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Suitability and Efficacy of *LaSota* Vaccine Strain of Newcastle Disease Virus for *in ovo* Vaccination

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ABSTRACT

Background: Newcastle disease is an important viral disease of poultry which has been causing menace in poultry industry despite routine vaccination. Conventional vaccination strategy has various disadvantages so *in ovo* vaccination approach was adapted for better protection.

Methods: In our study the *LaSota* strain of Newcastle disease virus was propagated in chicken embryo fibroblast till 10 passages and the presence of virus was detected by appearance of cytopathic effect and by PCR. Different dose of vaccine virus was inoculated in 18th day old embryos and their hatch rate and haemagglunition inhition (HI) titre was studied. The viral load was quantified by real time PCR.

Result: It was observed that 1/10000th dose of vaccine virus was suitable for *in ovo* vaccination. The highest viral load was detected in spleen, thymus and lungs indicating that the virus could replicate well in these three organs.

Key words: Chicken embryo fibroblast, *In ovo* vaccination, Haemagglutination inhibition, LaSota vaccine, Real time PCR, Newcastle disease virus.

INTRODUCTION

Poultry industry has been playing a major role in augmenting India's economy and also contributes towards meeting the nutritional requirements of its citizens by egg and meat production (Sharma *et al*., 2023). With the increase in intensive system of poultry farming infectious diseases poses a major problem in poultry production. One such fatal disease is Newcastle disease (ND) which poses a considerable threat to the poultry industry worldwide (Kapczynski *et al*., 2012). The disease is caused by virulent strains of Newcastle disease virus (NDV) (Alexander and Allan, 1974), which belongs to the genus Avulavirus and species avian avulavirus 1 abbreviated as Avian paramyxovirus 1 (APMV 1) (Amarasinghe *et al*., 2017) and is currently classified into the genus *Orthoavulavirus* of the subfamily *Avulavirinae* in the family *Paramxyoviridae* of the order *Mononegavirales* (Rima *et al*., 2019, Akter *et al*., 2023). The disease is highly contagious and they are classified as highly virulent (velogenic), moderately virulent (mesogenic), or low virulent (lentogenic) on the basis of their pathogenicity for chicken (Alexander, 1991). The virus can infect at least 236 bird species, including the vast majority of both wild and domestic bird species (Kaleta and Baldauf, 1988).

Epidemiological studies have revealed that the NDV genome is constantly evolving, with more than 21 genotypes identified thus far; however, regardless of genetic variability, they are all grouped into a single serotype known as avian paramxyovirus type-1 (Dimitrov *et al*., 2019). Due to this property the immunity developed against any virus strain is able to deliver cross protection against another strain (Bello *et al*., 2018). *LaSota* is the most widely used live vaccine strain due to superior immunogenicity. Vaccination failure is common due to non-maintenance of cold chain, poor selection of vaccine strain, insufficient dose, presence of maternal antibody and faulty vaccination schedule (Rathore *et al*., 1987) and also vaccine-induced immunity is short-lived. Parental immunity confer immunity in early stages of life but it can also interfere with vaccine effectiveness (Yosipovich *et al*., 2015). In order to overcome disadvantages in conventional vaccination strategy *in ovo* vaccination technique has been developed that has several advantages over the traditional methods (Sharma and Burmester, 1982; Sharma, 1985, Peebles, 2018). As the name implies, *in ovo* vaccine is administered in embryonated eggs with the help of an in-egg vaccine

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delivery system and soon after hatching birds are protected against the disease. *In ovo* vaccination has proved to be effective against marek's disease virus and infectious bursal disease (Sharma *et al*., 1995; Riaz *et al*., 2004). The efficacy of *in ovo* vaccine developed against NDV with recombinant DNA technology has been explored (Firouzamandi *et al*., 2016). ND DNA vaccine (expressing HN gene) along with *Centella asiatica* extract has been tried with successful delivery and immunoregulation (Subudhi, 2011). *In ovo* administration of lentogenic F-strain of Newcastle disease virus was done and it was observed that the virus replicated in embryos and newborn chicks and did not hamper hatchability, successfully induced antibody response and conferred protection against the disease in broiler birds (Manna *et al*., 2007). Suitability of *LaSota* vaccine strain for *in ovo* vaccination is not yet reported although its efficacy is superior as a lentogenic strain (Khanam *et al*., 2018) which prompted us to explore it for *in ovo* vaccination.

