Immune Response in Chickens Vaccinated with Freeze-Thawed or Warmed Water-in-Oil Vaccine

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This study investigated whether freezing or warming water-in-oil (W/O) vaccines affected the immune responses of chickens. One of the conditions affecting the efficacy of commercially available animal vaccines is the storage temperature range. Previous studies have shown that the properties of some inactivated vaccines change owing to freezing, leading to reduced immune responsiveness after inoculation. In this study, we first determined the freezing temperatures of a commercial W/O vaccine using freezers maintained at −10, −13, −15, and −20°C. The results showed that the W/O vaccine froze from −10 to −12°C. Next, we evaluated the effect on antibody level transitions (sample-to-positive ratio) in 46-day-old broiler chickens vaccinated with the W/O vaccine that was maintained at −20°C, 5°C, and −10°C, in that order. In addition, the effect on antibody value transitions was evaluated in 45-day-old broiler chickens vaccinated with the W/O vaccines that were frozen and thawed between −20°C and 5°C repeatedly or warmed to 42°C. In these experiments, no remarkable effect of the freeze-thawing or warming treatments on antibody value transitions was observed. These results suggested that the efficacy of the W/O vaccine was not significantly affected when placed in a frozen environment or left in a room temperature environment of 42°C or lower for approximately 5 d. These data indicate the possibility of expanding the temperature range for handling W/O vaccines.

Key words: adjuvant, cold chain, freeze-thaw, handling temperature, storage temperature, water-in-oil vaccine

J. Poult. Sci., 59: 378–383, 2022

Introduction

The storage temperature range is specified for commercially available animal vaccines to guarantee their efficacy. However, in Japan, it remains unclear whether these temperature ranges are strictly defined as handling conditions or as guidelines. Additionally, if freezing affects the quality of a vaccine, it must be avoided during handling and storage. In vaccine logistics, it is presumed that most of these precautions are observed; however, it is often impossible to handle vaccines within the stated temperature range. Previous studies have shown that it is difficult to maintain a specified storage temperature range during vaccine transport (Matthias et al., 2007; Kartoglu and Milstien, 2014; Das et al., 2019; Falcón et al., 2020). This is not only a problem in countries with underdeveloped cold chains, but also in countries and regions with developed cold chains (Kartoglu and Milstien, 2014; Falcón et al., 2020). In particular, the risk of inadvertent exposure to freezing temperatures has been overlooked worldwide (Falcón et al., 2020). This issue poses a severe problem for both veterinarians directing the use of vaccines, as well as for vaccine users. Veterinarians should instruct users not to vaccinate animals with these vaccines because they cannot guarantee the quality of vaccines whose storage temperature may have been outside the specified range. Furthermore, in the unlikely event that a handling error compromises the vaccine quality, the user suffers a direct disadvantage.

Currently, water-in-oil (W/O)-type vaccines are widely used as inactivated vaccines in chickens. This type of vaccine induces a strong and long-term immune response with a small amount of antigen (Aucouturier et al., 2001). This study...
determined whether handling W/O vaccines outside the prescribed temperature range affected their efficacy. Specifically, we determined the temperature range in which the commercially available W/O vaccine froze and investigated the changes in its properties after freezing or warming. Furthermore, to confirm the effect on the immune response, antibody value transitions of chickens were compared after inoculation with freeze-thawed, warmed, and untreated vaccines.

Materials and Methods

In this study, the commercially available inactivated combination vaccine for turkey rhinotracheitis (TRT), infectious bronchitis (IB), infectious bursa disease (IBD, Gumboro disease), and Newcastle disease (ND), “Novilis TRT + Ibmulti + G + ND” (500 mL/bottle, MSD Animal Health, Tokyo, Japan) was used as a W/O vaccine. The vaccine was delivered refrigerated by a veterinary drug wholesaler.

The Rakuno Gakuen University Institutional Animal Care and Use Committee approved Experiments 2 and 3 (No. DH20A4, No. DH21A4).

