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RESEARCH

Different culturing systems of IBV-Eg-F170-2015 strain of Egypt variant 2 infectious bronchitis virus

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ABSTRACT

Background: Infectious bronchitis virus (IBV) is an acute contagious disease cause severe economic losses in poultry production worldwide. Virus isolation is the golden standard for IBV diagnosis so the aim of this study was comparing the different biological systems for isolation of infectious bronchitis virus.

Methods: The Egyptian variant 2 IBV strain representative to the current circulating strain in Egypt was isolated in specific pathogen free (SPF) embryonated chicken eggs ECE), chicken embryo kidney cell (CEK) and in Vero cell line and was confirmed for IBV detection by Reverse Transcriptase Real time PCR (RRT-PCR) using IB specific primers. Comparison of different isolation methods was done by generating a standard curve by real time PCR.

Results: The results revealed that the propagated virus in CEK showed the highest virus titer (5.952×10^6 and 3.245×10^6) than Vero cell line (7.184×10^1 and 2.14×10^3) and ECE (7.536×10^3 and 5.444×10^5) after both 48 and 72 hours respectively.

Conclusion: The chicken embryo kidney cell (CEK) is the most successful and sensitive monolayer cell culture host system for isolation of the Egyptian variant 2 strain of infectious bronchitis virus (IBV)

Key words:
Infectious bronchitis virus, Chicken Embryo Kidney (CEK), Vero cell, isolation, standard curve, Embryonated Chicken Egg (ECE).

BACKGROUND

Avian infectious bronchitis (IB) is a major disease in the poultry industry worldwide. This disease frequently occurs in vaccinated and non-vaccinated flocks, and has caused severe economic loss over the last few years (Lee, *et al.*, 2003, Mase *et al.*, 2004, Pohuang *et al.*, 2009, and Yan *et al.*, 2009). Protection failure is mainly due to the numerous IB virus (IBV) serotypes and frequent emergence of new variants *(Yan et al., 2011 and Zou et al., 2010)*. Poultry farms in Egypt are currently suffering from substantial economic losses due to respiratory disease suspected to be predominantly caused by IBV infections. Despite the application of various vaccination strategies in poultry farms in different regions in Egypt, IBV has apparently established endemic status in chicken farms.

IBV belongs to Gamma corona virus genus in the family Coronaviridae, order Nidovirales. The virus is a single stranded positive sense, enveloped RNA virus of 27–32 kb length (Lai and Cavanagh, 1997) encoding four major structural proteins: a small envelope protein (E), integral membrane protein (M), phosphorylated nucleocapsid protein (N), and spike glycoprotein (S) (Spaan *et al.*, 1988). The S protein is cleaved into two subunits (S1 and S2).

The generation of genetic variants is thought to be resulted from few amino acid changes in the spike (S1) glycoprotein of IBV and recombination (Kingham *et al.*, 2000). The N protein is conserved and induces CTL as well as activated B cell responses, which are critical for preventing IBV infection in poultry (Ignjatovic and Sapats, 2005, and Tang, *et al.*, 2008). The M glycoprotein can induce the production of detectable antibodies and delayed type

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hypersensitivity responses (Ignjatovic and Sapats, 2005). IBV strains related to D3128, D274, D-08880, 4/91 and Egypt/Beni-Suef/01 genotypes have been detected at different poultry farms in Egypt (Sultan *et al.*, 2004; Abdel-Moneim *et al.*, 2002). Recently in Egypt, commercial chicken industry suffered from heavy losses due to the emergence of new IBV strains that was able to compromise immunity induced by most available vaccine (Abdel-Moneim *et al.*, 2012).

Growth of the infectious bronchitis virus often requires primary isolation in embryonated chicken eggs (ECE) and several passages. IBV can be isolated in various primary and secondary cells, such as chicken embryo kidney fibroblast and Vero cells, respectively (Otsuki *et al.*, 1979a and Arshad, 1993). CPE are characterized by rounding, development of syncytia, and subsequent detachment from the surface of the plate (Arshad, 1993). So the aim of this study is determination of the best and most sensitive isolation method for isolation of the infectious bronchitis virus Egyptian strains.