The study was conducted to study the effect of *in ovo* vaccination of *Lasota strain* on hatchability rate, development of immune response and replication of vaccine virus in vaccinated birds. The commercial *LaSota* vaccine strain was passaged in chicken embryo fibroblast cell culture system and the titre of the *LaSota* virus was determined. As the study is a new approach in NDV vaccination dose optimization was crucial for determining the suitable dose of *LaSota* vaccine and the hatchability and immune response was studied.

MATERIALS AND METHODS

Ethical approval

The research procedures were approved by Institutional Animal Ethics committee, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl. The study was conducted as a Phd research work from the year 2019-2024.

Propagation of the *Lasota* vaccine strain in chicken embryo fibroblast (CEF)

A commercial Newcastle disease vaccine was used for adaptation in cell culture system. The primary chicken embryo fibroblast cell culture was prepared from 9-11 day old embryonated White leghorn chicken eggs (Konwar *et al*., 2019). The cell viability test was done using trypan blue exclusion test and the cell suspension was adjusted to concentration of 0.5×10^6 per ml (Strober, 2015) which is standard for 25cc flask and dispensed in cell culture flask with 10 ml of Dulbecco's Modified Eagle's Medium containing 5% foetal bovine serum growth media and incubated at 37°C for 24 hours (Sarmah *et al*., 2024). The confluent CEF monolayer was used for virus inoculation. The cell culture flask thoroughly washed with HBSS and inoculated with 10 µl, 50 µl, 100 µl and 200 µl of virus inoculum per $25cm^2$ cell culture flask and kept at 37°C for 1 hour to facilitate proper adsorption of virus on to the cells. The un-adsorbed viruses were washed with HBSS and maintenance medium containing 2 per cent foetal bovine serum was added to the flask and incubated at 37°C for 3-4 days. Un-inoculated healthy monolayer was kept as control.

Trypsin treatment of *LaSota* virus

The passage 4(P4) virus inoculum was treated with 0.05% and 0.25% trypsin at the rate of 2.5µg/ml of inoculum in different time intervals for 1 hour, 45 minutes, 30 minutes and 15 minutes and was used for infection in different flask respectively. A neat inoculum without any treatment was also passaged in one flask. The time taken for appearance of CPE, degree of cytopathic changes was considered for the selection of virus for further passages.

Infection of the cell culture passaged virus in Chicken embryos

The P4 virus inoculums of 0.05% trypsin treated for 1 hour, 0.25% trypsin treated for 1 hour and neat virus was used for infection in 10day old chicken embryos. The death time of the embryos, types of changes in the embryos were studied for selection of the suitable virus for further propagation in CEF.

Confirmation of the cell culture adapted *LaSota* vaccine strain by Reverse Transcriptase- PCR

The 5^{th} passage and 10^{th} passage of virus in CEF was used for detection by RT-PCR. RNA extraction was done by Quiagen RNA mini kit and for cDNA synthesis was performed as per manufacturer's guidelines (Revertaid First strand cDNA synthesis kit). The RT-PCR was performed using NDV fusion gene published primers (F1- GCAGCTG CAGGGATTGTGGT, F2- TCTTTGAGCAGGAGGATGTTG, amplifying a 356 bp size) (Desingu *et al*., 2014) to amplify F0 cleavage site. The RT-PCR products were analyzed by electrophoresis in 1.5 % agarose gel stained with ethidium bromide (0.5 µg/ml) and amplification bands were observed and recorded in a Gel Doc System.

