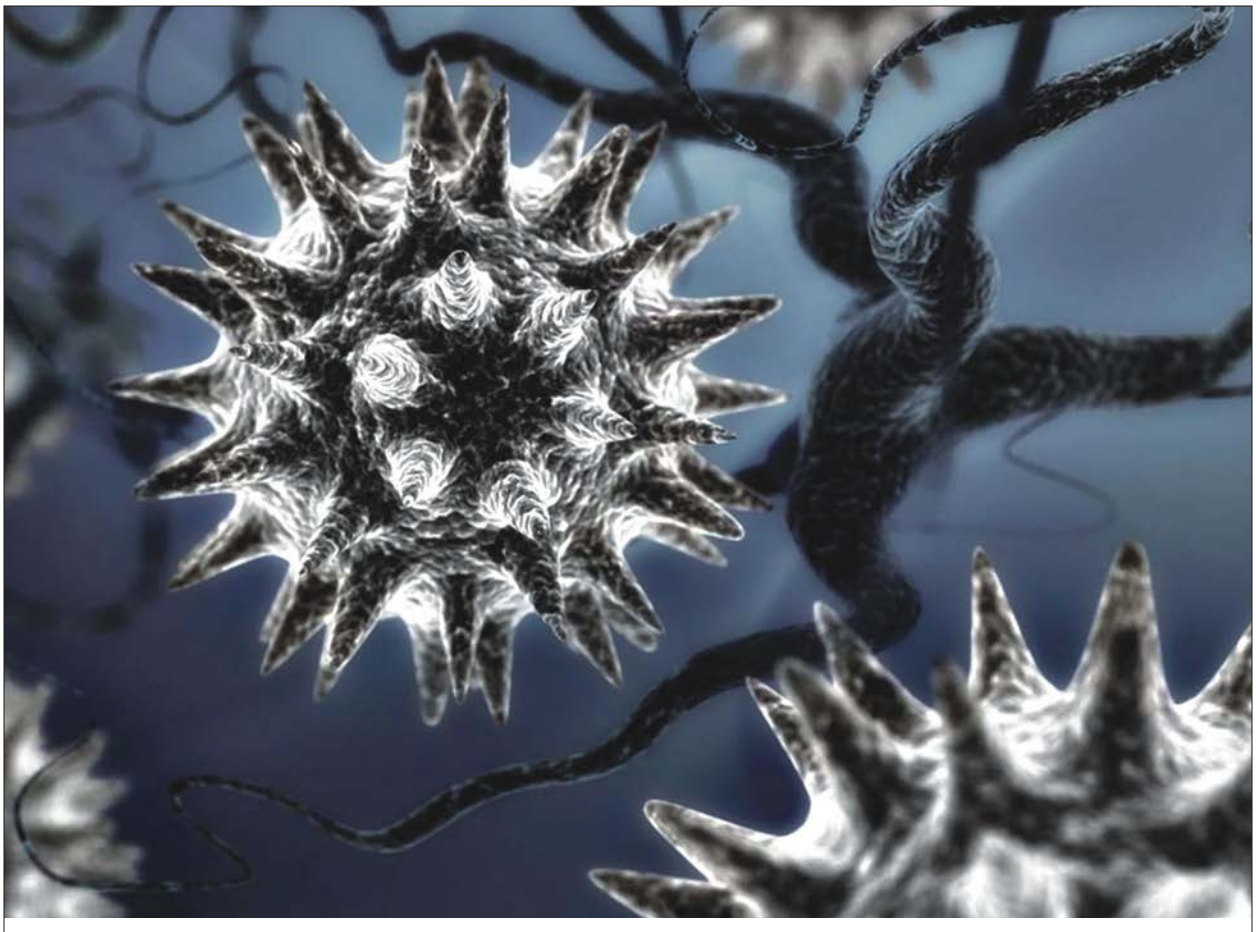

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Research Article
Comparative Study on Virus Inoculation Routes in Embryonated Chicken Eggs for Duck Plague Vaccine Production

