Sunflower seed oil containing ginseng stem–leaf saponins (E515-D) is a safe adjuvant for Newcastle disease vaccine

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ABSTRACT Vaccination is an effective method to prevent Newcastle disease (**ND**) in chickens. Marcol 52 and #10 white oil are mineral-based adjuvants and can be found in commercial inactivated ND virus vaccines. The present study demonstrated that a vegetable origin oil E515-D had lower polycyclic aromatic hydrocarbons and higher flash point than the commercial products Marcol 52 and #10 white oil. E515-D could be mixed with an aqueous phase containing ND virus antigen to form a stable water-in-oil vaccine emulsion and exhibited more potent adjuvant effects on the immune response than Marcol 52 and #10 white oil. Moreover, the absorption of E515-D–adjuvanted vaccine was faster than absorption of Marcol 52- and #10 white oil-adjuvanted vaccines when ND virus vaccines were injected in broilers. Therefore, E515-D was safe and could be a suitable adjuvant used in vaccines for food animals. In addition , E515-D is not easy to be flammable during shipping and storage owing to its higher flash point.

Key words: vegetable oil, ginseng stem–leaf saponin, adjuvant, Newcastle disease vaccine

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INTRODUCTION

Newcastle disease (**ND**) is caused by the ND virus (**NDV**) that is an avian paramyxovirus type I serotype of genus *Avulavirus* belonging to the Paramyxoviridae family (Hines and Miller, 2012; Orabi et al., 2017). When infected with the NDV, the chickens show clinical signs such as reduction in egg production, respiratory distress, listlessness, central nervous signs, and even death, causing huge amount of economic losses to the poultry industry (Kapczynski et al., 2013). Vaccination is one of the conventional approaches to the control of the disease (Kiril et al., 2017; Ma et al., 2019). To improve the immunization, inactivated NDV vaccine is usually supplemented with adjuvants, and the vaccine is usually made from emulsification of an aqueous phase containing antigen with an adjuvant oil phase. Generally, the adjuvant used for NDV vaccine originates from the mineral oil (Ben Arous et al., 2013; Lone et al., 2017). In China, immunization of NDV vaccine is compulsory for fowl (Yu et al., 2015). In accordance with the statistics, about 10 billion broilers are sold in the Chinese market in 2017 and 10.8 billion in 2018 (Fan et al., 2019; Ruidong Zhai et al., 2020). If each bird receives immunization of NDV vaccine with 0.3 mL for each shot, about 3 million L of NDV vaccine and 2 million L of adjuvant oil (aqueous phase: oil phase = 1:2) are consumed only for NDV vaccination annually. Although the adjuvant oil is made by refining and distilling, it is difficult to remove all the impurities from the oils, which has caused public concern (Kimber and Carrillo, 2016). And, the safety of mineral oil in vaccines is frequently questioned (Stone, 1993; Petermann et al., 2017). For example, polycyclic aromatic hydrocarbons (**PAH**) left in the oil was reported to be carcinogenic (Bulder et al., 2008; Pirow et al., 2019; Li et al., 2020). The metabolic fate of mineral oil in chickens has been found very slow, and the oil residue has been detected in the muscles, stomach, and other internal organs (Liu et al., 2010; EFSA Panel on Contaminants in the Food Chain (CONTAM), 2012; Liu et al., 2012). These adverse reactions have been even found at 12 wk after injection of mineral oil–adjuvanted NDV vaccine in chickens (Yamanaka et al., 1993).

The stem and leaves of *Panax ginseng* C.A. Meyer (**GSLS**) have been reported to display an adjuvant effect on poultry vaccines. Zhai et al. (2011a) showed that oral administration of GSLS could enhance ND vaccine and inactivated avian influenza vaccine (Zhai et al., 2011b), Ma et al. (2019) observed that GSLS and Se synergistically enhanced the immune effect of live bivalent vaccine, and Yu et al. (2015) demonstrated that oral