Quantitation of cell culture adapted virus

$TCID_{50}$ was determined following guidelines of Virology Methods Manual (Brian and Hillar, 1996) by Karber's method. The 10^{th} passaged NDV/LaSota/Neat infected cell culture harvest was processed for $TCID_{50}$. For performing TCID50 cells were cultured in 96 well cell culture plate with 100 µl of growth media per well. Cell free supernatants of virus infected cell culture fluid was used for estimation of virus quantity. A volume of 0.9 ml of diluents was dispensed into nine tubes and labelled. Using a new tip, 0.1 ml of stock virus suspension was added to the first tube. The contents of the first tube were thoroughly mixed by rigorous pipetting. Once mixed, 0.1 ml was withdrawn and transferred to the second tube. The same procedure was followed till all the dilutions (10-1 to 10-9) were completed. Cells were cultured on 96 well micro plate with 100 µl of growth media. After 24 hours, 10 µl of inoculum per well from different dilutions were added to all the wells of column

1-10, undiluted virus in column 11 and column 12 was kept as cell control. The plate was incubated at 37°C in the CO_2 incubator and monitored under inverted microscope daily for 4-5 days for appearance of CPE.

Comparison of 5th passaged neat virus and 10th passaged virus

The 5th passaged virus and 10th passaged virus was inoculated in 10th day old embryos to study the effect of the passaged virus in their hatchability and growth. The difference in the body weight and appearance of the embryo was observed and the suitable virus was used for dose optimization.

Dose optimization of NDV/Lasota vaccine strain for *in ovo* experiment

The 10th passaged chicken embryo fibroblast passaged *LaSota* strain was used for dose optimization for *in ovo* vaccination. Six numbers of experimental groups consisting of 6 embryos per group was used for the trial (Pakgohar and Mehrannia, 2024). *In ovo* vaccination was done as per the standard protocols (Abd El-Ghany, 2025). Vaccination was done with different doses of virus for dose optimisation. After inoculation the hole was sealed with paraffin or wax and transferred to the hatchery. The embryos were observed daily for any dead in shell or difficulty in hatching. The details of the different groups are presented in the Table 1.

***In ovo* vaccination with optimized dose of NDV lasota vaccine strain**

The optimized dose was used for vaccination of 18th day embryos. 30 number of embryos were inoculated with optimized dose of NDV *Lasota* vaccine and 10 embryos were kept as hatch control. The hatch rate, weight of birds at 0 day and HI titre of the vaccinated birds were studied in different time intervals.

Absolute quantification of virus by Real time PCR

Real Time PCR was performed to determine the virus copy number in the 0-day tissue samples of *in ovo* vaccinated chicks. The assay was performed in Applied Biosystem Quantstudio 5 system. The thymus, lungs, spleen, caecal tonsils, bursa and skin samples of 0-day chicks was collected aseptically for RNA extraction in RNALater (Sigma) solution. RNA extraction was done by trizole method and cDNA was prepared by cDNA synthesis kit. The cDNA concentration and purity were studied by using micro drop plate method of detection.

The *Lasota* specific primer sets used in the study were designed using DNASTAR and the primers used were: Lasota F-5′CCTGGGTTGTGATATGCTGTGCTC3′and Lasota R- 5′CCCAGTCCCCGAATAATGTTGTG3'.

Preparation of standard curve

The $TCID_{50}$ of 10th passaged virus in CEF was determined by Karber's method. Serial dilution of virus was done and RNA was extracted and quantified. cDNA was prepared and used for standard curve preparation for Real time PCR (Jonsson *et al*., 2009). Real time PCR was conducted using Maxima SYBR Green qPCR master mix (2x). Each sample was run in duplicate in 10 µl reaction. The reaction mixture consisted of 5 µl of Maxima SYBR Green qPCR master mix, 0.25 µl each of gene specific forward and reverse primers (10 picomol), 3.5 µl of Nuclease free water and 1 µl of template. The PCR was initiated by activation of Taq polymerase by incubating at 95°C for 10 min, followed by 40 PCR cycles including denaturation at 95°C for 15 seconds, annealing at 56°C for 15 sec and extension at 72°C for 15 sec. Ct values were determined and melting curve analysis was performed. After preparation of standard curve, the tissue samples were quantified.