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Abstract

Background and Objective: Duck Plague, caused by duck plague virus (DPV), leads to severe outbreaks in ducks, impacting poultry industries. Embryonated chicken eggs (ECEs) are widely used for DPV cultivation, but the optimal inoculation route remains unclear. This study evaluated the chorioallantoic membrane (CAM) and allantoic cavity (AC) inoculation routes in ECEs to optimize DP vaccine production by assessing harvested fluid yield and viral concentration from April to June, 2024. **Materials and Methods:** Ten days old ECEs were divided into six groups: E1, E2, E3, E4, E5 and E6. Each group had two categories: The CAM and the AC route of inoculation. Chicken fertile eggs were collected from the Government Central Poultry Farm, Mirpur and the Government Poultry Farm, Savar. The DPV, produced by the livestock research institute (LRI) at Mohakhali, had a titer of $10^{3.5}$ chicken embryo lethal doses 50 (ELD_{50}) per 0.1 mL used for inoculation. The virus concentration in harvested fluid was measured by titration. The mean virus concentration and harvested fluid amount from CAM and AC inoculation routes were analyzed using a paired t-test and chi-square test in SPSS (Excel Window 16), with significance set at $p \leq 0.05$. **Results:** The mean volume of harvested fluid for the CAM and AC routes of inoculation was 129.17 mL and 199.17 mL, respectively ($p<0.05$). The virus concentration of harvested fluid for the CAM route category in six groups, E1, E2, E3, E4, E5 and E6 was $10^{5.83}$, $10^{5.69}$, $10^{5.65}$, $10^{5.62}$, $10^{5.87}$ and $10^{5.42}$, respectively. On the other hand, the virus concentration of harvested fluid for the AC route category in six groups, E1, E2, E3, E4, E5 and E6 was $10^{4.71}$, $10^{5.17}$, $10^{4.51}$, $10^{4.54}$, $10^{4.55}$ and $10^{4.69}$, respectively. The mean virus concentration of harvested fluid in the CAM and AC route was $10^{5.68}$ and $10^{4.69}$, respectively ($p<0.05$). **Conclusion:** The CAM route of inoculation revealed a bit lower amount of fluid but had 10 times higher virus concentration than that of the AC route of inoculation, which could be beneficial for DP vaccine production.

Key words: Duck plague, inoculation routes, chorioallantoic membrane, allantoic cavity, harvested fluid, vaccine production

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The practice of duck farming is becoming more common among people in Bangladesh. Ducks have been traditionally raised in free free-ranging system as backyard poultry since ancient times. There are 68.26 million ducks in Bangladesh, which ranks second in poultry population[1]. Ducks are thought to be a very resilient bird. The duck plague virus (DPV), also known as duck viral enteritis, is very harmful to the ducks. The DPV is a double-stranded DNA virus belonging to the Herpesviridae family[2]. The etiological agent is a highly pathogenic and infectious pathogen for all age groups of ducks, which has been documented over the last decades[3], with high rates of morbidity and mortality in the poultry industries in Bangladesh[4]. Ducks that have recovered from DP disease become carriers and resistant to the disease; they can still shed DPV and spread the disease to susceptible ducks[5]. DPV was successfully isolated and characterized in Bangladesh[6] and outbreaks of the virus occur from March to June every year. The primary factor of spreading disease or an outbreak is interaction between a contaminated environment and susceptible ducks[5]. The DPV can cause substantial economic losses due to high mortality rates, reduced egg production and impaired meat quality. As a result, the vaccine plays a crucial role in preventing and controlling the spread of the virus. Ducks can be immunized against the DPV using either a live attenuated vaccine, which provides a strong immune response or an inactivated vaccine, which is safer for selective populations[7]. A live attenuated DP vaccine lowered the virulence of DPV by exposing it through multiple chicken embryos[8]. Such a live attenuated DP vaccine contained a minimum virus concentration of 10^3 embryo Lethal Doses 50 (ELD_{50}) per dose[9]. There are some comparisons among different routes of inoculation for virus replication[10]. The DPV can successfully replicate in the chorioallantoic membrane (CAM) and allantoic cavity (AC) route of inoculation in embryonated chicken eggs (ECEs)[9,11]. Though the AC route of inoculation is the easiest method for vaccine production, this method is commonly used for DPV propagation[12]. It is necessary to select a beneficial route of inoculation where the harvested fluid (embryo, fluid and chorioallantoic membrane) contains a high virus concentration. Highly virus-concentrated harvested fluid is needed for the voluminous production of the DP vaccine. Voluminous DP vaccine decreases the disease outbreak and economic losses. However, there is no extensive research done about comparing the CAM and AC route of inoculation for DP vaccine production. This research proposes for selection of a beneficial route of inoculation of DPV by comparing the fluid yield and virus concentration obtained in the chorioallantoic membrane (CAM) and allantoic cavity (AC) routes of inoculation for DP vaccine production.

