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Evaluation of optimum thawing temperature of the cell associated HVT+SB-1 bivalent vaccine

Abstract

The present study reports the determination of optimum thawing temperature and holding period of the Mareks Disease (MD) cell associated vaccine after reconstitution by plaque assay. The highest titre of MD HVT+SB-1 bivalent vaccine was obtained at the thawing temperature of 35°C for 45 sec followed by 26°C for 45 sec, 20°C for 60 sec and 40°C at 45 sec; when the vaccine was held on ice after reconstitution. The difference in virus titre among 30, 90 and 120 min holding period at 26°C and 35°C thawing for 45 sec was significant (P≤0.001). However the variation in titre between 30 and 90 min holding period was not significant for thawing temperatures of 20°C and 40°C (P>0.05). No plaques were seen when the bivalent HVT+SB-1 vaccine was thawed at 45°C for 45 sec. The titre obtained at 40°C was extremely low after a holding period of 120 min. After thawing at 35°C for 45 sec and when the reconstituted vaccine was held at RT, there was a drastic reduction in virus titre by 120 min. The study revealed that thawing at 20°C and 40°C for 45 sec would severely lower the titre of HVT+SB-1 vaccine. The complete loss of virus titre was seen at thawing temperature of 45°C for 45 sec. The study reasserts the importance of maintaining the cold chain, appropriate thawing temperature and holding period after reconstitution of HVT+SB-1, a cell associated MD vaccine in maintaining the requisite PFU/dose of vaccine.

Keywords: MD cell associated vaccine, plaque assay, thawing temperature

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Introduction

Marek's disease (MD) is a highly contagious lympho proliferative viral disease of chickens (*Gallus gallus domesticus*) caused by serotype 1 of Marek's disease virus (MDV) (family *Herpes viridae,* subfamily *Alphaherpes virinae,* genus *Mardivirus*). The disease is characterized by paralysis, neurologic signs, and the rapid onset of T-cell lymphomas in chicken. It is a significant concern in commercial poultry production due to its high prevalence rate and contagious nature. Several types of MD vaccines are in common use, both individually and in various combinations. The most widely used vaccines are attenuated serotype-1 MDV and naturally avirulent HVT or serotype-2 viruses. The serotype-2 virus was able to protect against pathogenic strains of MDV 1 on its own, but when administered with serotype-1, it had synergistic activity, providing improved protection.[1] MD vaccines are amongst the most delicate vaccines in veterinary use and this is especially the case with the widely used cell-associated MD vaccines since they need to be maintained at -196°C during transport and until used. Improper handling of the cell associated vaccines would lead to vaccination failures.[2] Thawing temperature and holding period of the vaccine after the dilution of vaccine constituents are important components of handling of MD cell associated vaccine. In order to maintain the potency of the vaccine, it is crucial that the right procedures are followed while thawing and dilution of the cell associated MD vaccine. Thawing the ampoules at temperature of more than 28°C, or prolonged holding can cause reduction in the titre of the vaccine virus.[2] Halvorson & Mitchell[3] reported that even a subtle deviation from the correct handling procedures of MD vaccine can result in 14 to 97 per cent loss of vaccine titer. Gimeno et al.,[4] reported that dilution of MD vaccines could lead to reduced protection against MD, reduced relative body weights, reduced load of vaccine DNA during the first three weeks, and increased field MDV DNA load. Reddy[5] opined that the thawing of the cell associated MD vaccine ampoules should be between 25-28°C and beyond this range will damage cells. The thawing temperature, maintenance of cold chain, holding period of vaccine after reconstitution impact the MD cell associated vaccine virus content per dose. There is a need to optimize the appropriate thawing temperature since the thawing temperature recommended by manufacturers across the globe for HVT and SB-1 live vaccines individually and in combination range from 20°C to 38°C. The current study reports the determination optimum thawing temperature for HVT+SB-1 cell associated vaccine by employing different thawing temperatures and holding periods through plaque assay.

Material and methods

Eggs

One hundred numbers of Specific Pathogen Free (SPF) embryonated chicken eggs were procured from Indovax Pvt. Ltd. Gurgaon, India, for CEF culture.