The chickens used in Experiments 2 and 3 were ROSS 308 broilers (45-day-old or 46-day-old) maintained in a floor pen (0.36 m²/bird) filled with sawdust and raised to commercial 25℃. For the experiment, a transponder was embedded in the femoral subcutaneous area was used to identify vaccinated chickens. Serum samples were collected on days 0, 21, 35, and 50 after vaccination, and cryopreserved until antibody testing.

The sample-to-positive (S/P) ratios of anti-ND, anti-IB, and anti-IBD antibodies in each serum sample were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (IDEXX Test Kits for Newcastle Disease Virus Antibody, Infectious Bronchitis Virus Antibody, and Infectious Bursal Disease Virus Antibody, Vaxxinova Japan, Tokyo, Japan).

The S/P ratios were obtained numerically using the following formula:

\[
S/P = \frac{(sample\ OD-negative\ control\ OD)}{(positive\ control\ OD-negative\ control\ OD)}
\]

where OD is the optical density measured using a microplate reader with a 650 nm filter. These ELISA kits interpreted S/P > 0.2 as positive.

Experiment 1. Freezing Temperature of the W/O Vaccine

Ten milliliters of the vaccine were placed in 24 glass test tubes (inner diameter 15 mm and wall thickness 0.8 mm) and an alcohol bar thermometer was inserted into four of them. The 24 vaccine tubes were grouped into four sets of six tubes, with each set containing one tube with a thermometer; the initial temperature was maintained at 5℃. The four sets were placed in freezers maintained at 5℃ and used as the control vaccine (Cont2). The untreated vaccine was stored at 5℃ and used as the control vaccine (Cont2).

Experiment 2. Immune Response to the W/O Vaccine Exposed to −20°C and −10°C

The vaccine was first exposed to −20°C for 12 h and then thawed at 5℃. Next, it was exposed to −10°C for 12 h, thawed again at 5℃, and used as the freeze-thaw-treated vaccine (FT1). The untreated control vaccine was stored at 5℃ (Cont1). The treated and untreated vaccines were prepared the day before inoculation and were warmed to the 20–25°C range before inoculation.

Seven 46-day-old broiler chickens were inoculated with 0.5 mL/bird (one dose) of FT1 and another seven chickens were inoculated with 0.5 mL/bird of Cont1. The inoculation site for the vaccine was the right pectoral muscle (musculus pectoralis). A transponder embedded in the femoral subcutaneous area was used to identify vaccinated chickens. Serum samples were collected on days 0, 21, 35, and 50 after vaccination, and cryopreserved until antibody testing.

The sample-to-positive (S/P) ratios of anti-ND, anti-IB, and anti-IBD antibodies in each serum sample were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (IDEXX Test Kits for Newcastle Disease Virus Antibody, Infectious Bronchitis Virus Antibody, and Infectious Bursal Disease Virus Antibody, Vaxxinova Japan, Tokyo, Japan).

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where OD is the optical density measured using a microplate reader with a 650 nm filter. These ELISA kits interpreted S/P > 0.2 as positive.

Experiment 3. Immune Response to the W/O Vaccines Repeatedly Exposed to −20°C or Exposed to 42°C for 5 d

Experiment 3 investigated the effects of subjecting the vaccine to more severe temperature changes than those in Experiment 2.

In the first treatment, the vaccine was exposed to −20°C for 12 h and then stored at 5°C for 12 h, which was repeated three times, and then was used as the freeze-thaw-treated vaccine (FT2). The second treatment involved exposing the vaccine to 42°C for 5 d, storing it at 5°C, and then using it as the warming-treated vaccine (W). The untreated vaccine was stored at 5°C and used as the control vaccine (Cont2). The treated and untreated vaccines were prepared the day before inoculation and were brought to the 20–25°C range before inoculation.

Fifteen 46-day-old broiler chickens were divided into three groups (n=5) and each group was inoculated with FT2, W, or Cont2. The inoculation site was the right pectoral muscle, and the inoculation amount was 0.5 mL/bird. A transponder embedded in the femoral subcutaneous area was used to identify vaccinated chickens.

Serum samples were collected on days 0, 15, 30, 42, 78, 86, and 98 after vaccination and cryopreserved until antibody testing. The S/P ratios of the anti-ND, anti-IB, and anti-IBD antibodies in each serum sample were measured using the ELISA kits used in Experiment 2.

