieved by staining the infected BK cells with peroxidase at 48 hours PI and this results correlated in parallel manner as by FAT (no data about sequential detection of BVD antigen in BK cells by IPT).

SUMMARY

The present study deals with the growth behaviour of BVD (Singer strain) virus in primary BK cells with regard to the rate of virus increase in cell free and cell associated virus, as well as detection of viral protein as assayed by Dot-ELISA, FAT and IPT. The results revealed the following:

* The optimum time to harvest the cell free virus was at 72 hours PI with titer ($10^{4.6}$) while for cell associated virus part its peak (10^{4}) was at 48 hours PI.

* The maximal development of viral antigens could be traced and titrated by Dot-ELISA. The most extensive reactions had been given by the cell free viral antigen 72 hours, its titer was found to be higher (2^{6}) than the intracellular (2^{5}) virus harvest 48 hours PI.

* The FAT proved to be sensitive and reliable technique for detecting and tracing the development of viral antigens. The best time to detect clear intracytoplasmic fluorescence in BK cells was at 48 hours PI.

* Regarding the comparative sequential studies between IPT and FAT it was found that both tests ran parallel concerning their sensitivity for detection and tracing of BVD viral antigens.

REFERENCES

1. Castrucci, G.; Cilli, V. and Gagliardi, G. (1968): Bovine virus diarrhea in Italy. I. isolation and characterization of the virus. Arch. Fur die gesm. Virusfors. 24, 48-64.

2. Coggins, L. (1964): Standardization of virus-neutralization test for bovine virus diarrhea. Am. J. Vet. Res. 25, 103-107.

3. El-Shahedy, M.S. (1988): Studies on some serological techniques on IBR virus. Ph.D. thesis, Cairo Univ.

4. Frey, H.R. and Liess, B. (1971): Growth curve studies and applicability of a highly cytopathogenic BVD-MD virus strain for diagnostic purposes using the micro-titer method. Zbl. Vet. Med. B, 18, 16-71.

5. Gillespie, J.H.; Baker, J.A. and Mcentee, K. (1960): A cytopathogenic strain of virus diarrhea virus Corn. Vet. 50, 73-79.

6. Gillespie, J.H.; Madin, S.H. and Darby, N.B.(1963): Studies on virus diarrhea virus of cattle with special reference to its growth in tissue culture and its other susceptibility. Corn. vet. 53, 276-282.

7. Hafez, S.M. and Liess, B. (1972): Studies on bovine viral diarrheamucosal disease virus. I. Cultural behaviour and antigenic relationship of some strains. Acta. Viral. 16 (5), 388-398.

8. Hawkes, R.E. Niday, E. and Gordon, J. (1982): A Dot-immunobinding assay for monoclonal and other antibodies. Analyt. Biochem. 114, 142-147.

9. Holm-Jensen, M. (1981): Detection of antibodies against hog cholera virus and bovine viral diarrhoea virus in porcine. A comparative examination using CF, PLA and NPLA assays. Acta Vet. Scand. 22, 85-98.

10. Hyera, J.M.K.; Liess, B. and Frey, H.R. (1987): A direct neutralizing peroxidase-linked antibody assay for detection and titrationof antibodies to bovine viral diarrhoea virus. J. Vet. Med. B 34 (3), 227-239.

11. Lee, K.M. and Gillespie, J.H. (1957): Propagation of virus diarrhea virus of cattle in tissue culture. Am. J. Vet. Res. 952-953.

12. Liess, B. (1966): Studies on rinderpest virus by the use of cell culture. Arch Exp. Vet. Med., 20, 157-257.

13. Nuttall, P.A. (1980): Growth characteristics of two strains of bovine virus diarrhaea virus. Arch. of Virol. 66 (4), 365-369.

14. Olafson, P., Maccallum, A.D. and Fax, F.H. (1946): An apparently New transmissible disease of cattle. Corn. vet. 36, 205-213.

15. Pagnini, P.; rossi, G.; Tommasis, B.D.E.; Mateo, R.D.I. and Martone, F. (1984): Resistance of bovine diarrhoea virus to plysical agents and various types of water. Acta Medica Vet. 30 (2), 277-284.

16. Parks, J.B.; Pritchett, R.E. and Zee, V.C. (1972): Buoyant density of bovine viral diarrhea virus. P.S.E.B.M., Vol. Vol. 140, 595-598.

17. Paruli, G.; Gregersen, J.P. and Luding, H. (1984): Plaque Focus immunoassay: A simple method for detecting antiviral monochonal or other antibodies and virual antigens in cells. J. Immunol. Meth. 74, 337-344.

18. Pethova, K. (1982): Kinetics of multiplication of bovine diarrhoea (mucosal disease) virus in homologous cells culture. Vet. Nauki 19 (8), 33-39.

19. Potter, M.L.; Corstuet, R.E. and Looney, C.R.(1984): Evaluation of bovine viral diarrhoea virus uptake by preimplantation embryos. Am. J. Vet. Res. Vol. 45, No. 9, 1778-1780.

20. Reed, L.T. and Muench, H. (1938): A simple method for estimating 50 percent end points. Am. J. Hyg. 27, 493-497.

21. Roeder, P.L. and Dre, T.W. (1984): Mucosal disease of cattle, A. late sequel to fetal infection. Vet. Rec. 31, 309-313.

22. Roizman, B. and Schluederherg, A.E. (1961): Virus infection of cattle in mitosis. II. Measles virus infection of mitotic HEP-2 cells. P.S.E.B.M. (N.Y.) 106, 320-323.

23. Schiff, L.T. and Storz, J. (1972): Kinetics of virus production in cells doubly infected with the virus of bovine diarrhoea and a bovine enterovirus. Arch. Fur. Die gesm. Virus fors. 36 No. 314, 218-225.

24. Taylor, D.O.N.; Gastafson, D.P. and Claflin, R.M. (1963): Properties of some viruses of the mucosal disease virus diarrhoea complex. Am. J. Vet. Res. 24: 98, 143-149.