Vaccines

Commercially available HVT+SB-1 cell associated vaccine

CEF cultures

Primary CEF cultures were prepared are as per the protocol of supplemental assay methods (2005), Centre for Veterinary Biologicals, USDA. In brief, nine to eleven day old chicken embryos

were decapitated, washed in PBS, minced and placed in a trypsinizing flask containing 50 ml growth medium without foetal bovine serum (FBS). After moderate stirring on magnetic stirror, the tissue was completely homogenized and the cells were allowed to settle and the supernatant was decanted. Trypnisation was carried out with 0.25% trypsin, followed by centrifugation at 250 x g at 10°C for 10 min, and the cells were suspended in growth medium. Concentration of the cells was adjusted to one million cells /ml of growth medium and dispensed into tissue culture flasks and incubated for three days at 37°C in an incubator with five per cent CO_2 and 75 per cent humidity to obtain confluent monolayer. Secondary CEF cultures were prepared from these primary monolayers by trypsinizing the cell sheet and resuspending the cells in growth medium to obtain a cell concentration of approximately 3,75,000 cells per ml. Four ml of secondary cell suspension was placed into 60-mm plastic tissue culture dishes and incubated in a humidified incubator at 37°C, with five per cent CO_2 to get confluent monolayers in 24 hour.

Thawing and reconstitution of vaccine

HVT+SB-1 vaccine was taken out from the liquid nitrogen storage container and thawed at 45 and 60 sec at different temperatures ranging from 20°C to 45°C and different holding intervals given in Table 1. Immediately the vaccine was diluted with the diluent, supplied by manufacturer, warmed to RT. It was done gently by aspirating the vaccine into a 10-ml syringe through an 18-gauge needle and then five ml of the diluent was collected into the same syringe and mixed gently. The contents of the syringe were offloaded into the diluent bottle. Two ml from the diluted vaccine was aspirated and used to rinse the ampoule once, then added back to the diluted vaccine. Diluted vaccine was mixed gently by slowly inverting the bottle. This mixture constituted "field strength" of the vaccine equivalent to one dose per 0.2ml.

Table 1 Determination of optimum thawing temperature: different temperatures, duration of thawing and holding period employed

Sl. No.	Thawing temperature	Duration of thawing	Holding period
1	20°C	60 sec	30, 90 & 120min on ice
2	26°C	45 sec	30, 90 & 120min on ice
3	35°C	45 sec	30, 90 & 120min on ice
4	35°C	45 sec	30, 90 & 120min at RT
5	40°C	45 sec	30, 90 & 120min on ice
6	45°C	45 sec	30, 90 & 120min on ice

Holding period

The diluted vaccine was tested for virus titer at different holding temperatures and at intervals of 30, 90 and 120 min on ice cubes. Shortly before the end of the holding period, a two ml of diluted virus was mixed with 8.0ml of growth medium to give an initial dilution of 1:5; further dilutions were carried out to get 1:25, 1:250 and 1:500.

Plaque assay

One ml of each of the final dilutions was seeded into 60 mm Petri dishes containing CEF culture. Five replicates were maintained for each dilution. The entire process of dilution and seeding was completed within two min to prevent cells from attaching to the surface of the dilution tubes. Further, the seeded plates were swirled gently to ensure uniform distribution of virus. The plates were incubated in a 75 per cent humidity atmosphere at 37ºC containing five per cent CO_2. Twenty-four hours post-inoculation, the medium from the plates was removed and replaced with 5ml maintenance medium. The same was replaced on day three. Petri plates with cells alone without seeding the vaccine virus were served as controls to monitor the integrity of monolayer. The plaques were counted on day five post inoculation using an inverted microscope. The cell controls were used to confirm the validity of the assay

Following formula was used to determine the titer (PFU/ml) of the vaccine per dose of 0.2 ml

$$\frac{No.\ of\ plaques \times V}{d \times 5} = PFU\ /\ dose$$

d=dilution factor;

V=volume of diluted virus added to the plate.