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administration of GSLS had capacity in increasing antibody responses of chickens to a bivalent NDV and avian influenza virus vaccine under the oxidative stress condition. In addition, Li et al. (2012) reported that GSLS combined with mineral oil adjuvant could promote the immune responses of NDV vaccine. Besides, compared with the mineral-originated oil, vegetable oil is renewable, edible, and safe. Although previous studies have demonstrated the adjuvant effect of some vegetable oil formulations in mice, swine, and sheep (Zhang et al., 2014, 2018; Cui et al., 2020), the vegetable oil as an adjuvant in poultry vaccine is barely reported. Our preliminary studies found that a vegetable origin oil E515-D, which comprised sunflower seed oil and ginseng saponins from GSLS, could form a stable water-in-oil (**W**/**O**) emulsion (Yuan et al., 2020). In the present study, E515-D, Marcol 52, and #10 white oil were first compared for their flash points and the concentrations of PAH; then, the NDV vaccines adjuvanted with E515-D, Marcol 52, and #10 white oil were characterized for their emulsions and induced immune responses in broilers. In addition, as broilers grow fast with approximately 50 D of growth period (Lee and Leeson, 2001; Broomhead et al., 2002) and fast absorption of the vaccine is important for the consumers, the residues were evaluated at the immunization sites. As Marcol 52 and #10 white oil were commonly used adjuvants for poultry vaccines, they were used for comparisons in this study.

MATERIALS AND METHODS

Animals

One-day-old yellow broilers were purchased from Ningbo Zhenning Animal Husbandry Co., Ltd. (Ningbo, China). Chickens were housed in separate cages and given free access to feed and water. Temperature was controlled at 35°C during the first 3 D and then gradually reduced to 26°C. The animals were acclimatized for 2 wk until the maternal antibody was less than 4 $\log_2$. The protocol on handling animals was approved by the Animals Ethics Committee (ZJU20160377) of Zhejiang University.

Antigen and Adjuvants

Inactivated NDV antigen (strain LaSota) was from Zhejiang Ebvac Biotechnology Co., Ltd. (Hangzhou, China); Marcol 52 was made in ExxonMobil, France; #10 white oil was from Sinopec Hangzhou Refinery (Hangzhou, China); E515-D was made from sunflower seed oil (Yihai Kerry Group Co., Ltd., China) and standardized ginseng stem–leaf saponins (GSLS) that contain ginsenosides Re (16.4%), Rd (9.0%), Rg1 (6.0%), Rb2 (3.8%), Rc (3.7%), Rb1 (2.4%), and Rf (0.1%) based on HPLC analysis (Hongjiu Ginseng Industry Co., Ltd., Jilin, China).

Diagnostic Kits

The NDV antibody test kit was the product of IDEXX Laboratories Inc. (Westbrook, MA). Newcastle disease virus antigen and positive control serum for hemagglutination inhibition (**HI**) test were purchased from Qingdao Yebio Biological Engineering Co., Ltd. (Qingdao, China).

Determination of PAH in Adjuvants

HPLC was used to assay PAH in the adjuvants in accordance with GB 5009.265-2016 (China State Bureau of Standards, 2016). Briefly, dichloromethane and N-hexane were used to extract PAH from the oil adjuvant and activate the solid-phase extraction column of florey silica soil to get the testing sample. Then, an ultra-performance liquid chromatograph–equipped Agilent 1,260 Infinity Fluorescence Detector was used to analyze the sample. The column was Agilent C18 (4.6 mm i.d., 250 mm long, particle diameter 5 μm). The mobile phase was a gradient of degassed water and acetonitrile at a flow rate of 1.5 mL/min. Naphthalene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo [a] anthracene, benzo [k] fluoranthene, and benzo [a] pyrene were determined using the fluorescence detector. The excitation/emission wavelengths were 270/324 nm for naphthalene, acenaphthylene, and fluorene; 248/375 nm for phenanthrene and anthracene; 280/462 nm for fluoranthene; 270/385 nm for pyrene and benzo [a] anthracene; and 292/410 nm for benzo [k] fluoranthene and benzo [a] pyrene.

Determination of Flash Point of Adjuvants

A flash point detector SYD-26-I (Shanghai, China) was used to test flash point of E515-D, Marcol 52, and #10 white oil, and the method was in accordance with GB/T 261-2008 (China State Bureau of Standards, 2008). Briefly, adjuvant oil was kept in the sample tank that was heated automatically to a candidate flash point temperature. When the temperature of the tank reached to 90°C, the flame was moved to the sample for every 2°C until there was a flame emerging in the sample. The flash point was recorded when the flame was observed in the sample.

Vaccine Preparation and Identification of Emulsion Type

Newcastle disease virus vaccines were made by emulsifying the aqueous phase with antigen with the oil phase E515-D, Marcol 52, or #10 white oil as previously described by Li et al. (2012). Two methods were used to identify the type of the vaccine emulsion. 1) Dilution method: 1 mL of NDV vaccine emulsion was dropped into a tube containing water or oil. The emulsion of W/O will be dispersed in oil while the emulsion of oil in water (**O**/**W**) will be dispersed in water. 2) Staining method: 0.5 mL of NDV vaccine emulsion was dropped on a slide. Then, a drop of eosin Y (water-soluble dye) or Sudan III (liposoluble dye) was added to the surface of emulsion. Eosin Y will be dispersed in O/W emulsion, whereas Sudan III dispersed in W/O emulsion.

