Acta virologica **56**: 155 – 157, 2012 doi:10.4149/av_2012_02_155

LETTER TO THE EDITOR

In vitro propagation of chicken anemia virus in chicken peripheral blood mononuclear cells

S. MANOHARAN, E. T. JAYALAKSHMI, K. KUMANAN

Department of Animal Biotechnology, Madras Veterinary College, Chennai - 600 007, India

Received October 3, 2011; accepted April 18, 2012

Keywords: chicken anemia virus; propagation; mononuclear cells; PCR

Chicken anemia virus (CAV) is worldwide in existence after the first report of isolation in Japan in 1979 (*10*). Since that time, the virus has been detected by isolation or serology in both laying and broiler chickens (*8*). In young chickens, CAV causes a transient to severe anemia and immunodeficiency, which leads to increased susceptibility to various viral and bacterial pathogens causing dermatitis in chickens infected before three weeks of age.

Outside the host, CAV can be propagated in embryonated chicken eggs and Marek΄s disease- or avian leucosis virus-transformed lymphoblastoid cell line-MDCC-MSB1 (*9*). CAV antigens have been detected in mature T-lymphocytes in the spleen (*1*) and in lymphoid aggregates of other organs (*6*). It was demonstrated that the mononuclear cells derived from various lymphoid tissues like spleen, thymus and bone marrow, supported CAV replication, but the titration revealed that the virus titers were lower than those obtained in CAV-infected MDCC-MSB1 cells (*4*). It is believed that the best host system for initial isolation of CAV may be the chicken, as a number of recently characterized CAV isolates cannot be propagated in MDCC-MSB-1 cell cultures (*2*). The present work was attempted to isolate CAV from PCR-confirmed outbreak samples from an easily available source – chicken peripheral blood mononuclear cells, under *in vitro* conditions for ethical reasons.

Tissue samples – thymus, liver, bone marrow and spleen – were collected during six different outbreaks that occurred between 2008 and 2010 in commercial poultry flocks in and around Namakkal town in Tamil Nadu state of India. The sample identity was given individually (Sample No.205 (2008); 339, 609, 583 (2009); 227, 488 (2010)). The pooled tissue samples from each outbreak were homogenized to get a final suspension of 10% (w/v) in serum-free RPMI medium and stored at -80°C for further use. Three experiments were done with the above samples according to the year of collection.

The mononuclear cells were harvested from CAV antibody-free chicken whole blood collected from the university poultry farm as per the method described by Chaturvedi *et al.* (*3*). Harvested PBMCs were seeded into 25 cm^2 tissue culture flask as well as in six-well plates with a final concentration of 5x10^6 cells/ml in duplicates and incubated at 39°C with 5% CO_2. The cells induced with Con-A at a concentration of 10 μg/ml along with uninduced cells were inoculated with the infected-tissue suspensions at the concentration of 10% (v/v) and harvested at day 5 post infection. The infection was verified by CAV VP1 gene PCR after freezing and thawing of the cell culture fluid, and by indirect immunofluorescence analysis (IFA) with an aliquot of cells collected before freezing and thawing of the cell culture fluid using CAV-positive serum raised in SPF chicks (Charles River Laboratories, USA) at the dilution of

Email: ulagaimano@yahoo.com; phone: 91-44-2536-9301, 91-94442-22615.

Abbreviations: CAV = chicken anemia virus; Con-A = concanavalin A; IFA = indirect immunofluorescence analysis; PBMC = peripheral blood mononuclear cells; VP1 = viral protein 1

1:50. A portion of the remaining cell culture fluid was used as inoculum for subsequent passages.

The total genomic DNA was isolated from PBMCs inoculated with CAV-suspected samples using DNAzol (Invitrogen, USA) and was used to amplify the VP1 gene (1353 bp) of CAV (forward primer 5′-TGGCAAGACGAGCTCGCAGACC-3′ and reverse primer 5′-CCCAGTACATGGTGCTGTTCG-3′) with the following cycle conditions: initial denaturation at 94°C for 2 min, 35 cycles of: 94°C for 1 min, 67°C for 1 min, 72°C for 1min, and final extension at 72°C for 10 min.

The isolated DNA from the first PBMC passage as well as from the fifth PBMC passage was 10-fold serially diluted in the range 10^{-1}–10^{-5}. Each diluted DNA sample was used as a template in PCR to confirm the CAV DNA replication in chicken PBMCs.

In each experiment, PBMCs were prepared from same age group of young chickens (4–6-week-old) to avoid the variation in cell susceptibility to CAV infection. The results of this study are presented in the table. The propagation of CAV from suspected field tissue samples, which were positive by PCR, by adapting to PBMCs derived from young chickens showed interesting results. The experiments were done at different time points as and when the samples were obtained. The first experiment was done with the sample No.205 and amplification of the VP1 gene was observed from the third passage to the 5th passage. Similarly, the IFA showed positive fluorescence in the whole cells from the third passage onwards.

Three samples were tested in the second experiment, two of which (Nos. 339 and 609) were positive for CAV both in PCR and in IFA from the first passage. In comparison, the third sample (No. 583) was positive from the third passage onwards. In the third experiment, two samples were processed for isolation and propagation (No. 227, 488) and both were found positive by IFA and PCR from the first passage.

