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MTT assay-based evaluation of avian avulavirus-1 pathogenicity in chicken embryo fibroblast cultures

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Abstract

Avian Avulavirus 1 (AAvV-1), the causative agent of Newcastle disease poses a significant threat to poultry industry. Assessing the pathogenicity of AAvV-1 isolates is crucial for understanding their impact on host cells. This study employs the MTT assay to evaluate the cytopathic effects of AAvV-1 strains isolated from various avian sources in chicken embryo fibroblast (CEF) cultures. The assay measures cell viability based on mitochondrial activity, providing a quantitative assessment of viral-induced cytotoxicity. Results indicate a significant reduction in cell viability in infected cultures, with varying degrees of pathogenicity among isolates. The MTT assay proves to be a reliable tool for evaluating AAvV-1 virulence *in vitro*, complementing traditional pathogenicity tests. These findings contribute to a better understanding of AAvV-1-host interactions and may aid in the development of targeted control strategies.

Keywords: Avian Avulavirus-1, MTT assay, velogenic, pathogenicity, chicken embryo fibroblasts

Introduction

Newcastle disease (ND) is a highly infectious disease of many avian species, leading to significant economic impairment to poultry industry. ND is caused by Newcastle disease virus (NDV), now called as Avian Avula virus-1 (AAvV-1) belongs to genus *Avian orthoavulavirus 1* (AOAV-1) within the subfamily *Avulavirinae* of the family *Paramyxoviridae* (ICTV, 2019) ^[1]. More than 241 species from 27 orders of the avian family are reported to be susceptible to ND (Kaleta and Baldauf, 1988) ^[5]. Virus strain and host species determines the severity and outcome of the disease. Basing on their pathogenicity for chicken, AAvV-1 strains can be differentiated into strains of high (velogenic), intermediate (mesogenic) and low (lentogenic) virulence (Dimitrov *et al.*, 2019) ^[2]. Velogenic AAvV-1 strains cause acute infections with high mortality, preceded by respiratory and neurological signs or haemorrhagic lesions in the gut, whereas mesogenic strains cause respiratory disease in young birds and decreased egg production in laying flocks. In contrast, lentogenic strains may produce only mild respiratory signs in young chickens (Orynbayev *et al.*, 2018) ^[9]. Virulence of a given NDV isolate can be determined under laboratory conditions by assessing the intracerebral pathogenicity index (ICPI) in 1-day-old chicks or by mean death time (MDT) in 9-day old embryonated chicken eggs. It is quintessential to understand the potential risks of transmission of domestic and wild-bird origin AAvV-1 into poultry. The aim of the present study was to estimate the ability of AAvV-1 strains isolated from various avian sources to induce pathogenicity in chicken embryo fibroblast cultures.

Materials and Methods

Virus strains

Five AAvV-1 isolates, CHKN5 (AAvV-1/Chicken/Amaravathi/2018), CHKN10 (AAvV-1/Chicken/Hyd/2018), PGN1 (AAvV-1/Pigeon-P1/Tanuku/2017), MIG9 (AAvV-1/Pelican/Telineelapuram/2018) and EMU5 (AAvV-1/Emu/LFC-RJNR/2018) maintained as virus repository in the Department of Veterinary Microbiology were used in the study.

The chicken isolates, CHKN5 and CHKN10, were isolated from commercial broiler flock and backyard chicken respectively, both the flocks exhibiting clinical signs like diarrhea, nervous symptoms and death. During necropsy, lesions observed in the dead carcasses were petechial haemorrhages in the proventriculus and ulcers in the intestine. PGN1 strain was isolated from a pigeon, a free-range bird and is apparently healthy. EMU5 strain was obtained from an emu raised in a mixed poultry farm that also houses chicken, ducks and geese. Pelican strain (MIG9) was isolated from cloacal swabs collected from clinically healthy spot-billed pelicans at Telineelapuram wild bird sanctuary, Srikakulam district, Andhra Pradesh, India.

Virus propagation and pathogenicity tests

The virus isolates were propagated in 9 day old embryonated chicken eggs using standard procedures (Alexander, 1989) [1]. Virus identity was confirmed by haemagglutination-inhibition (HI) assay with polyclonal chicken antiserum raised against LaSota and also by reverse transcription-polymerase chain reaction (RT-PCR) of the FPCS sequence (Nantha Kumar *et al.*, 2000) [7]. The mean death time (MDT) and intracerebral pathogenicity index (ICPI) were determined using standard procedures (OIE, 2012) [8].