Test for Stability of the Vaccine Emulsions

Newcastle disease virus vaccines were emulsified by E515-D, Marcol 52, and #10 white oil. Then, stability of the vaccine emulsions was evaluated by centrifugation method as per the Chinese Veterinary Pharmacopoeia. Briefly, 10 mL of vaccine emulsions samples were centrifuged at 3,000 rpm for 15 min, and emulsions were regarded stable when volume of the aqueous phase at the bottom of the centrifugal tube was less than 0.5 mL after centrifugation.

Test for the Immune Responses of Broilers to NDV Vaccines

One hundred twenty broilers at 14 D of age were randomly divided into 10 groups (n = 12/groups) and received subcutaneous (**s.c.**) or intramuscular (**i.m.**) injection with 0.3 mL of NDV antigen in saline or adjuvanted with E515-D, Marcol 52, or #10 white oil. Birds injected with saline solution served as a control. Blood samples were collected at 1, 2, 3, 4, 5, and 6 wk after immunization for testing NDV-specific HI titers. At 6 wk after immunization, blood was collected to test NDV-specific antibody titer and neutralizing antibody titer.

Observation of Residue of NDV Vaccine in the Injection Sites

Fifty-four broilers at 14 D of age were randomly divided into 5 groups. Four groups (n = 12/groups) were i.m. injected with 0.3 mL of a commercial NDV vaccine (Marcol 52 as adjuvant) or the vaccine adjuvanted with E515-D, Marcol 52, or #10 white oil at the left chest muscles. Birds injected with saline (n = 6) served as a control group. Two birds from each immunized group and 1 bird from control group were euthanized once a wk for checking the vaccine residue at the injection sites.

Hemagglutination Inhibition Test

Serum NDV-specific HI titer was measured mainly as described by Zhai et al. (2011a). In brief, serum was diluted in double (from 1:2 to 1:1024) in a 96-well microtiter plates with V-shaped bottom, NDV antigen (25 μl; 4 hemagglutination units) was added to each well. After incubation at 37°C for 30 min, 25 μl of 1% rooster erythrocyte suspension was added and incubated at 37°C for 30 min. Hemagglutination inhibition titer was defined as the reciprocal of the final dilution of serum, which would completely inhibit hemagglutination. Positive and negative serum controls were set on each plate, and all samples were tested in triplicate. Data were presented as $\log_2$ of the highest dilution of HI.

ELISA for NDV-specific Antibody Titer

An ELISA kit was used to detect serum NDV-specific antibody titer as previously described by Wang et al. (2013). Briefly, 100 μL of diluted serum sample (1:500)

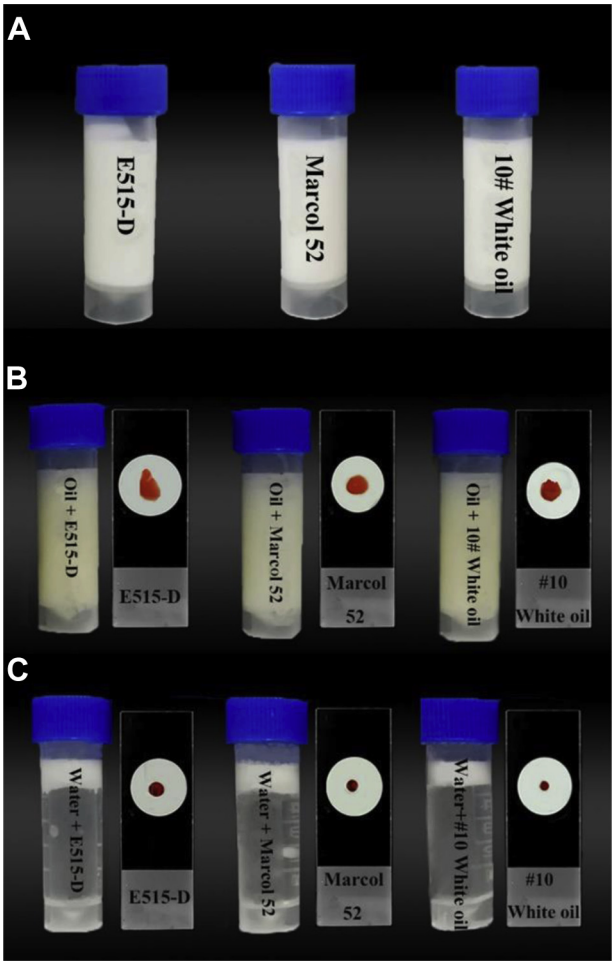

Figure 1. Identification of vaccine emulsion forms. NDV vaccines were emulsified in E515-D, Marcol 52 and #10 white oil (A). The vaccine emulsion was dispersed in vegetable oil or stained with liposoluble dye Sudan III (B) but not dispersed in water or stained with water-soluble dye eosin Y (C), indicating all the vaccine emulsions were water in oil (W/O). Abbreviation: NDV, Newcastle disease virus.