MATERIALS AND METHODS

Viruses:

The selected IB virus strain is IBV-Eg-F170-2015 Egyptian variant 2 strain obtained from broiler farm in Alexandria, Gene Bank Accession No. is KY119259

Virus isolation in ECE:

Amount of 0.2 ml of IB virus was inoculated in five specific pathogen free embryonated chicken eggs (SPF ECE) of 9-11 days old via allantoic sac with daily candling up to 3 days for embryonic deaths. After 48 and 72 hours the embryos was examined for embryonic changes and macroscopic lesions, and the allantoic fluid was harvested for real time PCR confirmation of IB virus (Beaudette and Hudson, 1937).

Virus isolation in chicken embryo kidney cells (CEK):

Primary chicken embryo kidney cell fibroblast was prepared from 17 days old chicken embryo. After a confluent monolayer was grown in T-25 tissue culture flasks, the cells have been washed 3 times using PBS and inoculated with 0.2 ml of IB virus. The inoculum was allowed to be adsorbed for 1 hour at 37ºC and 5% CO2, then maintenance media (Dulbecco's Modified Eagle Medium (DMEM) + 5% Fetal Bovine Serum (FBS) + 1% Penicillin/Streptomycin Solution (Pen/Strep)) was added. The infected cells were observed daily for cytopathogenic effect (CPE) and harvested after 48 and 72 hours for IB virus confirmation by using real time PCR (Gillette, 1973 and Otsuki *et al.*, 1979a, b).

Virus isolation in Vero cell line:

A percent of 80% Confluent monolayer of Vero (African green monkey kidney) adherent cell line was grown in T-25 tissue culture flask. The growth media was decanted and cells were washed by PBS 3 times. 0.2 ml of IB virus was inoculated and allowed to be adsorbed for 1 hour at 37ºC / 5% CO2 for 1 hour then the maintenance media was added and incubated in CO2 incubator up to 3 days with daily observation for CPE. The cells were harvested after 48 and 72 hours and confirmed for IB virus by real time PCR (Yasumura & Kawakita, 1963).

Viral RNA extraction:

IB virus RNA extraction was done from allantoic fluid, infected CEK cells and infected vero cells harvested after 48 and 72 hours, by using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA) according to the manufacturing instructions as procedures.

Real time RT-PCR and Standard curve:

Real time PCR for infectious bronchitis virus was performed with Quantitect probe RT-PCR kit (Qiagen, Inc. Valencia CA) and Stratagen MX3005P machine (Stratagene, USA) by using IBV specific primers and probe according to **Callison *et al.*, 2005 and 2007), (table 1)**.

Table (1): Primers and probe used in IBV amplification

Primer	Sequence (5′-3′)
IBV5-GU391	GCT TTT GAGCCT AGC GTT
IBV5-GL533	GCC ATG TTG TCA CTG TCT ATT G
IBV5-G probe	FAM-CAC CAC CAG AAC CTG TCA CCT C-BHQ1

10 fold serial dilution of the original IBV isolate of $10^{5.2}$/ml EID50 was done and the standard curve was obtained by by plotting the CT values *vs* log of 10-fold serial dilutions of the standard original IBV isolate to identify the threshold of detection and the viral copy number from infected isolates was calculated.

RESULTS

Virus isolation in ECE

After 5 days post inoculation, the ECE was opened and the embryo was observed and showed curling and dwarfing in all the inoculated eggs, which are the characteristic lesions of the IBV. The allantoic fluid was harvested and IBV was confirmed and identified by real time RT-PCR using specific primers and probe.

Virus isolation in chicken embryo kidney cell (CEK):

The CPE appeared on the infected CEK cells after 48 hours post inoculation (PI) showed partial sloughing of the cells while after 72 hours PI, Complete sloughing of the cell and large plaque formation were observed **(Fig. 1)**.