Preparation of primary chicken embryo fibroblast cultures

The CEF culture was prepared as described by Freshney (2000) [3] with minor modifications. Briefly, 9-11 days old ECE's were swabbed with 70% ethanol and the shell was crack opened using sterile forceps. After removing the chorioallantoic membrane (CAM), the embryo was removed using forceps by grasping around the neck and placed in a sterile petri plate containing sterile PBS. The head, feet and visceral organs were removed using sterile scissors, leaving the skin. The skin was washed thrice in sterile PBS and fragmented into smaller pieces with the help of scissors in a glass beaker. The fragmented tissue was transferred to another beaker, 30-50 ml of 0.5% trypsin was added and further macerated with a magnetic stirrer for approximately 5 min. The supernatant solution was removed leaving behind the macerated tissue. 30-50 ml of fresh trypsin solution was once again added and stirred for 10 more minutes until a single cell suspension is obtained. Two ml of FBS (Fetal bovine serum) was added to stop the trypsin activity. The supernatant containing cells was transferred to a sterile centrifuge tube containing FBS and centrifuged at 10,000 rpm for 15 min. Supernatant was discarded and the cells were resuspended in 100 ml growth media. The cells were washed once again by centrifugation and finally re-suspended in fresh growth medium. A cell suspension of 0.5 ml was diluted with an equal amount of trypan blue solution and the cells were counted in haemocytometer. Concentration of the cells was adjusted to 10^6 cells / ml of growth medium and dispensed into tissue culture flasks and incubated for 48 h at 37°C in an incubator with five per cent CO₂ for formation of confluent monolayer.

Calculation of TCID₅₀ for the AAvV-1 strains

Chicken embryo fibroblast cells were seeded into 96-well cell culture plates at a seeding density of 5×10^4 cells/well in DMEM with 10% FBS. The cells grew to 100% confluence within 24 h. Serial 10-fold dilutions from 10^{-1} to 10^{-10} of the virus were made with a serum free infection medium. To the confluent monolayers, 50 µl of the virus inoculums for each

dilution was added to ten wells. After one hour of adsorption at 37°C, the virus inoculum was removed and 100 µl of infection medium was added to all the wells including controls. Later the plates were sealed and incubated at 37°C in a CO₂ incubator. The infected wells along with the control wells were observed for CPE regularly. TCID₅₀ per ml of virus inoculum was calculated as per the method described by Reed and Muench (1938) [10].

In vitro Pathogenicity test in CEF cultures by MTT Assay

Measurement of metabolic activity of primary cells following infection with AAvV-1 isolates (CHKN5, CHKN10, PGN1, MIG9 and EMU5) was carried out by 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide reduction (MTT) assay. Primary chicken cells were seeded in 96 well cell culture plates at a seeding density of 5000 cells per well. Four wells were left without adding cells to serve as negative control. Following 24 h incubation at 37°C, medium from the wells was removed and the cells were rinsed 3 times with PBS. Cells were infected with AAvV-1 isolates at MOI ranging from 0.005 to 1, using quadruplicate wells for each MOI. 50 µl of maintenance medium containing appropriate amounts of virus was added to the wells. Plates were incubated for 24 h PI at 37°C. The medium was removed from each well, washed with PBS and replaced with 100 µl of serum free medium. 10µl of the dye solution (12mM MTT) was added to all the wells. Negative control: 10 µl of the dye was added to 100 µl of the medium alone without cells. Plates were incubated at 37°C for 4 h. MTT containing medium was removed by inverting, flicking and blotting. 50 µL of DMSO was then added to each well, the plate was tapped for mixing the contents before incubating the plate for 10 min at 37°C. Following incubation, contents were mixed and absorbance was recorded in a plate reader, Multiscan Go (M/s Thermo Fischer Scientific) at 540 nm. Four wells were sham infected with infection medium without virus. High absorbance value indicates high cell viability.

Statistical analysis

Each sample was tested in quadruplicate and data from MTT assay was expressed as the mean $\pm$ SD. Statistical analysis was performed using SPSS Version 17.0. (Chicago: SPSS Inc.). Differences in the mean absorbance values of the monolayers infected with AAvV-1 isolates of different pathogenicities were evaluated using one way ANOVA followed by Duncan LSD Alpha post-hoc correction method. Significance was considered when $p < 0.05$ was observed.

Results and Discussion

The present study aims to assess the pathogenicity of AAvV-1 strains isolated from various avian sources in chicken embryo fibroblast cultures employing MTT assay. MTT assay provides a useful tool for evaluating the cytotoxic effects of virulent AAvV-1 on monolayers of chicken embryo fibroblasts.

AAvV-1 isolates (CHKN5, CHKN10, PGN1, MIG9 and EMU5) propagated in 9-day old embryonated eggs through allantoic route was confirmed by haemagglutination, haemagglutination inhibition test and RT-PCR targeting FPCS sequence. All the NDV strains used in this study were velogenic with mean death time (MDT) ranging between 42-60h and Intracerebral pathogenicity index (ICPI) ranging between 1.475-1.8 (Table 1).