MATERIALS AND METHODS

Study area: The study was conducted at the DP vaccine production section, Livestock Research Institute (LRI), Mohakhali, Dhaka, during April to June, 2024.

Ethical approval: This experiment is related to comparing the beneficial route of virus inoculation for DP vaccine production and was conducted according to the guidelines of the Experimental Ethics Committee of Livestock Research Institute.

Virus antigen: A lyophilized DPV working seed stored at -40°C was used for experimental trials. It had a titer of $10^{3.5}$ chicken ELD_{50} per 0.1 mL. Working seed virus produced by the DP Vaccine Production Section, LRI, Mohakhali, Dhaka, was free of bacterial and fungal contamination as determined by culture on nutrient agar and Sabouraud dextrose agar. Penicillin, gentamycin, streptomycin and antimycotic were added to the stock during egg inoculation.

Selection of ECEs: Ten days old ECEs were used for this experiment. Fertile chicken eggs were maintained in an incubator that provided stable humidity (45-60%) and temperature (36.5-37.5°C). Fertile chicken eggs were obtained from the Government Central Poultry Farm, Mirpur and the Government Poultry Farm, Savar.

Experimental design: A total of 366 fertile chicken eggs were collected from the Government Central Poultry Farm, Mirpur and divided into three groups: E1, E2 and E3 (Table 1). Each group had two categories. Category 1 was for the CAM route and Category 2 was for the AC route of inoculation. Each category contained 30 eggs for inoculation and 31 eggs for titration. Eggs were washed with Timsen disinfectant powder. Two grams of Timsen powder were mixed with 1 L of water to prepare the disinfectant solution. Eggs were submerged for 10 sec and air-dried. Then Eggs were placed in an incubator at 36.5-37.5°C for 10 days. Ten days old ECEs were used for inoculation either by CAM or AC route after candling. Eggs and equipment were disinfected with a 70% ethanol spray. Dead ECEs were discarded after 24 hrs of inoculation. A similar experimental design was also applied to 366 ECEs in three groups, E4, E5 and E6, being collected from the Government Poultry Farm, Savar (Table 1).

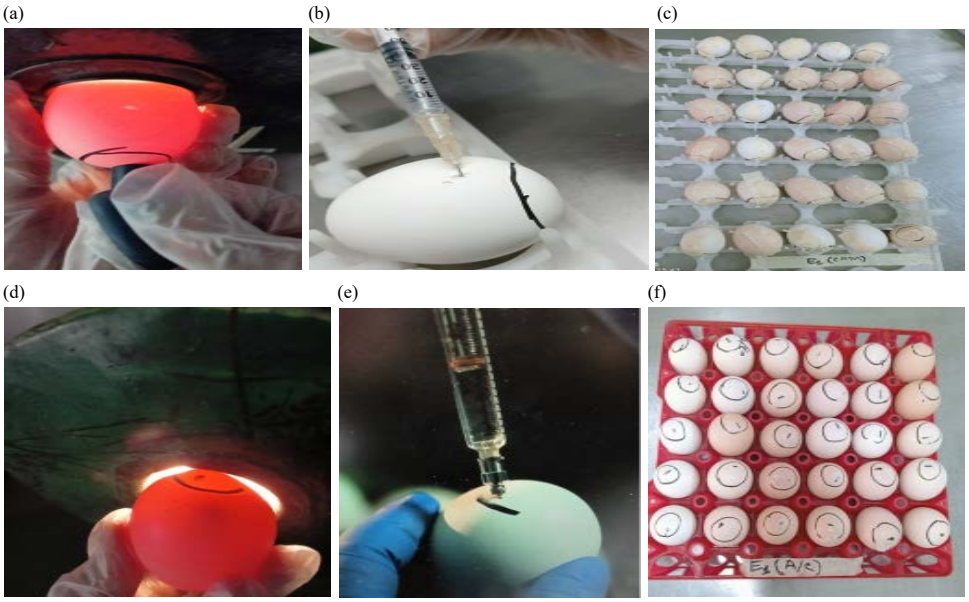

Fig. 1(a-f): DPV inoculation process, (a-c) Egg candling, artificial sac making, inoculation and sealing for the CAM route and (d-f) Egg candling, inoculation and sealing for the AC route

