Enumeration Techniques of Newcastle Disease Virus (NDV) for Oncolytic Virotherapy

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ABSTRACT

The present work was carried out to evaluate different quantitation techniques when Newcastle disease virus was intended to use as an oncolytic agent. The R_2B Mukteshwar strain of NDV was procured as lyophilized vaccine. Then application were carried out like haemagglutination test, tissue culture infective dose-50 ($TCID_{50}$), plaque forming unit (PFU) calculation and real time PCR to enumerate the number of viruses. The HA titre was obtained as 1:128 across the dilutions. While $TCID_{50}$ and PFU counts were obtained as $1 \times 10^{8.16}$/ ml and 4.2×10^7 PFU/ ml, respectively. Based upon comparison with standard NDV RNA, Real time PCR also revealed the number of virus 10^8/ ml. HA was found consistent but indirect; contrastingly $TCID_{50}$ suffered with subjectivity of interpretation. PFU counts were found within a range and lease possibility of interpretation error than $TCID_{50}$. Though real time was found automated, highly specific and sensitive assay but handling of RNA and cost were limiting factors.

Keywords: Newcastle disease Virus (NDV), Haemagglutination test, Tissue culture infective dose-50 ($TCID_{50}$), Plaque forming units (PFU), Real time PCR, Virotherapy

Since last decade of 20th century, use of viruses has been resurged as a novel form of tumour therapy (Guo *et al.,* 2014). Among the candidate oncolytic viruses, Newcastle Disease Virus (NDV has emerged as a favoured oncolytic virus (Tayeb *et al.,* 2015). At present many strains of NDV have been well characterized as oncolytic virus at different parts of world and presently at different phases of clinical trials (Mataveeva *et al.,* 2015). In India, Kumar *et al.* (2012) and Kumar & Kumar (2015) reported in vitro evaluation of velogenic field strains for oncolytic potential. Here, instead of searching velogenic field strains, R_2B Mukteshwar strain of NDV was chosen as a model due to its mesogenic nature and easy availability. Virotherapy, like other form of therapies acts in dose dependent manner but virus quantitation is not straight forward and carried on indirect evidences of viral activities. Even though virus enumeration is age old techniques but concerned literature is not easily available. These are counted on the basis of various classical techniques such as Haemagglutination test and its inhibition, Tissue culture infective dose 50 ($TCID_{50}$) and Plaque forming unit (PFU) determination. Recently, real time PCR has emerged as highly sensitive and specific quantitative technique (Wise *et al.,* 2004). Therefore, in the present communication, application of these techniques has been described when NDV is purposed for virotherapeutic agent, though considerations are equally applicable to diagnostic purposes.

MATERIALS AND METHODS

Newcastle Disease Virus (NDV), R_2B Mukteshwar strain was procured as commercial vaccine vials from Venketshwara hatcheries limited, Pune, India. Virus had been propagated in SPF eggs. The vials belonged to same manufacturing batch and each vial was labeled to contain 10^8 EID_{50} viruses.

Haemagglutination (HA) test

HA test was carried out to determine the HA activity and titre of virus as described in OIE terrestrial manual (OIE, 2012). One vial was reconstituted with 1 ml of sterile PBS. Twenty five µl of virus was put in to first well of V bottom microtitre plate, serially diluted (1:2) in PBS across the column (1A-1G) to make dilution from 1:2 to 1:128. Then each well of column 1 was diluted serially across the rows from 2-11. Further, 25 µl of PBS was dispensed all working wells. Thereafter, 25 µl of 1% chicken RBC suspension was placed to all the working wells. After shaking for 5 min, the plate was put at 4°C for one hour and formation of serrated edged mat and button were recorded as negative and positive results, respectively. The HA activity was further confirmed through blocking of HA activity with known antiserum (Haemagglutination inhibition test).

Tissue Culture Infective Dose 50 (TCID$_{50}$)

The Tissue Culture Infective Dose 50 (TCID$_{50}$) was calculated as per protocol described by Ahamed *et al.* (2004). Vero cells were trypsinized and viable cell count was adjusted to 1×10^6 cells /well of 24 well cell culture plate and let them in suspension till use. One hundred eighty microlitre of pre warmed HBSS solution was placed in each well of plate. Twenty microlitre of virus suspension was put in to first well mix well and then it was diluted as 10 fold serial dilution across the wells (A1-A6 and then B1- B-4), except the last two control wells (B5 and B6) where no virus and undiluted virus suspension was inncoluated. Then 80 µl of suspension was poured off from the each well. Thereafter 1 ml of cell suspension having required number of cells was put in to each working well of the plate, mixed gently and incubated in CO_2 Incubator under defined growth conditions for 48 hours and observed the development of CPE under inverted microscope and its percent was estimated for each well. TCID$_{50}$ was calculated as per method of Karber (1931).