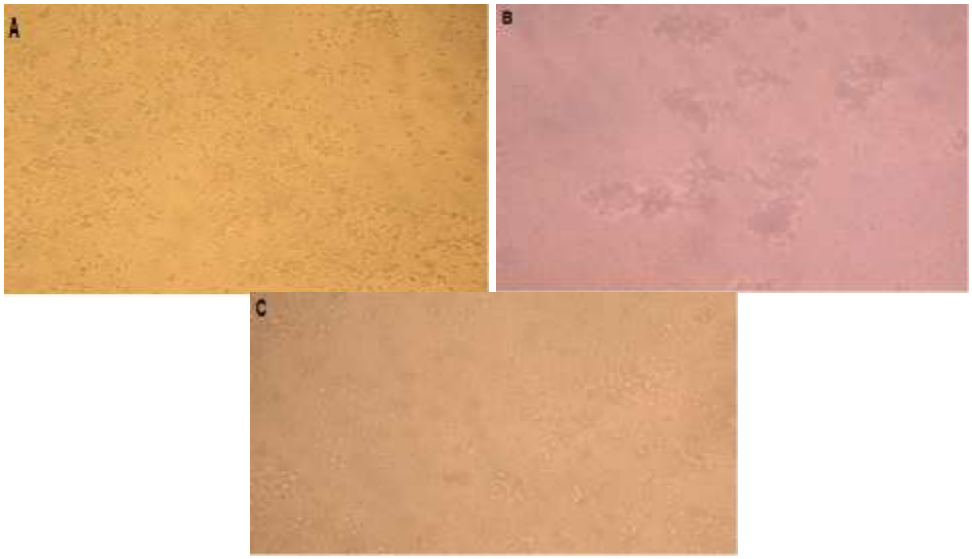

Fig. 1: showing the CPE in CEK after inoculation of IB virus. **(A)**: Partial sloughing of the cells 48 hr. PI, **(B)**: Complete sloughing of the cell and large plaque formation 72 hr PI, and **(C)**: Negative control 72 hr. PI with normal CEK cells

Virus isolation in Vero cell line:

Observation of the CPE in the inoculated Vero cell line with infectious bronchitis virus showed slight sloughing of the cells after 48 hours, followed by destruction of the cell monolayer and complete detachment of the cells after 72 hours post inoculation **(Fig. 2)**

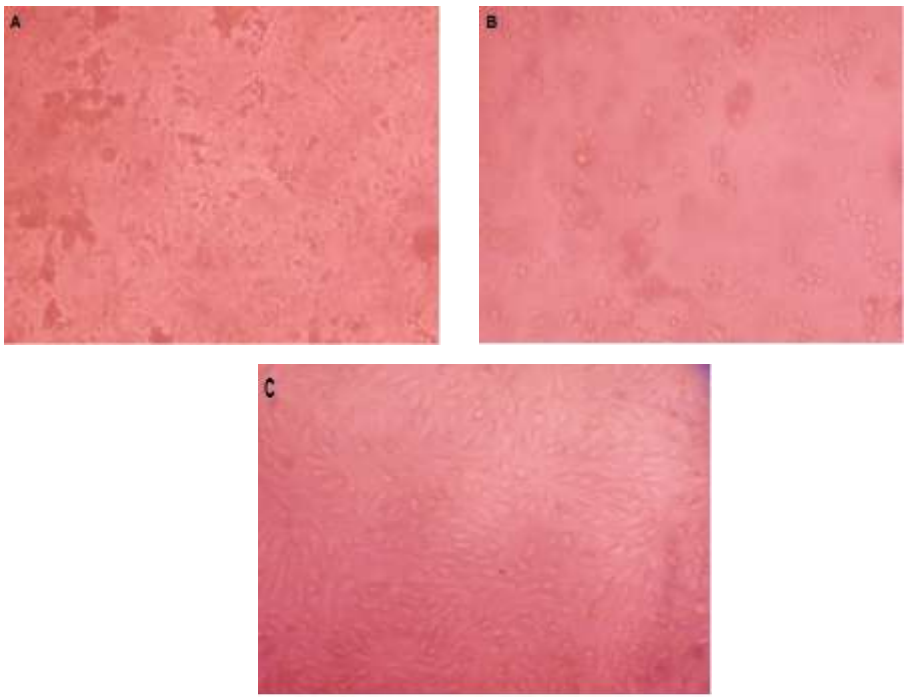

Fig. 2: showing CPE in Vero cell line after inoculation of IB virus. (A): Slight Sloughing of cells 48 hr. PI, **(B):** Sloughing of the cell 72 hr. PI, and **(C):** Negative control 72 hr. PI with normal Vero cells

Real time RT-PCR and virus quantitation

Standard curve is generated by plotting Ct values against log transformed concentrations of serial tenfold dilutions of the standard original IBV isolate, each plot corresponds to a particular input target copy number.

Results showed that the isolate obtained from CEK was of the highest virus copy number 5.952×10^6 and 3.245×10^6 after both 48 and 72 hours respectively followed by the isolates obtained from Vero cell line.