was added to an NDV antigen–coated 96-well plate, and the well was set in duplicate. After incubation at room temperature for 30 min, the plate was washed, and 100 μL of goat anti-chicken antibody-horseradish peroxidase conjugate was added. After 30 min incubation at room temperature, 100 μl of 3,3',5,5'-tetramethylbenzidine solution was added and incubated at room temperature for 15 min. Afterward, 100 μl of stop solution was added to terminate the reaction. The plate was read at 650 nm using an automatic ELISA reader for optical density value. The ratio of sample to positive (S/P) was calculated by (mean of sample optical density-mean of negative control optical density)/(mean of positive control optical density−mean of negative control optical density), and the endpoint titer was estimated by the equation log10 titer = 1.09 (log 10 S/P) +3.36 (Flock check program, IDEXX).

Determination of Neutralizing Antibody Titer

Neutralizing antibody was carried out as previously described by Ma et al. (2019). In brief, serum was diluted in serial four-fold dilution beginning at 1:4 with medium

Table 1. Flash point (°C) of adjuvant oils.

	E515-D	Marcol 52	#10 white oil
Flash point	228	158	155

The flash point was determined by a flash point detector SYD-26-I in accordance with GB/T 261-2008.

in tubes. Then, 100 $TCID_{50}$ (50% tissue culture infective dose) of NDV (Strain LaSota) was mixed with an equal volume of the diluted serum samples. After incubation at 37°C for 1 h, the mixtures were added to chicken embryo fibroblasts in 96-well plates. The plates were incubated at 40°C in 5% CO_2 for 144 h, and then, the cytopathic effect was recorded and calculated as per the Reed–Muench method. Serum virus neutralizing titers were expressed as mean ± SD.

Statistical Analysis

One-way ANOVA was used to analyze the data by GraphPad Prism 7.0 software (San Diego, CA). P value < 0.05 was considered as statistically significant difference. Data were expressed as mean ± SD.

RESULTS

Emulsion Type and Stability of NDV Vaccines

Newcastle disease virus vaccines adjuvanted with E515-D, Marcol 52, and #10 white oil were the stable white emulsion, and no separation was found after centrifugation at 3,000 rpm for 15 min (Figure 1A). All 3 vaccines were W/O emulsion as they stained with liposoluble dye Sudan III (Figure 1B) but not with water-soluble dye eosin Y (Figure 1C).

Flash Point of Oil Adjuvants

To investigate the inflammability the adjuvant oils, flash point was measured. Table 1 shows that the flash points from high to low were E515-D (228°C) > Marcol 52 (158°C) and #10 white oil (155°C).

Table 2. Polycyclic aromatic hydrocarbons (PAH) detected in E515-D, Marcol 52, and #10 white oil (μg/kg).

PAH	E515-D	Marcol 52	#10 white oil
Naphthalene		5,090	567
Acenaphthylene			
Fluorene	12.5	64.5	32.1
Phenanthrene	61.4		533
Anthracene			28
Fluoranthene	10.7		46.3
Pyrene	14.9		20.9
Benz [a] anthracene			
Benzo [k] fluoranthene	2.34	7.6	4.4
Benzo [a] pyrene			
Total	101.84	5,162.1	1,231.7

The level of PAH was determined by HPLC in accordance with GB 5009.265-2016.

Polycyclic Aromatic Hydrocarbons in Oil Adjuvants

Polycyclic aromatic hydrocarbons are a class of organic compounds containing 2 or more benzenes and include naphthalene, acenaphthylene, fluorene, phenanthrene, phenanthrene, fluoranthene, pyrene, benzo [a] anthracene, benzo [k] fluoranthene, and benzo [a] pyrene. Table 2 shows that a total of PAH in E515-D was 50- and 12-fold lower than that in Marcol 52 and #10 white oil, respectively.