Determination of haemagglutination inhibition (HI) antibody titre against NDV post vaccination

The HI test was performed as per the standard protocol (OIE, 2008). The serum samples of chickens which were collected on 0, 10th, 21st and 30th day post-hatch was used for the detection of antibodies against NDV by hemagglutination inhibition assay. The validity of results was assessed against a negative control serum and a positive control serum.

RESULTS AND DISCUSSION

Propagation of the *Lasota* vaccine strain in chicken embryo fibroblast (CEF)

For propagation of the *LaSota* vaccine strain Chicken embryo fibroblast cells were prepared. After 24 hrs of incubation the CEF monolayers with 60-70% confluency observed under microscope (Fig 1) was inoculated with 10 µl, 50 µl, 100 µl and 200 µl virus inoculums per 25 cm^2

Table 1: Different doses of cell culture passaged vaccine virus and groups used for *in ovo* vaccination.

Group	Dose of vaccine inoculated	No. of embryos
1	1/10th dose of vaccine virus	6
2	1/50th dose of vaccine virus	6
3	1/100th dose of vaccine virus	6
4	1/1000th dose of vaccine virus	6
5	1/10000th dose of virus	6
6	Hatch control	6

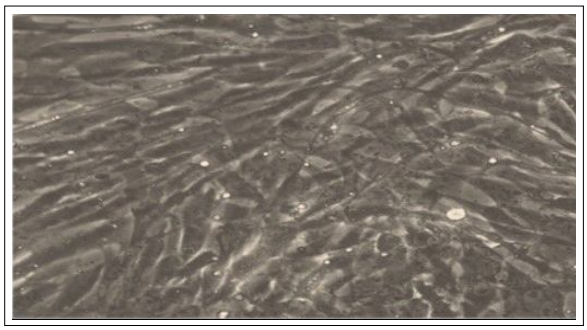


Fig 1: Uninfected cell monolayer of chicken embryo fibroblast after 96 hours.

cell culture flask. It was observed that 10µl of viral inoculums was efficient for production of cytopathic changes as there was appearance of cytopathic changes from the first passage onwards within 96 hours (Fig 2). Typsin treatment was given to passage 4 (P4) with both 0.05% and 0.25% concentration and at different time intervals and compared with neat virus. It was observed that 15 minutes of trypsin treatment is sufficient for appearance of CPE. The characteristic CPE of NDV like syncytia formation, plague formation and ballooning of cells was observed and CPE was comparative in both 0.25% trypsin treated flask and untreated neat virus flask. There was appearance of CPE in both the flask within 72 hours.

For further investigation the trypsin treated cell culture adapted P4 virus and neat virus was inoculated in 10 days old embryos and it was observed that embryo inoculated with 0.25% trypsin treated virus died in 86 hours (Fig 3) and the embryo was stunted with haemorrhagic skin. The embryo inoculated with untreated neat virus died within the same time period but the embryo was cyanosed in appearance and stunted (Fig 3). The 0.05% trypsin treated virus inoculated embryo died on 8th day (Fig 3) and growth was similar to the control embryo (Fig 4). The time taken for appearance of CPE and changes in embryos are depicted in the Table 2. The degree of CPE, time taken for CPE of the trypsin treated virus was comparable with the neat virus, so the untreated virus was used for further passages in cell culture.

The NDV virus Fusion gene specific primers were used for confirmation of the virus in the fifth and eleventh passaged cell culture fluid in CEF. The amplified product had a size of 356 bp (Fig 5).

$TCID_{50}$ of NDV adapted in cell culture

The 10th passaged *LaSota* virus adapted in CEF was used for $TCID_{50}$ calculation by Karber's method was used. The $TCID_{50}$ value was calculated to be $10^{8.11}$/ml (Table 3).

Comparison of 5th passaged neat virus and 10th passaged virus

100 µl of both the passages were inoculated by allantoic route in 10th day old embryos. It was observed that embryos of both the groups were alive and on 18th day both the group was harvested. The P5 infected embryos (Fig 6) were stunted in growth in comparison to the control (Fig 7) and P10 inoculated embryos (Fig 8). In case of P10 infected embryo the sizes of the embryo was almost similar to the control and the skin was slightly haemorrhagic. Therefore, the P10 virus was used for dose optimization for *in ovo* vaccination as the embryo was comparable with the uninfected control.