Experiment 4. Microscopic Observation of Frozen or Warmed Vaccines

The tip of a disposable plastic needle (CS-NEE, Kanto Chemical, Tokyo, Japan) was dipped in FT2, W, and Cont2, and spot-smeared onto glass slides. Cover glasses were placed on the smeared vaccines, which were then observed under an optical microscope (BX41; Olympus, Tokyo, Japan). In addition, to confirm the effect of repeated freeze-thaw cycles on the properties of the vaccine solution, a freeze-thaw was performed 0–5 times at −20°C and 5°C, and the vaccine solutions were observed using an optical microscope, as above.
Statistical Analysis

In Experiment 2, the antibody S/P ratios of chickens inoculated with FT1 and Cont1 were compared using the Wilcoxon rank-sum test for each collection day after inoculation. In Experiment 3, the antibody S/P ratios of chickens inoculated with FT2, W, and Cont2 were compared using the Steel–Dwass test for each collection day. All statistical analyses were performed using R version 4.1.2 (R Foundation for Statistical Computing, 2021). The significance level was set at p<0.05.

Results

Freezing Temperature of the Vaccine

After placing the vaccine tubes in a −20°C freezer, −12°C was attained in 30 min, and five of the six tubes froze. After that, −14°C was attained in 40 min, and all six tubes froze. After placing the vaccine tubes in a −15°C freezer, −10°C was attained within 30 min, and one of the six tubes froze. Subsequently, the temperature reached −12°C in 40 min, and all six tubes froze. In the −13°C freezer, the vaccine tubes attained −12°C in 50 min, and one of the six tubes froze. After 70 min, all six tubes were frozen at −12°C. The vaccine tubes reached −10°C in 60 min in the −10°C freezer and did not freeze for more than 70 min after reaching −10°C. Based on these results, the freezing temperature of the vaccine was estimated to range from 10°C to −12°C.

Immune Response to the FT1 and Cont1 Vaccines

The distribution and transition of antibody S/P ratios (ND, IB, and IBD) by day of serum collection in chickens vaccinated with FT1 or Cont1 are shown in Fig. 1A. The average antibody S/P ratios of ND, IB, and IBD increased with time after inoculation in both FT1 and Cont1 vaccinated chickens. No significant differences in antibody S/P ratios were observed between the FT1 and Cont1 vaccinated chickens on all serum collection days.

Immune Response to the FT2, W, and Cont2 Vaccines

Figure 1B shows the distribution and transition of antibody S/P ratios (ND, IB, and IBD) by day of serum collection in chickens vaccinated with FT2, W, or Cont2.

Transition of the ND antibody S/P ratio: The average ND antibody level in the Cont2 vaccine group peaked 73 d after inoculation and then gradually decreased. In contrast, ND antibody levels in the FT2 and W groups increased until 98 d after inoculation. At 73 d after inoculation, a significant difference was observed between ND antibody levels in the W and Cont2 groups (p<0.05); however, no significant difference was observed beyond 86 d after inoculation. Moreover, no significant difference was observed between the FT2 and Cont2 groups or between the FT2 and W groups on each day of serum collection.

Transition of the IB antibody S/P ratio: IB antibody levels in two birds of the Cont2 group increased gradually until 98 d after inoculation. The IB antibody level in one bird in the FT2 group was elevated slightly, peaked 73 d after inoculation, and then diminished thereafter. The average IB antibody level in one bird from the W group increased rapidly, peaked 30 d after inoculation, and then diminished gradually thereafter. No significant differences were observed between the FT2 and Cont2 groups, W and Cont2 groups, and FT2 and W groups on each day of serum collection.

Transition of the IBD antibody S/P ratio: Average IBD antibody levels increased from 30 d after inoculation to 98 d after inoculation in the Cont2, FT2, and W groups. A significant difference was observed between the FT2 and W groups at 42 and 86 d after inoculation (p<0.05). No significant differences were observed between the Cont2 and FT2 groups or between the Cont2 and W groups on each day of serum collection.