Plaque Forming Unit (PFU) calculation

PFU calculation of stock NDV was done according to Kournikakis and Fildes (1988) with slight modifications. Culture of Vero cell was carried out in 6 well tissue culture plates (Costar, Corning). Ten fold virus dilutions were made in 0.5 ml sterile Eppendorf tube using HBSS as diluents. 100 µl of viral suspension was transferred in respective wells of cell culture plate and well number 6 was kept as uninnoculated control. The volume was made to 1 ml using basal DMEM. Viruses were allowed to adsorbed on cell for I hour in CO_2 incubator. After that the medium was aspirated from each well and wells were washed thrice. Then double strength maintenance medium ($2\times$ basal DMEM with 1% FBS without antibiotics) with 0.3 % agarose solution was overlaid quickly. After solidification plate was kept in CO_2 incubator at 37°C with 5% CO_2 for 4 days to develop visible plaques. After careful removal of solidified media, monolayer was stained with 0.1 % crystal violet methanol solution for 20 minutes and then washed with dripping distilled water. The number of plaques in each well for each plate was counted after drying of plate and PFU was obtained with the use of following formula. PFU /ml = number of plaques × 10 / dilution strength

Real Time PCR of Viral RNA

Viral RNA was isolated from vaccine using purelink DNA/RNA isolation mini kit (Invitrogen, USA) following the manufacture's instruction. Viral RNA was quantified with nanodrop spectrophotometer (Thermo). Vetmax NDV real time PCR kit (Life technologies, USA) was used for the purpose of detection of viral RNA. This Taqman chemistry based kit was used as per manufacturer's instruction. The reaction mixture was added with sample RNAs at the concentration of 100 ng /reaction and then final volume of reaction was made by nuclease free water. The NFW and standard RNA (10000 copies /µl) was used negative and positive control.

The standard RNA was 10 fold serially diluted to get 10^4 to 1 copy of RNA/ µl to obtain standard curve. The Real time PCR machine (Applied Biosystem- 7500) was programmed as per manufacturer instruction. BHQ and VIC were included as reporter and quencher dye and annealing temperature was set at 55°C. Amplification graphs were visualized after completion of reaction. Both presence and copy number was determined using standard curve of NDV RNA. PCR products were electrophorsed to confirm the specific PCR amplification of targeted gene.

RESULTS AND DISCUSSION

When one vial of vaccine was reconstituted in 1 ml of sterile PBS, its 25 µl original suspension gave the titre of 1:128 across the dilutions (Fig. 1). On technical ground, consistent HA obtained after incubation of plate at 4°C/ 1 hr rather than for 30 minutes at room temperature. HI test with known serum was found to inhibit the haemagglutination activities of virus which reconfirmed the virus identity.

HA test is a very simple yet reliable way to detect the presence of NDV and recommended as diagnostic test by OIE (OIE, 2012). This property is based upon the presence of haemagglutinin neuraminidase peplomers which facilitate the entry of virus in the host cell (Khattar *et al.*, 2009). In connection to oncolytic potential, modification of HN protein is looked upon an important tool in enhancement of oncolytic activity of virus (Vigil *et al.*, 2007).

The titre (1:128) obtained was compared with other quantization techniques like EID_{50} or PFU calculation then the present value was found two log less than reports of Karmakar *et al.*, 2015 (512 : $10^{8.1}$) EID_{50} but seemingly higher than Gopinath *et al.*, 2011 (4: 10^{7}) EID_{50}. The variation might be caused by absolute number of RBC in 1% suspension which acted as second functional factor in the test. As application of this test to virotherapy work, it was used by comparatively fewer workers for dose calculation during virotherapy experiment. Previously, Lehner *et al.*, 1990 and Schlag *et al.*, 1992 etc. used HA titre for virotherapy dose determination or Walter *et al.*, 2012 for *in vitro* cytotoxicity test. But, HA test is considered less sensitive method of enumerating virus particles than PFU (Wilcox, 1959) or EID_{50} and quantitative PCR (Gopinath *et al.*, 2011).

The quantity of virus in a one vial was further determined by calculating $TCID_{50}$ and PFU. This was done by assessing the percent development of CPE on cell culture as per dilution of virus (Table 1, Fig. 2). Thereafter $TCID_{50}$ was calculated as $10^{7.16}$ $TCID_{50}$ for 100 µl or $10^{8.16}$ $TCID_{50}$ / ml. In the dilution range of 10^{-1} to 10^{-5}, countable plaques were obtained at 10^{-4} and 10^{-5} and PFU at highest dilution was calculated as 4.2×10^{7} PFU/ml (Fig. 3).