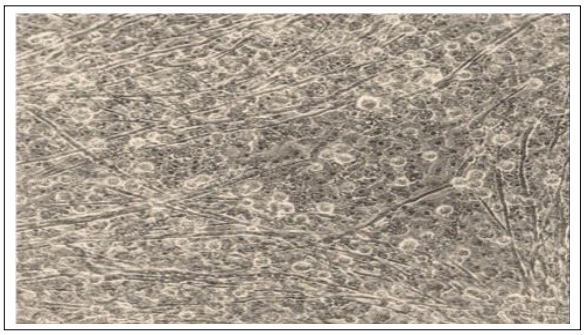
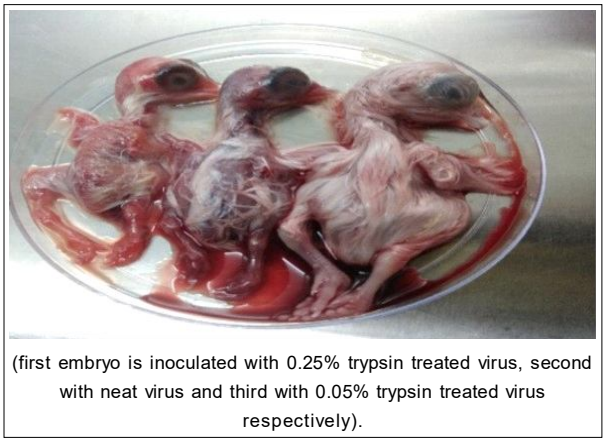


Fig 2: Infected cell monolayer with NDV vaccine virus after 96 hours.



(first embryo is inoculated with 0.25% trypsin treated virus, second with neat virus and third with 0.05% trypsin treated virus respectively).

Fig 3: Depicting the embryos inoculated with NDV by allantoic route.

Fig 4: Control embryo.

Table 2: Comparison between trypsin treated vaccine virus and untreated vaccine virus in CEF culture and embryos.

Virus used for infection in CEF	Time taken for appearance of CPE	Changes in embryos
0.05% trypsin treated P4	96 hrs	Embryo died on 8th day
0.25% trypsin treated P4	72 hrs	Embryo died within 86 hrs, haemorrhagic and stunted growth
Neat inoculums P4	72 hrs	Embryo died within 86 hours and is cyanosed but bigger in size than 0.25%
Control	No changes	Normal growth of embryo

Dose optimization of NDV / *LaSota* vaccine strain for *in ovo* experiment

The 10th passaged *LaSota* vaccine was used for *in ovo* vaccination in 18-day old embryos. The hatch rate obtained on 21st day of incubation in different groups is as follows (Table 4). It was observed that the hatch rate of group 4, 5 was 100% and comparable with hatch control. The 1/10000 dose was considered the suitable dose for *in ovo* vaccination as the hatch rate was 100% and the average body weights of all the chicks were 35-37 gram at hatch which was comparable to the weight of hatch control chicks. The serum samples of all the groups were collected on 0,10th day, 21st day and 30th day post hatch and tested for haemagglutination inhibition titre. The titres are presented in the Table 5. Maternal HI antibodies were detected in all the groups at high levels >6. In all the vaccinated groups the HI titre was >5 (log2) between 7th and 30th day of age demonstrating a strong humoral immune response. In contrast there was less HI titres in the hatch control and seroconversion was observed.

Hatch rate and HI titre after *in ovo* vaccination with optimize dose of NDV / *LaSota* vaccine

The hatch rate of the vaccinated group was 100% and similar to hatch rate of the hatch control group. HI titre of both control and vaccinated group was tested and presented in Table 6. The HI titre of both groups on 0 day was high about 5 log2 but HI titre of control birds started decreasing and was about 2 log2 on 30th day. The vaccinated group maintained a steady decline with days but till 30th day a protective titre was present. The average body weight of all the vaccinated chicks were 35-36 grams which was comparable with the control groups.