The dilution of the DNA template, which was isolated from CAV-infected PBMCs, in the PCR proved that CAV replicated in PBMCs. In samples that were positive in the first passage, the amplification was noticed only in the undiluted template compared to the 10^{-3}-diluted templates from the fifth passage. Similarly, in samples that gave positive results in the third passage, amplification was seen in the undiluted template from third passage compared to the 10^{-2}-diluted DNA template of the fifth passage. In all the three experiments, Con-A induction enhanced the sensitivity of the detection by one passage in IFA and also by additional one log dilution of CAV DNA template in PCR.

As the target cells for CAV are lymphoid cells, the present study was aimed at using the PBMCs from chickens, which are the susceptible host for CAV. The source of PBMCs were live birds as described in the study of McNeilly *et al.* (*4*), wherein they used mononuclear cells from various organs after sacrificing the birds. For ethical reasons, mononuclear cells from peripheral blood of chickens have been used in this study. Since 3–4-week-old chicken is highly sensitive for CAV infection, PBMCs were collected from this age group. The tissues used in the first experiment were collected during the year 2008, which might have resulted in a drop in virus titer, leading to a later detection of the virus (at the third passage). The detection methods used, viz. indirect immunofluorescence analysis and PCR, are reported to be sensitive methods for detecting CAV (*5*). The signal intensity in IFA and PCR was found to increase as the passage level increases, which could be due to the increase in virus titer. In the second experiment, one sample out of three was found positive only at the third passage, which indicates that the virus titer was very low and required some blind passages before being detected. Although virus titration could not be done, the serially diluted template DNA in the PCR gave an indication of the quantitative level of replicating CAV DNA. However, the PCR positivity was observed up to the dilution 10^{-3} in the five times passaged virus compared to the undiluted template positivity in the first passage. The production of low virus titer was already shown (*4*), but in this work it was shown that there is an increase in the CAV DNA replication as the passage number increases.

The use of the mitogen Con-A, which selectively stimulates T-cells (*7*), might have stimulated T-cell population

Table. Results of IFA and PCR for VP1 gene in the six outbreak samples positive for CAV and propagated in PBMCs for five passages

Sample No. and year received	Passage number									
	1		2		3		4		5	
	IFA	PCR	IFA	PCR	IFA	PCR	IFA	PCR	IFA	PCR
205 (2008)	–	–	–	–	+	+	+	+	+	+
339 (2009)	+	+	+	+	+	+	+	+	+	+
609 (2009)	+	+	+	+	+	+	+	+	+	+
583 (2009)	–	–	–	–	+	+	+	+	+	+
227 (2010)	+	+	+	+	+	+	+	+	+	+
488 (2010)	+	+	+	+	+	+	+	+	+	+

in chicken PBMCs, thereby increasing the cell population susceptible for CAV replication. This has enhanced the sensitivity of detection by one passage and also resulted in one log increase in the dilution of the DNA template for PCR detection.

It is concluded that, although the *in vitro* cultured chicken PBMCs could not be used to increase the virus titer, they could be used for the initial isolation and propagation of CAV to prepare a virus stock for the future research to be used in places, where MDCC-MSB1 cells are not available.

Acknowledgement. This work was supported by the Indian Council of Agricultural Research, Govt. of India, New Delhi through the ′Niche area of Excellence in Animal Biotechnology′ program.

References

1. Adair BM, McNeilly F, McConnell CDG, McNulty, MS, Avian Dis. 37, 943–950, 1993. http://dx.doi.org/10.2307/1591898

2. Calnek BW, Lucio-Martinez B, Cardona C, Harris RW, Schat KA, Buscaglia C, Avian Dis. 44, 114–124, 2000. http://dx.doi.org/10.2307/1592514

3. Chaturvedi U, Kalim S, Kumar S, Sawant P, Tiwari S, Khurana SK, SahooAP, Palia S,Tiwari AK, Indian J. Exp. Biol. 48, 1175–1180, 2010.

4. McNeilly F, Adair BM, McNulty MS, Avian Pathol. 23, 547-556, 1994. http://dx.doi.org/10.1080/03079459408419024

5. Natesan S, Kataria JM, Dhama K, Rahul S, Baradhwaj N, Virus Res. 118, 78–86, 2006. http://dx.doi.org/10.1016/j.virusres.2005.11.017

6. Smyth JA, Moffett DA, McNulty MS, Todd D, Mackie DP, Avian Dis. 37, 324–338, 1993. http://dx.doi.org/10.2307/1591656

7. Toivanen P, Toivanen A, J. Immunol. 111, 1602–1603, 1973.

8. von Bulow V, Schat KA, In Diseases of Poultry. Chapter 30. Iowa State University Press, Ames, IA, pp. 739–756, 1997.

9. Yuasa N, Taniguchi T, Goda M, Shibatini M, Imada T, Hihara H, Institute of Animal Health Q (Tokyo), 23, 75–77, 1983.

10. Yuasa N, Tanigushi T, Yoshida I, Avian Dis. 23, 366–385, 1979. http://dx.doi.org/10.2307/1589567