Table (2): Ct values and viral copy number of IBV after isolation in different cell lines

Code	Results of RT-PCR	Ct	Conc. (EID50 / 1 ml)
Origin	Positive	15.19	1.896×10^7
ECE 48 hrs.	Positive	22.02	7.536×10^3
CEK 48 hrs.	Positive	**16.19**	**5.952×10^6**
Vero 48 hrs.	Positive	30.11	7.184×10^1
ECE 72 hrs.	Positive	18.28	5.444×10^5
CEK 72 hrs.	Positive	**16.72**	**3.245×10^6**
Vero 72 hrs.	Positive	23.12	2.14×10^3

DISCUSSION

Virus isolation is the gold standard for the diagnosis of Infectious bronchitis virus (Beaudette and Hudson, 1937, and Stephensen *et al.*, 1999). Primary isolation of infectious bronchitis virus usually requires inoculation in specific pathogen free (SPF) embryonated chicken eggs (ECE) and/or primary cell culture derived from chicks (Dhinakar & Jones, 1997). The use of mammalian cell cultures for primary isolation of IBV has been unsuccessful (Otsuki *et al.*, 1979b). Isolation of IBV has been attempted in various primary and secondary cells, such as chicken embryo kidney fibroblast and Vero cells, respectively (Otsuki, *et al.*, 1979a, and Arshad, 1993)

In this study, the infectious bronchitis virus strain F170/2015 Egyptian variant2 representative to the current circulating strains in Egypt was propagated in SPF-ECE, chicken embryo kidney cell (CEK) and in Vero cell line.

The isolated virus was confirmed for IBV detection by Reverse transcriptase Real time PCR (RRT-PCR) using IB specific primers and then the standard curve was done for comparing the sensitivity of different isolation methods used in propagation of the IBV strain.

Specific pathogen free embryonated chicken egg (SPF-ECE) is used for primary isolation of IBV. In this study the inoculation of the selected IB virus strain (F170/2015 Egyptian variant2) in ECE induced the embryonic characteristic lesions of IB virus in which is curling, stunting and dwarfing after 72 hours and this is in accordance to OIE, 2013 which reported that these lesions are induced by the field strains of IBV. Also this was agreed with Darbyshire *et al.*, 1975 who reported that IBV grows well in the developing chicken embryo, the maximum virus titer in allantoic fluid is obtained after 24 to 48 hours post-inoculation and the peak can be delayed for non-egg-adapted field strains.

Inoculation of infectious bronchitis virus strain in CEK showed cytopathic effects (CPE) characterized by Complete sloughing of the cells and large plaque formation 72 hours post inoculation in accordance to Lukert, 1965, 1966, Gillette 1973, and Otsuki *et al.*, 1979b

The possibility that IBV might replicate in African green monkey kidney cell line (vero) was reported by Yasumura & Kawakita, 1963. In our study, the inoculated Vero cell line with infectious bronchitis virus showed CPE characterized by complete sloughing of the cells after 72 hours and this was in accordance to Arshad, 1993 who reported that the infected Vero cell cultures showed CPE of rounding, plaque formation, and subsequent detachment and sloughing of the cells. Also our results agreed with Cunningham *et al.*, 1972 who reported the CPE of IBV after 2 days of inoculation on Vero cells

Quantification and calculation of exact IB viral copy numbers in the infected isolates from ECE, CEK, and vero cell line was obtained by using the standard curve which was generated by 10 fold serial dilution of standard original IBV isolate and the quantification limits were determined using CT values obtained for each dilution of the standard isolate, The values were plotted against the log of the number of template copies and the viral copy number was calculated.

The results of our study revealed that The propagated virus in CEK showed the highest virus titer than Vero cell line and ECE after both 48 and 72 hrs (Table 2) and this is consistent with Gillette 1973 who reported that the most successful monolayer cell cultures for IBV studies are chicken kidney (CK) cells and chick embryo kidney (CEK) cells. Our results also were agreed with Lukert, 1965, 1966, and Otsuki *et al.*, 1979b, who reported that Chicken embryo kidney (CEK) cells and chicken kidney cells show the highest sensitivity for adapted IBV strains

In conclusion, the results of this study revealed that the most successful and sensitive monolayer cell cultures for isolation of the Egyptian variant 2 strain of infectious bronchitis virus (IBV) is the chicken embryo kidney cell (CEK).

AUTHOR DETAILS

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