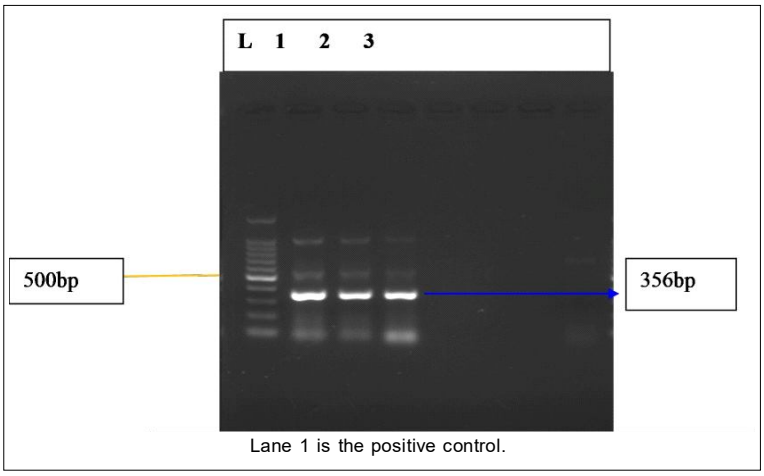


Fig 5: PCR amplification at 356bp which shows the propagation of NDV virus in 5th (lane 2) and 11th (lane 3) passaged CEF.

Table 3: Titration of 10th passaged NDV virus.

Dilution	CPE	No CPE	Cumulative total			CPE%
			CPE	No CPE	Total	
10^{-1}	8	0	53	0	53	100
10^{-2}	8	0	45	0	45	100
10^{-3}	8	0	37	0	37	100
10^{-4}	8	0	29	0	29	100
10^{-5}	8	0	21	0	21	100
10^{-6}	7	1	13	1	14	92.8
10^{-7}	4	4	6	5	11	54.5
10^{-8}	2	6	2	11	13	15.38
10^{-9}	0	8	0	19	19	0
10^{-10}	0	8	0	27	27	0

Difference of Log = (% showing CPE at dilution immediately above 50% - 50 %)/ (% showing CPE at dilution immediately above 50% - % showing CPE at dilution immediately below 50%).

= (54.5 – 50)/ (54.5 – 15.38) = 0.11

Log_{10} 50% endpoint dilution = Log10 of dilution showing CPE immediately above 50% - (difference of Logarithm × Logarithm of dilution factor).

Log_{10} 50% endpoint dilution = -7 – (0.11×1) = -7.11 Titre of virus/ 0.1ml. Therefore, the virus concentration per ml is $10^{8.11}$ $TCID_{50}$/ml.

Absolute quantification of virus by real time PCR

The Real Time PCR assay could detect upto 10^{-8} dilution of the cDNA. Regression analysis of the Ct values generated by the serial ten-fold dilutions produced a correlation coefficient over 0.991 for the reaction (Fig 9). The viral copy number was analysed from thymus, lungs, spleen, caecal tonsils, bursa and skin samples of 0day chicks and it was observed that highest titre of virus was detected in spleen ($10^{5.8}$), followed by thymus ($10^{5.2}$), lungs ($10^{4.3}$), bursa ($10^{3.7}$), caecal tonsils ($10^{3.5}$) and skin ($10^{2.6}$) (Fig 10).

In India NDV is an enzootic disease and vaccination is the major intervention to control the disease along with other biosecurity measures. The lentogenic strains of NDV such as *Hicthner, B1* and *LaSota* strains were most commonly used live vaccines for the control of the Newcastle disease (Peeters *et al*., 1999). Lasota vaccine strain can provide protection to the birds against various heterologous genotypes (Cornax *et al*., 2012). *In ovo* vaccination technology is a relatively new method and cause earlier stimulation of immunity as well as reduction in chicks handling and labour costs (Sharma and Burmester, 1982). It has been reported that commercial live NDV vaccines are not safe for *in ovo* vaccination due to high lethality for chicken embryos (Kapczynski *et al*., 2012). Different approaches were used to attenuate the live NDV vaccines for *in ovo* use. In this study the *LaSota* vaccine strain of Newcastle disease virus was used for *in ovo* vaccination in White leghorn breed of chickens. The commercial *LaSota* vaccine was initially propagated in chicken embryo fibroblast cell culture system till 10^{th} passage.