Table 1: Experimental design to compare the different routes of virus inoculation in ECEs from April, 2024 to June, 2024 for DP vaccine production

Farm	Group	Subgroup (n = 30)	Titration (n = 31)	ECEs in each subgroup
Mirpur	E1	E1 (CAM)	E1T1	61
		E1 (AC)	E1t1	61
	E2	E2 (CAM)	E2T2	61
		E2 (AC)	E2t2	61
	E3	E3 (CAM)	E3T3	61
		E3 (AC)	E3t3	61
Savar	E4	E4 (CAM)	E4T4	61
		E4 (AC)	E4t4	61
	E5	E5 (CAM)	E5T5	61
		E5 (AC)	E5t5	61
	E6	E6 (CAM)	E6T6	61
		E6 (AC)	E6t6	61

Chorioallantoic membrane (CAM) and allantoic cavity (AC), Groups: E1 E2, E3, E4, E5 and E6

Routes of DPV inoculation: The DPV inoculation was attempted in chicken eggs by both CAM and AC routes (Fig. 1).

- **CAM route:** Ten to eleven days old ECEs were candled; the air sac and the penetrating area, avoiding blood vessels, were located (Fig. 1). The air sac region and the penetrating area were sterilized with 70% ethanol. With the help of an egg drilling machine, a hole was made in the penetrating area so as not to puncture the shell membrane and another hole was made on the eggshell over the air sac. A drop of PBS was placed in the hole of the penetrating area. An artificial air sac was made while applying negative pressure with a rubber pump onto the natural air sac hole. Then, 0.1 mL of inoculum was inoculated through the CAM route. Both openings were sealed using wax material. Control eggs were treated with 0.1 mL PBS instead of the inoculum. Eggs were incubated at 36.5-37.5°C for 48-96 hrs. After inoculation, ECEs were observed twice daily. Dead ECEs were discarded within 24 hrs
- **AC route:** Ten to eleven days old ECEs were candled to locate the air sac (Fig. 1a-f). The air sac area was sterilized with 70% ethanol. A hole was made over the air sac using an egg drilling machine. Then 0.1 mL of inoculum was inoculated using a tuberculin syringe and 22 22-gauge needle. The opening site was sealed with wax material. Control eggs were similarly treated with 0.1 mL PBS instead of inoculum. Eggs were incubated at 36.5-37.5°C for 48-96 hrs. After inoculation, ECEs were observed twice daily. Dead ECEs were discarded within 24 hrs

Fluid harvation: The ECEs were disinfected at the air sac region. The air shell was cut using sterile scissors and collected harvested fluid (embryo, fluid and CAM) using sterile forceps. The collected harvested fluid was stored at -40°C in a 3 mL vial for further study.

DNA extraction and PCR: The DNA extraction kit Wizard® (Promega, United States) was used to extract DNA. The primers were used for making copies of the specific DNA segments of the DPV. A 25 µL reaction mixture was prepared by mixing Buffer (12.5 µL), nuclease-free water (3.5 µL), probe (1 µL), forward primer (1 µL), reverse primer (1 µL) and DNA template (5 µL). The following thermal conditions were used to amplify DNA polymerase chain reactions, after adding all materials: An initial denaturation at 94°C for 2 min, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C and 2 min at 72°C, with a final extension at 72°C for a total of 7 min.

Virus concentration: The ELD_{50} per 0.1 mL of the DPV from the harvested fluid was determined following the method of Tripathy *et al.*[13]. A ten-fold serial dilution of 1 mL harvested fluid of each subgroup was made (10^{-4}-10^{-9}) and 5 ECE were inoculated with 100 µL of the diluted virus through the CAM route of inoculation; each group of 5 ECE was inoculated with each virus dilution. Then all ECEs were incubated in an egg incubator at 36.5-37.5°C and observed twice daily for 3 to 4 days. Death patterns were recorded and the 50% chicken embryo lethal dose (ELD_{50}) was calculated by the method of Reed and Muench[14].

Statistical analysis: Results of the mean value of virus concentration and amount of harvested fluid in the CAM route of inoculation and AC route of inoculation were analyzed by paired t-test and chi-square test for statistical significance using the Statistical Package for Social Science (SPSS) for Microsoft Excel window 16. A p-value of $\leq$0.05 was considered significant.

RESULTS

Detection of DPV by PCR: The extracted DNA samples were used to amplify DNA Polymerase genes by Polymerase Chain Reaction (PCR). All the positive samples (6) showed the expected amplicon size of 446 bp (Fig. 2).