Microscopic Changes between Frozen or Warmed Vaccines and Untreated Vaccines

No visible differences in appearance were observed among the frozen, warmed, and untreated vaccines (Fig. 2A). In addition, no visible differences in the emulsion state were observed, even for the vaccine that was frozen at −20°C and thawed 0–5 times at 5°C (Fig. 2B).

Discussion

Currently, vaccines containing liquid paraffin and surfactants as adjuvants have become mainstream inactivated vaccines for chickens marketed in Japan. The W/O vaccine used in this study was a commercially available oil-adjuvant-inactivated vaccine product. The instruction manual of the vaccine states, “Avoid direct sunlight or freezing as it affects quality.” In this study, repeated freezing experiments under different low-temperature conditions showed that the freezing temperature of the commercial W/O vaccine was in the range of −10 to −12°C. Additionally, the emulsion state of the vaccine solution did not change significantly because of repeated freezing and thawing or exposure to increased temperatures. Assuming that freezing reduces the quality of W/O vaccines, the freezing temperature of the vaccine observed in this study was more than 12°C below the lower limit of the storage temperature range. Hence, we speculated that the lower limit of the storage temperature range of the vaccine could be set below 2°C. The 2–8°C temperature range indicated for the vaccine used in this experiment is consistent with what is generally considered the recommended temperature range for vaccines. However, the basis for recommending this range for an individual product is unclear, and may not be the optimum handling temperature range for this individual product.

In Experiment 2, we found that inoculation with the W/O vaccine subjected to frozen and semi-frozen conditions had no significant effect on antibody production. In Experiment 3, there was no difference in immunoreaction after inoculation with the W/O vaccine that was subjected to repeated freeze-thaw cycles or continuous warming compared to that of the untreated W/O vaccine. The results of these two vaccination tests showed that freezing (regardless of freezing pattern) or warming (to 42°C) the W/O vaccine used in this study had almost no effect on the immune response in chickens, compared to the immune response from the control vaccine. The stability of these immune effects against freezing and warming may be related to the emulsified state of the vaccine,
Fig. 1. A: Distribution and transition of antibody sample-to-positive (S/P) ratios (ND, IB, and IBD) by the day of serum collection in chickens vaccinated with freeze-thaw-treated (FT1) or control (Cont1) vaccines. B: Distribution and transition of antibody sample-to-positive (S/P) ratios (ND, IB, IBD) by the day of serum collection in chickens vaccinated with the freeze-thaw-treated (FT2), warming-treated (W), or control (Cont2) vaccines. The line graph shows the transition of the average value of the S/P ratio. Circles are antibody S/P ratios for individual serum samples. ND, Newcastle disease; IB, infectious bronchitis; IBD, infectious bursa disease. * indicates a significant difference in the mean value of the enzyme-linked immunosorbent assay antibody S/P ratio between the groups on the same day after vaccination ($p < 0.05$).
which was almost unchanged by freezing and warming, as shown in Experiment 4.

Warming the W/O product improves fluidity, facilitates injections, and reduces the probability of a cold shock during vaccination. However, depending on the warming procedure, the time and temperature applied to the W/O vaccine may damage antigen formulation (de Wit and Montiel, 2022). Stone et al. (1978) have shown that an oil emulsion vaccine prepared by homogenizing one volume of antigen containing 4% (v/v) polysorbate 80 with four volumes of mineral oil containing 10% (v/v) sorbitan monooleate is stable at 37°C for at least 12 weeks. Moreover, Herbert (1968) elucidated the mechanism by which the inoculated W/O vaccine is maintained in the body for a relatively long period, with a sustained release of antigen-maintaining immunity. These studies support the notion that the W/O vaccine used in the present study may maintain its long-term performance even when placed in a temperature range equivalent to the chicken body temperature. However, it should be noted that the sustained release of antigens may vary among chickens. Therefore, further studies based on the mechanism of individual differences in antigen release and immune responses are needed to thoroughly assess the impact of high temperatures on vaccine quality.