E515-D–Enhanced NDV-specific Antibody Responses

To compare the adjuvant effects of E515-D, Marcol 52, and #10 white oil on the immune responses to NDV vaccines, broilers were s.c. or i.m. immunized with NDV vaccines. Blood samples were collected after immunization to analyze antibody responses. Figure 2 showed that E515-D, Marcol 52, and #10 white oil had an adjuvant effect with the highest serum HI titer detected in the E515-D group and then was in Marcol 52 and #10 white oil groups. At 6 wk after immunization, HI titers in the Marcol 52 (1:14 for s.c.; 1:13 for i.m.) and #10 white oil (1:12 for s.c.; 1:12 for i.m.) groups decreased lower but in the E515-D group (1:37 for s.c.; 1:33 for i.m.) remained higher than 4 $\log_2$. Antibody titers measured by ELISA were significantly higher in the E515-D group (2,717 for s.c.; 2,407 for i.m.) than in HI titers in the Marcol 52 (1,384 for s.c.; 1,288 for i.m.) and #10 white oil groups (1,400 for s.c.; 1,198 for i.m.) (Figures 3A and 3B). Importantly, the neutralizing antibody titer (Figures 3C and 3D) in the E515-group (1:113 for s.c.; 1:76 for i.m.) was significantly higher than in the Marcol 52 (1:41 for s.c.; 1:22 for i.m.) and #10 white oil (1:25 for s.c.; 1:13 for i.m.) groups.

Residues in the Injection Sites after Immunization With NDV Vaccines

To investigate the safety of the oil adjuvant NDV vaccines, the emulsion residue in the injection site was observed. Birds received i.m. injection with different NDV vaccines at the chest. In Figure 4, we observed that the NDV vaccine formulated by E515-D began to be absorbed by bird from 3 wk after the injection. However, bird inoculated with the commercial NDV vaccine and the vaccines adjuvanted with Marcol 52 and #10 white oil showed poor absorption ability until 5 wk after the injection. Moreover, the residue of mineral oil adjuvant Marcol 52 and #10 white oil could induce observed severe inflammatory reaction than the E515-D in the injection site. These observations indicate that the NDV vaccine adjuvanted with E515-D is a safe adjuvant for chickens.

DISCUSSION

The present study showed that a vegetable origin oil E515-D had lower PAH (Table 2) and higher flash point