Chicken embryo fibroblast cells cultured in 25cm² flask were subcultured every 3 days with a split ration of 1:3 depending

on the density of the monolayer. Healthy CEF monolayers after 24h was presented (Figure 1). The five velogenic isolates showed characteristic cytopathic changes in CEF cultures at first passage and the cytopathic changes observed in the monolayers were depicted (Figure 2). The uninfected control displayed a rich network of long, branched spindle-shaped morphology of adherent fibroblasts and rounded refractile (dead) cells. Cytopathic effect was not obvious until 36-48 h of infection varying with the isolate. The characteristic cytopathic effects noted in the infected monolayers included increased granularity, rounding of cells, cytoplasmic vacuolation, discrete clumping of cells, formation of syncytia, plaque formation, coalescence of plaques, large areas of cell destruction, disintegration of the monolayer and large cell clumps floating in the superficial media. Generalised lysis was observed over the entire monolayer. Infected fibroblasts were completely lysed and detached from the substrate at 84 h post inoculation and 2 to 3 days before control monolayers. Cytopathic changes observed in the infected monolayers were recorded at regular time intervals (Figure 2).

NDV virus strains are readily adapted to primary cultures of chicken embryo fibroblasts and cytopathic effects are observed from the first passage. Clarified cell culture fluid was initially tested for presence of AAvV-1 by spot haemagglutination test. The titre of each AAvV-1 isolate was estimated by microtitre haemagglutination test and were found to be ranging from 2^6 to 2^8 . TCID₅₀ of AAvV-1 isolates (CHKN5, CHKN10, MIG9, PGN1 and EMU5) ranged from 1×10^7 to 3.6×10^7 /ml (Table 1).

Metabolic activity of chicken embryo fibroblast cells was measured by MTT assay following infection with AAvV-1 isolates of varying pathogenicity (CHKN5, CHKN10, PGN1, MIG9 and EMU5). Differences in growth kinetics of AAvV-1 isolates at different MOI (0.005, 0.05, 0.5, 0.75, and 1.0) were quantified and the cell viability was measured in terms of absorbance values. MOI was calculated based on the TCID₅₀ titres of the isolates.

At 24 h following virus infection, viability of cells was high at low MOI, and the viability reduced with increasing MOI (Figure 3 & 4). The reduction in cell viability was virus dose dependent, with increasing MOI, inducing significant reduction in cell metabolic activity ($p < 0.01$). All the velogenic isolates consistently induced significantly lower viability in chicken embryo cells over a range of multiplicities of infection. This finding is in concordance with Kumar and Kumar (2015) [6] who during their study with an oncolytic NDV on MCF-7 cell lines proved that NDV-induced cytotoxicity correlated linearly with the virus titre in the infected supernatants and that cell death is because of apoptosis and not due to necrosis. Similar experiment was carried out to measure differences in cell viability of different tumor cell lines (HCT116, HeLa, A549, and MCF7) infected with two mesogenic (NDV/Altai/pigeon/770/2011 and NDV/Altai/pigeon/777/2010) and two lentogenic strains (NDV/mallard/Amur/264/2009 and NDV/ teal/Novosibirsk region/320/2010) at different passage levels (first and tenth passages). Only mesogenic strains exhibited relative changes in cell viability with different viral titres and that lentogenic strains could not be adopted to grow in the tumor cells as efficiently as the mesogenic strains (Yurchenko *et al.*, 2018; 2019) [13, 14]. The MTT assay was employed by several authors to determine the immune potency of rec-F and rec-HN antigens of AAvV-1 (Gowrakkal *et al.*, 2015) [4], to determine toxicity and anti-viral activity of fucoidan against

AAvV-1 (Trejo-Avila *et al.*, 2016) [12] and to determine the oncolytic potential of AAvV-1 isolates (Yurchenko *et al.*, 2018; 2019) [13, 14].