Results and discussion

The cell associated HVT+SB-1 bivalent vaccine was titrated in secondary CEF cell culture after thawing at temperatures ranging from 20°C to 45°C. The results are given in the Table 2. The mean PFU/ 0.2ml dose with standard error (SE) obtained at 20°C thawing for 60 sec at a holding period of 30, 90 and 120 min on ice was $2.02x10^3$±37.416, $1.88x10^3$±58.309 and $1.62x10^3$±58.309 PFU respectively. The difference in virus titre was not significant between 30 and 90 min holding period ($P>0.05$) whereas, it was significant between 30 and 90 min and, 90 and 120 min. ($P<0.001$). The values obtained at thawing temperature of 26°C for 45 sec, for similar holding periods were $3.6x10^3$±63.245, $3.2x10^3$±44.721 and $2.92x10^3$±37.416 PFU respectively and the variation in titre was found to be significant among the three holding periods ($P<0.001$). Whereas at 35°C for 45 sec thawing the mean values with SE were $4.28x10^3$±48.989, $3.98x10^3$±86.023, $3.4x10^3$±50.99 for 30, 90 and 120 min of holding time respectively and the difference in the titre between 30 and 90 min was less significant in comparison to the holding period between 30 and 120min. The variation in titer was also significant between 90 and 120 min holding period ($P<0.001$). The mean PFU/0.2ml dose obtained at 40°C thawing for 45 sec at a holding period of 30, 90 and 120 min with SE were $1.46x10^3$±87.177, 1.32 $x10^3$±73.484 and $0.12x10^3$±48.989 respectively and the variation in the titer was not significant between 30 and 90 min ($P>0.05$). However no plaques were seen when the thawing temperature was increased to 45°C keeping the same holding temperatures. After thawing at 35°C for 45 seconds, the diluted vaccine was kept at RT and mean plaques, enumerated at 30, 60 and 120 min were $3.12x10^3$±48.989, $1.8x10^3$±54.772 and $0.84x10^3$ plaques respectively and the variation of the virus titer amongst the three holding periods were highly significant ($P<0.05$). The vaccine titres were significantly different ($P<0.001$) among the different thawing temperatures of 20°C, 26°C, 35°C (held on ice as well as RT), 40°C and 45°C. However, the difference in vaccine titre was insignificant ($P>0.05$) between thawing temperature of 20°C at 90 min holding period on ice and thawing temperature of 35°C at holding period of 90 min at RT. The highest titre of the vaccine was obtained at the thawing temperature of 35°C for 45 sec followed by thawing at 26°C, 20°C and 40°C for 45sec. The titre obtained at 40°C was extremely low after holding period of 120 min. The uninfected CEF monolayer was free from cytopathic changes.

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Table 2 Mean Plaque forming units/0.2ml dose of HVT+SB-1 vaccine at different thawing temperatures and holding period

Holding time on ice	Thawing temperatures and time				35°C, 45 sec
	20°C, 60sec	26°C, 45sec	35°C, 45sec	40°C, 45sec	(holding at RT)
30 min	$^{u}2.02\ x10^{3} \pm 37.416^{a}$	$3.6\ x10^{3} \pm 63.245^{a\ w}$	$4.28\ x10^{3} \pm 48.989^{ax}$	$1.46\ x10^{3} \pm 87.177^{a\ y}$	$3.12\ x10^{3} \pm 73.484^{a}$
90 min	$^{u}1.88\ x10^{3} \pm 58.309^{a\ v}$	$3.2\ x10^{3} \pm 44.721^{b\ w}$	$3.98\ x10^{3} \pm 86.023^{bx}$	$1.32\ x10^{3} \pm 73.484^{bu}$	$1.8\ x10^{3} \pm 54.772^{a}$
120 min	$^{u}1.62\ x10^{3} \pm 58.309^{b\ v}$	$2.92\ x10^{3} \pm 37.416^{aw}$	$3.46\ x10^{3} \pm 50.990^{ax}$	$0.12\ x10^{3} \pm 48.989^{cy}$	$0.84\ x10^{3} \pm 67.8233^{b}$

Note: No Plaques were recorded at thawing temperature of 45°C.