Virus propagation: The propagation of the DPV was confirmed by detecting lesions after 53-57 hrs of inoculation on CAM and the embryo. The CAM (Fig. 3a-b) and embryo (Fig. 4a-c) were hemorrhagic, necrotic and edematous, that was compared among the CAM route, AC route and normal category.

The amount of harvested fluid from both the CAM route and AC route of inoculation categories is presented in Table 2. The t-test shows a significant difference between the mean values of CAM and AC fluid volume. The t-statistic is much higher than the critical values for both one-tailed and two–tailed tests. The very low p-values ($p<0.05$ for one-tailed and two-tailed) indicate that this difference is statistically significant. Hence, reject the null hypothesis of equal means and conclude that there is a significant difference in the mean values of CAM and AC fluid volume.

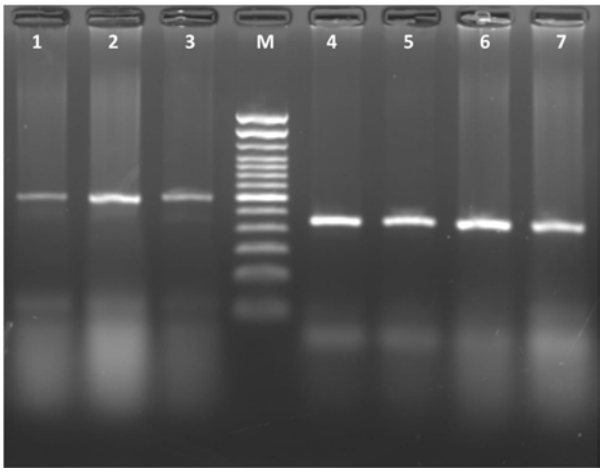

Fig. 2: Representative PCR for the DPV
Lane 1-3 and 5-7: Positive samples, Lane 4: Positive control and Lane M: Ladder 100 bp

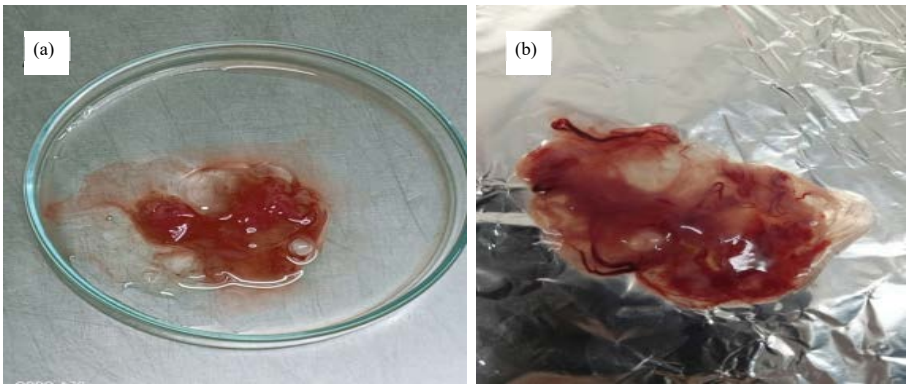

Fig. 3(a-b): Chicken chorioallantoic membrane (CAM), (a) Normal CAM in the control group and (b) Degenerative blood vessels and edematous CAM in 13 days old embryos

Fig. 4(a-c): Chicken embryo condition, (a) Normal embryo in the control group and (b-c) Hemorrhagic and edematous embryos at 13 days old

Table 2: Amount of harvested fluid from different routes of inoculation

			Fluid volume (mL)		Mean volume (mL)	
Farms	Groups	ECEs (n)	CAM route of inoculation	AC route of inoculation	CAM route of inoculation	AC route of inoculation
Mirpur	E1	30	50	250	129.17	299.17
	E2	30	200	375		
	E3	30	180	300		
Savar	E4	30	70	280		
	E5	30	155	320		
	E6	30	120	270		

Chorioallantoic membrane (CAM) and allantoic cavity (AC), Groups: E1 E2, E3, E4, E5 and E6