Studies has shown that the Mesogenic and velogenic strain of NDV can effectively produce cythopathic effect in various cell culture systems, but lentogenic strains require trypsin for replication in chicken embryo fibroblast or mammalian cell types (Seal *et al.,* 1995; Wambura, 2006). The virulence of NDV virus is determined by the sequence of amino acid at the protease cleavage site of the F precursor (Panda *et al*., 2004, Kochiganti *et al*., 2024). The lentogenic strains contain fewer basic amino acids compared to other strain and it can only be cleaved by trypsin-like extracellular proteases. In our study trypsin treatment was given to passage 4 of virus at the rate of 2.5 µg/ml (Kournkiakis and Fildes, 1988) for different time intervals but we observed that neat virus without any trypsin treatment was capable of producing cythopathic effects similar to the tyrpsin treated virus. This may be due to certain changes in the amino acid sequence of the virus due to repeated passaging in cell culture system, however further research will be required to study the genetic changes. The vaccine virus was propagated till 10^{th} passage level and its $TCID_{50}$ was calculated to be $10^{8.11}$ (Mehrabanpour *et al*., 2007).

In our study optimization of vaccine virus doses was done and the hatchability was studied. Several live vaccinations cannot be delivered *in-ovo*, mostly because the vaccine virus results in significant embryonic mortality (Wakenell *et al*., 1986) , decreased hatchability (Sharma *et al*., 1995) or the development of clinical illness after hatching

Fig 6: P5 inoculated embryo.

Fig 7: Control embryos.

Fig 8: P10 inoculated embryo.

Table 4: Hatch rate of *in ovo* vaccinated embryos in various group.

Group	Dose of vaccine inoculated	No. of embryos	No. of chicks hatched	Hatchability percentage (%)
1	$1/10^{th}$ dose of vaccine virus	6	2	33.33
2	$1/50^{th}$ dose of vaccine virus	6	3	50
3	$1/100^{th}$ dose of vaccine virus	6	5	83.33
4	$1/1000^{th}$ dose of vaccine virus	6	6	100
5	$1/10000^{th}$ dose of vaccine virus	6	6	100
6	Hatch control	6	6	100

Table 5: HI titre of *in ovo* vaccinated birds with different doses.

Group	HI titres on 0 day	HI titres on 10th day	HI titres on 21st day	HI titres on 30th day
1	6.5±0.22	5.83±1.6	5.66±0.25	5.5±0.22
2	6.33±0.21	5.66±0.21	5.5±0.22	5.33±0.21
3	6.22±0.12	5.5±0.22	5.0±0.36	5.16±0.1
4	6.66±0.21	6.16±0.16	5.33±0.21	5.8±0.16
5	6.33±0.42	6.33±0.21	5.83±0.16	6±0.2
6	6.05±0.55	4.16±0.2	3.16±0.21	3±0

Table 6: HI titre of *in ovo* vaccinated birds with optimized dose.

Group	HI titres on 0 day	HI titres on 10th day	HI titres on 21st day	HI titres on 30th day
Control	5.0±0.25	4.5±0.22	3.33±0.21	2.33±0.210
Vaccinated	5.66±0.210	6.16±0.16	5.83±0.16	4.5±0.34

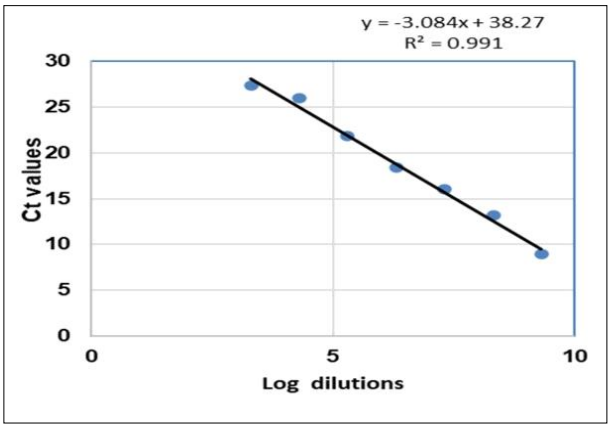


Fig 9: Standard curve showing the correlation between log value of $TCID_{50}$ and Ct values.