$TCID_{50}$ and PFU are the widespread means of virus particle enumeration (Chia *et al.*, 2012). As commercially available NDV vaccine vials were intended to use for virotherapy purpose, there might be chances of inadvertent discrepancies in actual viral load and indicated dose on label because of mistakes at production, transport or storage stage etc. so, these exercises confirmed the presence of active virus, their probable numbers and commercial claim. $TCID_{50}$ is dose determination method derived from statistical extension of PFU (Karber, 1931) and it is well applicable to use with CPE producing viruses like NDV (Ahamed *et al.*, 2004).

In the present study, result of PFU was derived as 4.2 $\times 10^{7}$ PFU/ml and as per Poisson distribution curve (Zar, 2010) the relation between PFU and $TCID_{50}$ should be 0.5 to 0.7 $TCID_{50}$ equals to one PFU. On this basis and holding the commercial claim as true, the result fell below to theoretical relationship with marginal error. The cause behind obtaining lower count in PFU experiment may be missing of small plaques during counting or probability that two virus particles produced a coalesced plaque at few places. Further the plaque formation is dependent upon virus strain and indicator cell type (Chia *et al.*, 2012).

For virotherapy work, several researchers such as Silberhumer *et al.*, 2010; Li *et al.*, 2011; Zaher *et al.*, 2013 and Yan *et al.*, 2014 choose PFU as viral counting method. Whereas other workers preferred $TCID_{50}$ for viral dose determination (Buijs *et al.*, 2015).

$TCID_{50}$ is among the most popular viral counting method along with PFU. But this assay is also dependent upon some inherent limitations and major is TCID values are discontinuous and coverage between the class intervals is sometime remain uncovered (Grigorov *et al.*, 2011). *Vis-a-vis* more number of researchers preferred PFU over $TCID_{50}$ as PFU is considered gold standard test for virus enumeration (Chia *et al.*, 2012).

Prior to estimation of test RNA quantity the linear relation between different dilutions of standard NDV RNA molecule (Standard curve) was drawn. The attributes of real time PCR like slope of line, Y intercept, R^2 and efficiency were found as -3.016, 42.521, 1 and 114.59 %, respectively. The C_t values range of standard RNA were 24 and 29 for 10000 and 100 molecules per reaction, respectively. Lower detection limit of assay were 100 molecules per reaction.

As C_t Value of Xeno RNA (10000 molecules/reaction) was found at 23 which equated to the standard RNA Ct Value, therefore, the RNA isolation efficiency was also found to be approximately at 100%. The test RNA gave the least Ct value of 12 and then proportionately increased. As the standard relation between copy number and Ct value is the copy number was estimated to more than 10^{8}/ml (Fig. 4). When PCR product was run the 2% agarose gel and a single band of approximately 128 bp was found which confirmed the specificity of real time PCR for detection of NDV RNA.

Real time PCR technology is the latest method of detection of pathogen including NDV (OIE, 2012). This experiment could detect the presence of NDV in vaccine alongwith estimation of number of virus particles in suspension. This technique is more sensitive than RT-PCR, less time consuming and omits the use of live animal experiment (Gopinath *et al.*, 2011).

In the present study, one step Taqman probe based real time assay was used to detect and quantified the virus. Avoidance of cross contamination occurs during cDNA synthesis in two step based methods is the greatest advantage of such technique (Wise *et al.*, 2004; Farkas *et al.*, 2007). As we could detect 10^{8} EID_{50} of virus at Ct Value 12 by using 10,000 molecules of standard RNA at Ct value-24 which means it was able to detect RNA according to theoretical relation of Ct Value and copy number (change of 2.5 Ct value means change of 10 log of copy number) where standard curve should show slope of -3.2 which was also approximated during the experiment. Comparable results were obtained by Antal *et al.*, 2007 with Lux primers & Lasota strain (Ct value 13 for 10^{8} EID_{50}) and Farkas *et al.*, 2007 (Ct value 13.8 for 10^{8} EID_{50}) with Taqman assay & Mukteshwar strain during the standardization process of their real time PCR. Though, Gopinath *et al.*, 2011 found higher (40- Ct) values with similar EID_{50} of velogenic strains, but they admitted some background signals might be there.

The more important criterion was lower limit of detection which was 100 RNA molecules in the present study. Different workers reported varied detection limit of virus like Gopinath *et al.* (2011) reported it to 10^{4} viruses but Wise *et al.* (2004) and Farkas *et al.* (2007) claimed that 10 viruses could be detected by their methods when using M

gene as target and 1000 when target was F gene. Variations are caused by target gene, primer sequence, real time chemistry and whether plasmid or RNA from biological fluid is used for detection etc. Amplification of one gene product confirmed the specificity of assay also and it might be gene sequence flanked within M gene (Wise *et al.*, 2004) which was used for differentiation of lentogenic strain with other two strains.