Table 3: Concentration of live attenuated DPV in ECEs obtained from titration

			ELD_{50} per 0.1mL		Mean Value	
Farms	Group	ECEs (n)	CAM route of inoculation	AC route of inoculation	CAM route of inoculation	AC route of inoculation
Mirpur	E1	31	$10^{5.83}$	$10^{4.71}$	$10^{5.68}$	$10^{4.695}$
	E2	31	$10^{5.69}$	$10^{5.17}$		
	E3	31	$10^{5.65}$	$10^{4.51}$		
Savar	E4	31	$10^{5.62}$	$10^{4.54}$		
	E5	31	$10^{5.87}$	$10^{4.55}$		
	E6	31	$10^{5.42}$	$10^{4.69}$		

Chorioallantoic membrane (CAM) and allantoic cavity (AC), Groups: E1 E2, E3, E4, E5 and E6

The results of titration in both CAM and the AC route of inoculation categories are presented in Table 3. It shows that the highest virus concentration of the CAM route of inoculation categories was $10^{5.87}$ in the fifth group (E5) among six groups and the AC route of inoculation categories was $10^{5.17}$ in the second group (E2) among six AC subgroups. The t-test shows a significant difference between the mean titration values of the CAM route and the AC route of inoculation. The t-statistic of -5.0000 is beyond the critical values for both the one-tailed and two-tailed tests. The very low p-values ($p<0.05$) indicate that this difference is statistically significant. Therefore, reject the null hypothesis of equal means and conclude that there is a significant difference in the mean values of the titration of harvested fluid of CAM route and AC route of inoculation categories.

DISCUSSION

Live attenuated DP vaccine effectively controls the DPV outbreak internationally[15,16]. But there was no study comparing the route of inoculation of DPV for DP vaccine production. This study is the first step for the selection of a beneficial route of inoculation on the basis of the amount of harvested fluid and virus concentration for DP vaccine production.

During the period of this present investigation, the CAM and AC route of inoculation categories of ECEs from the Government Central Poultry Farm, Mirpur and the Government Poultry Farm, Savar, were titrated. Table 3 showed that the highest virus concentration of the CAM route of inoculation category was $10^{5.87}$ in the fifth group (E5) among six groups and the AC route of inoculation category was given as $10^{5.17}$ in the second group (E2) among six AC subgroups, where it was the highest virus concentration. The mean value of virus concentration in the CAM route and the AC route of inoculation categories was $10^{5.68}$ and $10^{4.695}$, respectively. There was a significant difference ($p<0.05$) in the mean values of the titration of harvested fluid when inoculated via CAM and AC routes. In our research, we found live attenuated DPV grew well in ECEs, which was dissimilar from the observation of Panickan[17]. This researcher found that there was no virus replication in the CAM or AC route of inoculation in ECEs. The CAM route was a suitable route for the virus replication with the highest virus concentration, which was Similar to the findings studied by some researchers[18,19]. Kong *et al.*[20] noted that ECEs died when they were inoculated with DPV through the CAM route of inoculation compared with the AC route of inoculation, as virus distribution was higher in the CAM route of inoculation. The AC route of inoculation of chicken embryos was unsuccessful in this investigation, as we searched for a beneficial route of inoculation for vaccine production. However, some researchers grew well in the AC route of inoculation of ECEs[20]. Butterfield *et al.*[21] noted that virulent DPV inoculating material gave given highest virus growth in the AC route of inoculation, but this route of inoculation may be employed for the production of antigenic materials only where the CAM is still the route of choice. However, Kong *et al.*[20] observed that the virus concentration where the DPV replicated better in the AC route than the CAM route of inoculation, even not using the virulent inoculating antigen. Although the mean volume of fluid obtained from the CAM route of inoculation was lower than that of the AC route of inoculation, the mean titration value of the CAM route of inoculation category was higher than the mean titration value of the AC route of inoculation.

CONCLUSION

The harvested fluid obtained from the CAM route of inoculation revealed a bit lower volume compared to the AC route. However, the DPV concentration in harvested fluid obtained from the CAM route of inoculation was ten times higher than fluid received from the AC route. Therefore, the CAM route of inoculation could be beneficial for increased production of the DP vaccine when the virus concentration is adjusted by adding stabilizers.

SIGNIFICANCE STATEMENT

This study evaluated the fluid yield and viral concentration from the CAM and AC routes of inoculation in ECEs to optimize DP vaccine production. The mean volume of harvested fluid for the CAM route was a bit lower than that of the AC route of inoculation. However, the CAM route revealed a much higher virus concentration than that of the AC route of inoculation. This result suggests that the CAM route could be beneficial for DP vaccine production if the virus concentration is adjusted by adding a stabilizer.

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