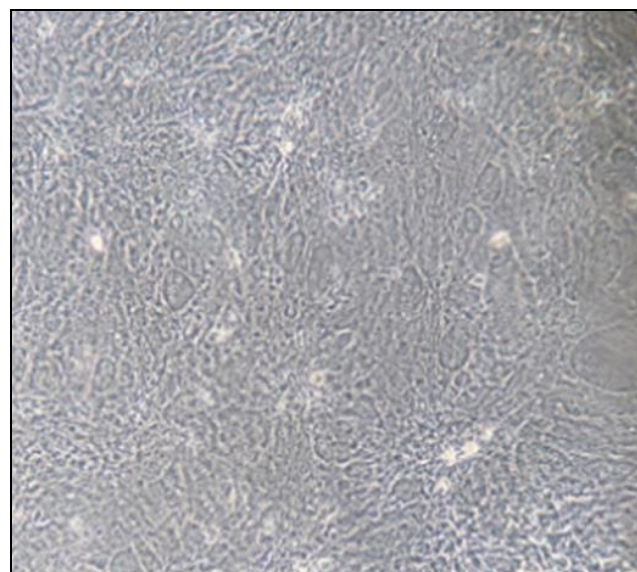


Fig 1: Uninfected Chicken embryo fibroblast monolayer (20 X)



Fig 2: Chicken embryo fibroblast monolayers infected with AAvV-1 showing syncytia and vacuole formation

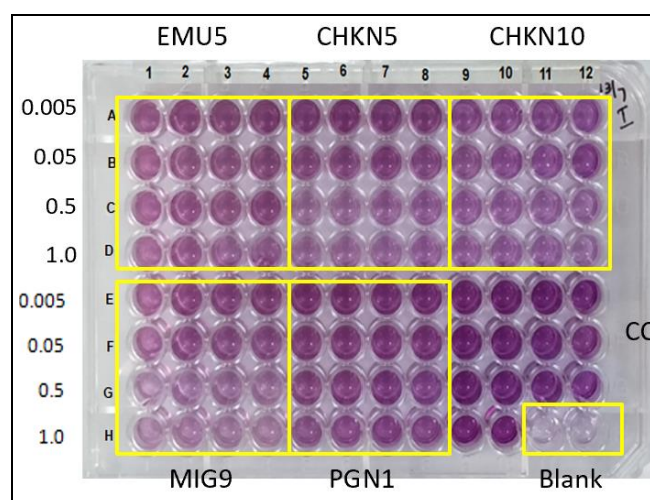


Fig 3: MTT assay showing cell viability in CEF monolayers infected with various AAvV-1 strains at different MOI. (Rows: A, E-0.005 MOI; B, F-0.05 MOI; C, G-0.5 MOI; D, H-1.0 MOI; CC-Cell controls)

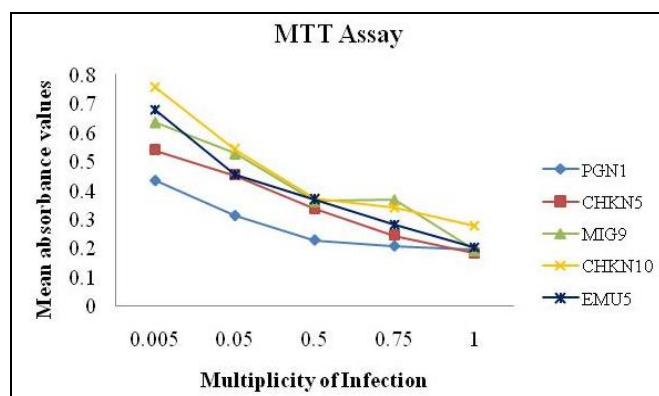


Fig 4: Measurement of cell metabolic activity following infection with velogenic AAvV-1 isolates by MTT assay

Chicken embryo fibroblast cells showing significantly higher cell death with increasing MOI ($p < 0.01$). Data points are the mean of quadruplicate wells.

Table 1: Biological characteristics and titres of AAvV-1 strains used in the study

S. No	Virus Isolate	Pathotype	Spot HA	HA titre (log ₂)	MDT (hrs)	ICPI	TCID ₅₀ /ml
1.	CHKN5	Velogenic	Positive	2 ⁸	42	1.75	3.6 x 10 ⁷
2.	CHKN10	Velogenic	Positive	2 ⁶	46	1.6	1.57 X 10 ⁵
3.	MIG9	Velogenic	Positive	2 ⁷	60	1.475	3.2 X 10 ⁴
4.	PGN1	Velogenic	Positive	2 ⁸	48	1.85	2.45 X 10 ⁷
5.	EMU5	Velogenic	Positive	2 ⁶	48	1.82	1 X 10 ⁷

Conclusion

The MTT assay proved to be a reliable method for assessing the pathogenicity of AAvV-1 isolates in chicken embryo fibroblast cultures. The observed reduction in cell viability reflected the cytotoxic effects of different viral strains, highlighting variations in their pathogenic potential. This *in vitro* approach offers a quantitative and efficient alternative to traditional pathogenicity tests, aiding in the evaluation of AAvV-1 virulence. These findings enhance our understanding of AAvV-1-host interactions and may support the development of improved diagnostic and control strategies for avian infections. Further studies incorporating molecular and *in vivo* analyses will help validate and expand upon these results.

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Conflict of Interest

Not available

Financial Support

Not available

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