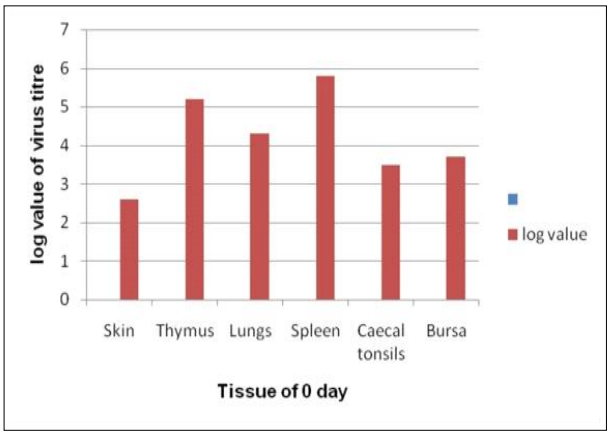


Fig 10: Viral load in various organs of vaccinated chicks.

(Sharma, 1985). The development of a live vaccination for *in-ovo* use depends on choosing a highly attenuated virus strain, virus modification and inoculum dose that have the least detrimental effects on embryos or newborn chicks (Okwor *et al.*, 2014). It has been proved that *in ovo* vaccination in appropriate dose does not adversely affect the hatchability of chicks (Riaz *et al.*, 2004). Manna *et al.*, 2007 reported that when high dose of Lentogenic F strain vaccine was used singly, the hatchability was affected and, in the present study, we have observed that in 1/10th ,1/50th and 1/100th dose of virus the hatch rate was less. When high dose of Lentogenic vaccine strain is inoculated the virus replicates very aggressively in various organs and as the immune system of the chicks are still developing there is very less development of immune response and causes mortality of the embryos in shell or known as dead in shell. There was 100% hatch rate when an inactivated ND vaccine was used *in ovo* (Baksi *et al.*, 2017). This indicated that *LaSota* strain could be used safely for *in-ovo* vaccination with proper attenuation and suitable dose. The optimized dose was used for vaccination in 30 embryos and it was observed that the hatchability was 100% and the average weight of the birds were similar to that of control birds. In the vaccinated groups the HI titre was >5 (log2) between 7 and 30 days of age demonstrating a strong humoral immune response (Kapczynski *et al.*, 2012). In contrast there was less HI titres in the hatch control and seroconversion was observed due to virus shedding (Manna *et al.*, 2007). The optimized virus dose was sufficient to induce high protective titre till 30th days post hatch and similar observation were found by Okwor *et al.* (2014) and Baksi *et al.* (2017).

Real Time PCR was performed and it was observed that the highest viral load was detected in spleen, thymus and lungs indicating that the virus could replicate well in these three organs (Ailing *et al.*, 2014). The virus was also detected in skin which indicates the virus replication in the fibroblast cells as the virus was passaged in CEF.

CONCLUSION

In ovo vaccination technique has proved to be beneficial over conventional vaccination methods. In India *in ovo* vaccination is not a common practice compare to foreign countries. In this study we have propagated the *lasota* vaccine strain in CEF cell culture till 10th passage level and made it suitable for *in ovo* vaccination. Dose optimization is crucial for *in ovo* vaccination and satisfactory hatch rate and immune response were elicited when proper vaccination dose was used. In the study conducted, 1/10000th dose of *Lasota* strain virus was found to be suitable

for *in ovo* vaccination. However large-scale field trials and vaccination trials in both layers and broilers will provide important data on the feasibility of the vaccines commercially. A challenge study will provide more knowledge regarding the immune-mechanism of *in ovo* vaccine optimized in this study.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Author's contribution

All the Authors have equally contributed for the research article.

Conflict of interest

The authors declare that there is no Conflict of Interests regarding the publication of this research article

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