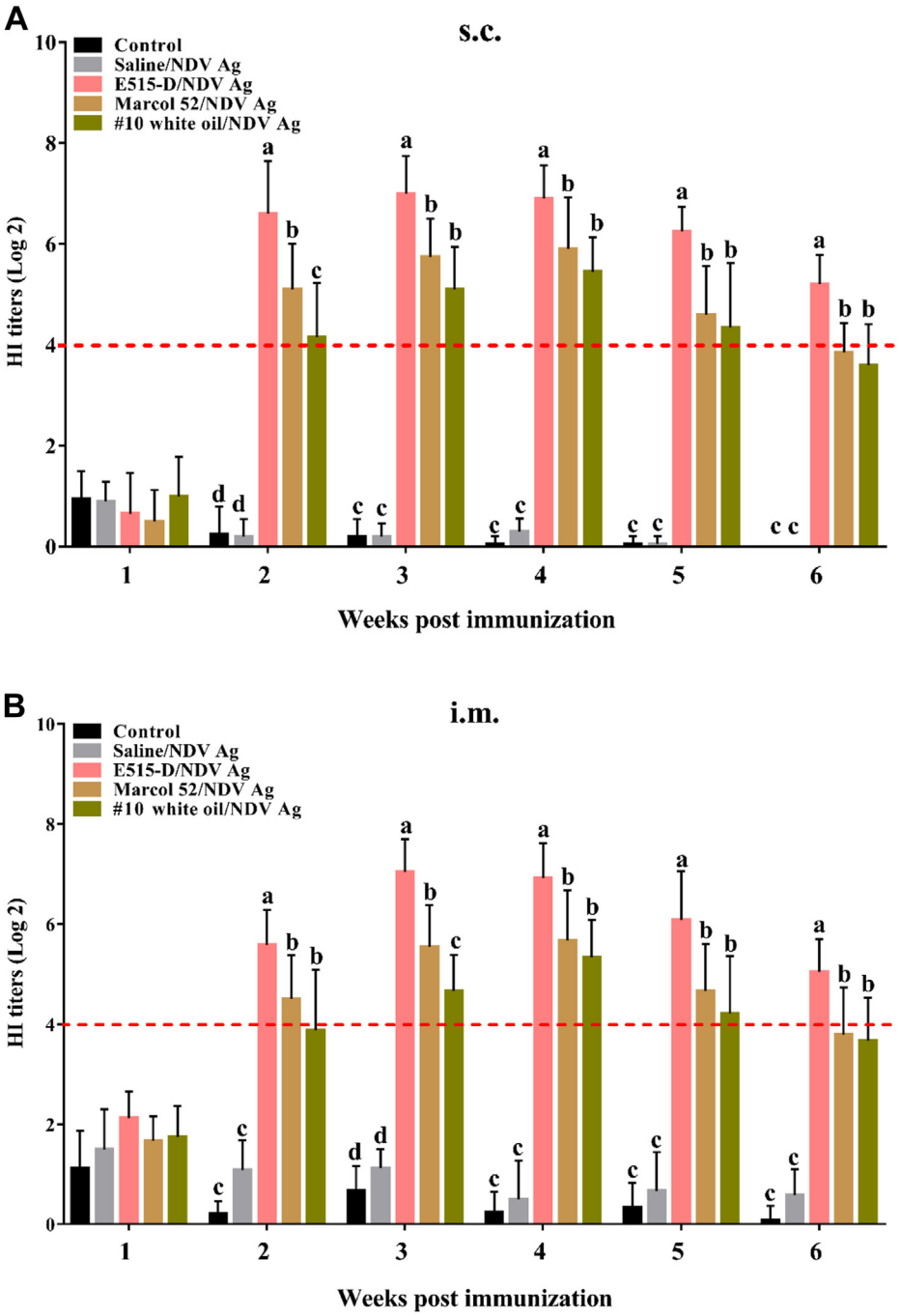

Figure 2. HI titers induced by NDV vaccines. Broilers (n = 12/group) were s.c. (A) or i.m. (B) injected with NDV antigen (Ag) in saline or emulsified in E515-D, Marcol 52, or #10 white oil. Birds injected with saline alone served as a control. Blood samples were collected at 1, 2, 3, 4, 5, and 6 wk after immunization and used for analysis of NDV-specific HI titer. Data are represented as mean ± SD. Bars with different letters are statistically different ($P < 0.05$). Abbreviations: HI, hemagglutination inhibition; i.m., intramuscularly; NDV, Newcastle disease virus; s.c., subcutaneously.

(Table 1) than the commercial products Marcol 52 and #10 white oil. E515-D could be mixed with an aqueous phase containing NDV antigen to form a stable W/O vaccine emulsion and exhibited more potent adjuvant effect on the immune response than Marcol 52 and #10 white oil (Figures 1–3). Moreover, the absorption of E515-D–adjuvanted vaccine was faster than Marcol 52– and #10 white oil–adjuvanted vaccine when NDV vaccines were injected in broilers (Figure 4).

Today, most adjuvants for inactivated poultry vaccines are mineral oil based. Marcol 52 and #10 white oil are the mineral oils found in many commercial poultry vaccines in China. These products contain hydrocarbons including normal, isoalkanes, and cycloalkanes as well as PAH (Kimber and Carrillo, 2016). In 1995, the scientific community on foods evaluated the safety of mineral oil in rats and found that accumulation of hydrocarbons was observed in the liver and lymph nodes associated with a granulomatous response (FAO/WHO, 1995). In 2002, the Joint FAO/WHO Expert Committee on Food Additives found all mineral oil products accumulated in tissues in dose- and time-dependent manners, causing hepatic