Superscripts a, b and C for within column comparison;

Superscripts u,v,w,x and y for within row comparison;

Mean±SE values within the same column with different superscipts vary significantly (P≤0.001);

Mean ±SE values in row with different superscript vary significantly (P≤0.001).

In the present study, the highest titer for HVT+SB-1 vaccine was obtained at a thawing temperature of 35°C for 45 sec held on ice is in accordance with the findings of Geerligs and Hoogendam (2007) who investigated the effect of the thawing procedure on live virus titer of the CVI988 vaccine and reported that the highest titers were found with diluent at a temperature of 30°C to 37°C. The reconstituted vaccine after thawing at 35°C for 45 sec when held at RT, there was a drastic loss of virus titre at 120 min of holding period, which is in accordance with the observation of Gerlings & Hoogendam[6] who also reported that the diluted vaccine should be stored at cold conditions to prevent the loss of titer. It was found that when the vaccine was kept on ice after thawing at 35°C for 45 sec there was no significant loss of titer up to two hour holding period, which is in agreement with the opinion of Morrow & Fehler[2] who recommended that MD vaccine should be used within two hours after reconstitution. M/s Venkateshwara Hatcheries Pvt. Ltd. which produces HVT+SB-1 vaccine in India recommends that reconstituted vaccine should be stored on ice and to be used completely within 60 min. On thawing at 26°C for 45 sec, there was a significant reduction in virus titer, compared to 35°C; however the vaccine titre was more than the minimum PFU required per bird and still can be used for vaccination. Morrow & Fehler[2] opined that thawing the ampoules at temperatures of more than 28°C, or prolonged incubation before dilution, can cause damage to MD vaccine virus. However, the current study contradicts this opinion, wherein we found thawing at more than 28°C but within the 35°C does not have any deleterious effect on virus activity. HVT+SB-1 bivalent vaccine produced by Bioimmune Company, Kansas USA, recommends thawing temperature of 26.5°C and usage of the diluted vaccine at least within one hr to avoid loss of titer. At 20°C thawing for 60 sec, the virus titre was significantly reduced compared to 35°C and 26°C thawing but still the titer was above the required standard titre per bird recommended by the OIE. It was found that 45 sec duration of thawing at 20°C was not sufficient to completely dissolve the vaccine and minimum of 60 sec duration was required for complete thawing at 20°C. In North America, MD vaccine manufacturing companies prescribe a wide range of thawing temperatures ranging from 15°C to 30°C and it looks the vaccine manufacturers are averse to increasing the thawing temperature more than 30°C fearing the damage to virus and loss of virus titre. Our results showed that 40°C and 45°C thawing for 45 sec is undesirable as it lead to loss of titer, especially at 45°C where there was no viral plaques at all, an indication of loss of viral infectivity which is in agreement with the claims of M/s Venkateshwara hatcheries Pvt. Ltd. India, that thawing at more than 37°C would cause damage to the virus. Fifteenth Western Meeting of Poultry Clinicians and Pathologists held at USA in 2004 has suggested thawing of MD vaccine at around 27°C for 90sec. However in the present study, thawing at 26°C for 45 sec yielded lesser PFU than thawing at 35°C for 45sec. The optimum thawing temperature of 35°C for 45 sec found in the present study is in accordance with the recommended thawing temperature of M/s Venkateshwara hatcheries Pvt. Ltd. in India. In many parts of India, during summer season, the temperature exceeds 40°C and this necessitates proper handling of the MD cell associated vaccines.

Summary

The highest titre of MD bivalent HVT+SB-1 vaccine was obtained at the thawing temperature of 35°C for 45 sec followed by 26°C for 45 sec, 20°C for 60 sec and 40°C at 45 sec when the vaccine was held on ice after reconstitution. No plaques were seen when the bivalent HVT+SB-1 vaccine was thawed at 45°C for 45 sec.

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None.

Conflict of interest

The authors declare that there is no conflict of interest.

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