Table 1. Per cent CPE scores at different dilution of virus for $TCID_{50}$ calculation

Sl. No	Dilution	Percent CPE in corresponding wells	Numerical score (Percent CPE/10	Proportion to maximum response
1	10^{-1}	100+100+100+100	10+10+10+10	40/40 (1)
2	10^{-2}	100+100+100+100	10+10+10+10	40/40 (1)
3	10^{-3}	100+90+90+90	10+9+9+9	37/40 (0.925)
4	10^{-4}	90+90+90+90	9+9+9+9	36/40 (0.9)
5	10^{-5}	80+90+80+70	8+9+8+7	32/40 (0.8)
6	10^{-6}	70+70+70+70	7+7+7+7	28/40 (0.7)
7	10^{-7}	70+60+70+60	7+6+7+6	26/40 (0.65)
8	10^{-8}	40+50+50+40	4+5+5+4	18/40 (0.45)
9	10^{-9}	20+10+20+20	2+1+2+2	9/40 (0.225)
10	10^{-10}	0+0+0+0	0	0/40 (00)
11	No virus	0	0	0/40 (0)
12	10^{0}	100	1	1
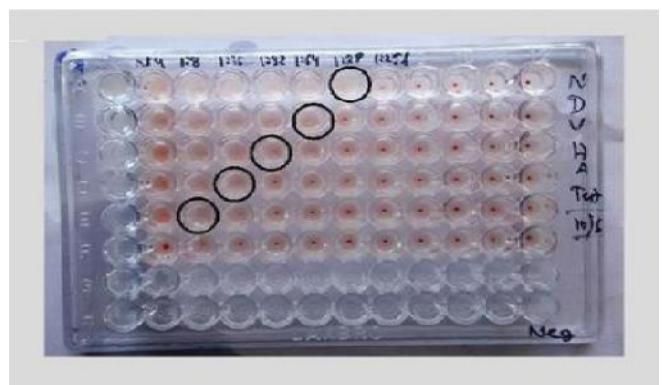
**Fig. 1:** Haemagglutination test of NDV showing titre as 1:128.
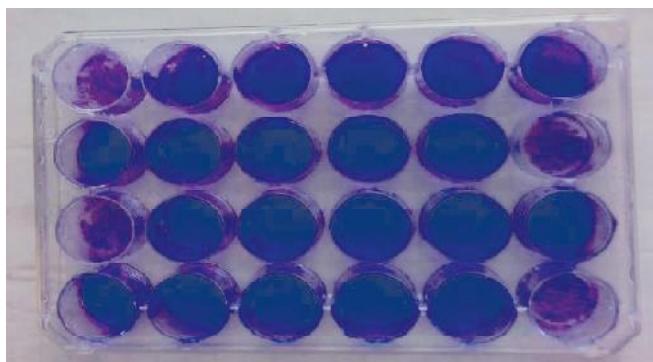
Fig. 2: TCID$_{50}$ calculation of NDV in 24 well cell culture plate. A-1 and C-1 wells having 10^{-1} dilution, B-5 and D-5 used as no virus control and B-6 and D-6 show well with original virus suspension, crystal violet staining.
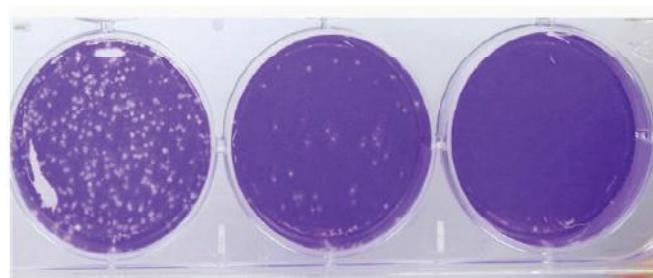

Fig. 3: Plaque forming units calculation of NDV. From left to right dilution are 10^{-4}, 10^{-5} and no virus control, crystal violet staining.
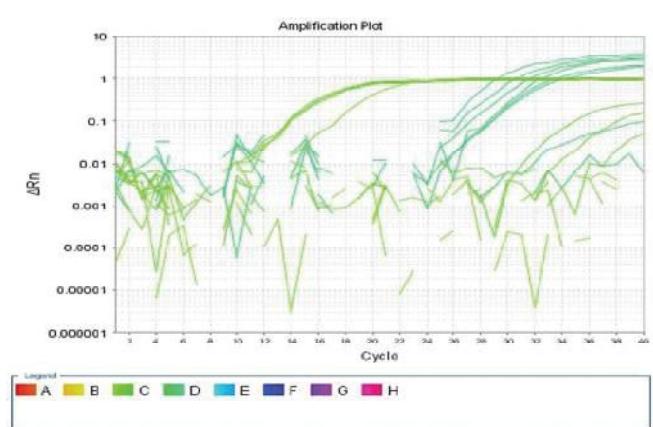

Fig.. 4: Real time PCR of NDV RNA using Taqman assay. Test RNA sample quantity was estimated with standard RNA.

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