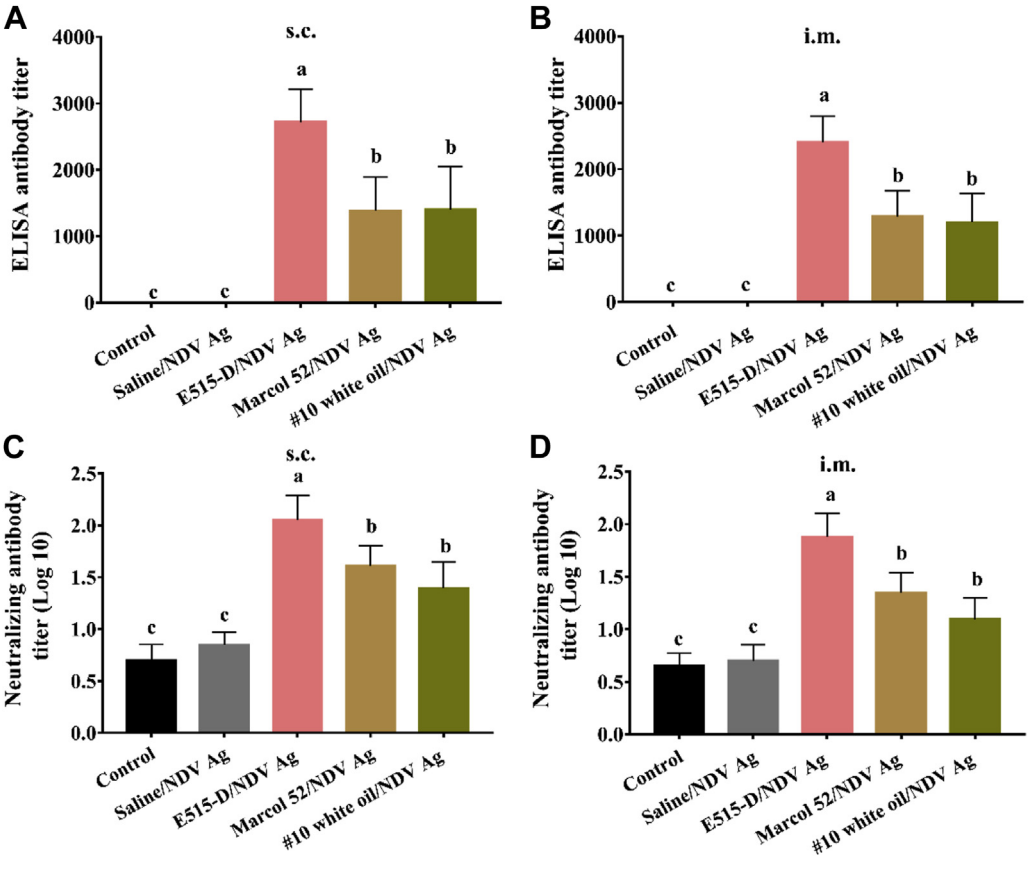

Figure 3. NDV-specific antibody and neutralizing antibody induced by NDV vaccines. Broilers (n = 12/group) were s.c. or i.m. immunized with NDV Ag in saline or emulsified in E515-D, Marcol 52, or #10 white oil. Birds injected with saline solution alone served as a control. Blood samples were collected at 6 wk post immunization for analysis of NDV-specific antibody by ELISA (A, B) and neutralizing antibody titers (C, D). Data are represented as mean ± SD. Bars with different letters are statistically different ($P < 0.05$). Abbreviations: i.m., intramuscularly; NDV, Newcastle disease virus; s.c., subcutaneously.

damage in Fischer rats (FAO/WHO, 2002). Polycyclic aromatic hydrocarbons are formed by the organic compounds that possess 2 or more fused aromatic rings of carbon and hydrogen atoms, and some of them have carcinogenic and mutagenic potentials (Bansal and Kim, 2015). Although the mineral oils used as a vaccine adjuvant are highly refined, the present study found that Marcol 52 and #10 white oil remained to have the concentrations of PAH (5,162.1 and 1,231.7 μg/kg, respectively), which were 50- and 12-fold higher than those of E515-D (101.84 μg/kg), suggesting that E515-D is much safe for food. Flash point is another concern that should be addressed when oil adjuvants are selected for the use in poultry vaccine. The flash point is usually used to measure the tendency of the test specimen to form a flammable mixture with air in response to heat and an ignition source. Higher flash point means it less volatile and safer regarding the storage and transport (Mullin and Champ, 2003; Gulum et al., 2015). The present study showed that E515-D had higher flash point (228°C) than those of Marcol 52 and #10 white oil (158°C and 155°C, respectively), indicating that E515 is not easy to flammable in shipping and storage.

Three types of oil emulsion including O/W, W/O, and water in oil in water can be formed depending on the ratio of oil and aqueous phases and the surfactants used. It was reported that the W/O emulsion showed more potent adjuvant on vaccines against the avian influenza virus, infectious bronchitis virus, or NDV than O/W or water-in-oil-in-water emulsion in chickens (Jansen et al., 2006; Lone et al., 2017). The reason is because W/O emulsion sustainably releases the antigen to stimulate the lymph tissues from the vaccine depot at the injection sites (Spickler and Roth, 2003; Bonam et al., 2017). Figure 1 shows E515-D, Marcol 52, and #10 white oil could develop a stable W/O emulsion when emulsified with an aqueous phase.