This study showed for the first time that there was no significant effect of the freeze-thawed W/O vaccine on the immune response of vaccinated chickens, compared to that of chickens inoculated with the control vaccine. However, the limitations of these results are that the vaccination experiments were conducted with younger broiler chickens in a conventional rearing environment and that the data were obtained from immunoresponses to only one vaccine product (the vaccine lots used in Experiments 1 and 2 were different). Additionally, in the present study, the vaccine solution was inverted and homogenized immediately before vaccination. When the W/O vaccine was kept stationary, liquid-phase separation occurred slowly over time. Therefore, it is necessary to investigate the effects of freezing or warming when the liquid phase is separated after it is kept stationary. In the two inoculation experiments, the vaccine was administered to

![Fig. 2. A: Microscopic emulsion state of freeze-thaw treated (FT2), warming-treated (W), and control (Cont2) vaccines. Bar = 50 µm. B: State of a microscopic emulsion of the vaccine without freezing or after repeated freeze-thawing at -20°C and 5°C, 1–5 times. Numbers indicate the count of repeated freeze-thaws. Bar = 20 µm.](image-url)
chickens on the day after the final day of freezing or warming. The potential for the accelerated degradation of quality by freezing or warming was not considered. Thus, veterinarians and users should avoid using the results as sole evidence when prescribing or using vaccines stored outside the storage temperature range.

In conclusion, it is suggested that for W/O vaccine products, freezing and warming may not significantly impair quality and may have little effect on immune function when inoculated into chickens. This may be the basis for increasing the availability of W/O vaccines. Evidence-based optimization of the storage and handling temperature ranges will help dispel concerns regarding the potential risks of vaccine handling operations and the economic potential of the product. This will increase the distribution potential of these vaccines.

Acknowledgments

The authors would like to thank all poultry farm staff of Rakuno Gakuen, who cooperated with raising and health management work during the experiment. We would also like to express our deep gratitude to MSD Animal Health for providing the vaccine formulation as an experimental sample. This study was funded by KAWAVET LLC, the owner of the Research Office Concerning the Health of Humans and Birds, and was supported in part by the Japan Society for the Promotion of Science (JSPS) KAKENHI, Grants-in-Aid for Scientific Research (C) (No. 21K05907, TI; No. 21K05908, YH; No. 21K05942, TW), and by a Grant-in-Aid for Cooperative Research Fund (2021-03) from Rakuno Gakuen University.

Author Contributions

T.K. and T.I. contributed to the design and execution of experiments, analysis of results, and writing of the manuscript.

T.W., M.H., and Y.H. assisted and discussed the interpretation of the data obtained from the experiments.

M.Y. contributed to poultry raising, health observation, and care.

Conflicts of Interest

The authors declare no conflict of interest.

References

Aucouturier J, Dupuis L and Ganne V. Adjuvants designed for veterinary and human vaccines. Vaccine, 19: 2666–2672. 2001.

Das MK, Arora NK, Mathew T, Vyas B, Sindhu M and Yadav A. Temperature integrity and exposure to freezing temperature during vaccine transfer under the universal immunization program in Three States of India. Indian Journal of Public Health, 63: 139–142. 2019.

de Wit JJS and Montiel E. Practical aspects of poultry vaccination. In: Avian Immunology (Kaspers B, Schat KA, Göbel T and Vervelde L eds.). 3rd ed. pp. 469–488. Academic Press. London. 2022.

Falcón VC, Porras YVV, Altamirano CMG and Kartoglu U. A vaccine cold chain temperature monitoring study in the United Mexican States. Vaccine, 38: 5202–5211. 2020.

Herbert WJ. The mode of action of mineral-oil emulsion adjuvants on antibody production in mice. Immunology, 14: 301–318. 1968.

Kartoglu U and Milstien J. Tools and approaches to ensure quality of vaccines throughout the cold chain. Expert Review of Vaccines, 13: 843–854. 2014.

Matthias DM, Robertson J, Garrison MM, Newland S and Nelson C. Freezing temperatures in the vaccine cold chain: a systematic literature review. Vaccine, 25: 3980–3986. 2007.

Stone HD, Brugh M, Hopkins SR, Yoder HW and Beard CW. Preparation of inactivated oil-emulsion vaccines with avian viral or Mycoplasma antigens. Avian Diseases, 22: 666–674. 1978.