Newcastle disease virus–specific antibody immune responses exert a vital role in protecting chickens from NDV infection. Usually, HI assay was conducted to detect levels of specific antibody against hemagglutinin neuraminidase protein (Bello et al., 2018). In Figure 2, vaccine adjuvanted with E515-D induced significantly higher serum NDV-specific HI titers than vaccines formulated by Marcol 52 and #10 white oil did in chickens. Generally, chickens are believed to be susceptible to infections of NDV when the serum HI titer level is less than 4 $\log_2$ (Li et al., 2004). Compared with the E515-D group, serum NDV-specific HI titers in both the Marcol 52 and #10 white oil groups were less than 4 $\log_2$, whereas the E515-D group had higher HI titers than 4 $\log_2$ at 6 wk after immunization. This result means that NDV vaccine emulsified in E515-D could

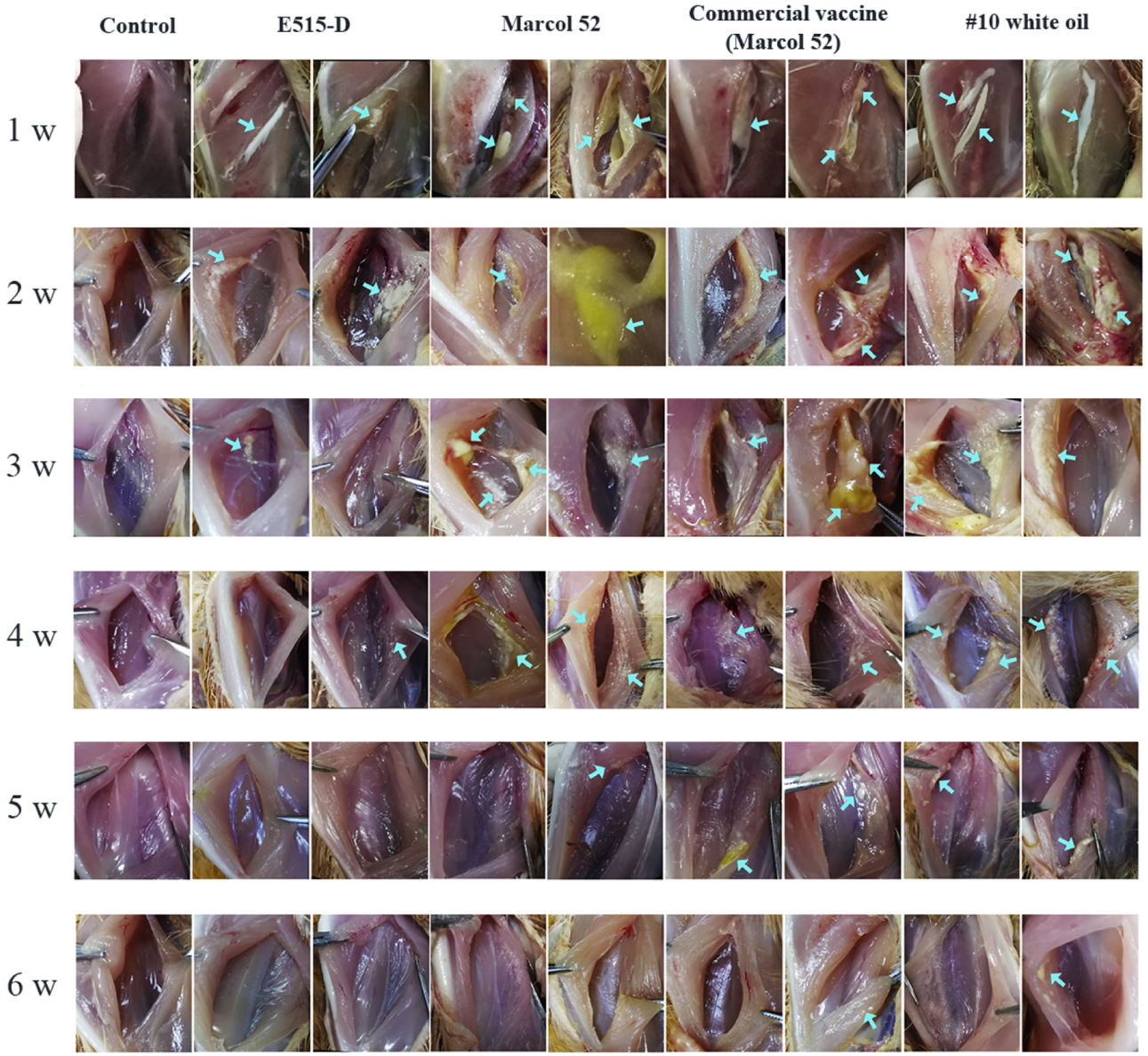

Figure 4. The residues of different NDV vaccines in the injection sites. Broilers (n = 12/group) were i.m. injected in the left chest with commercial NDV vaccine (emulsified in Marcol 52) or NDV vaccines emulsified with E515-D, Marcol 52, and #10 white oil and broilers (n = 6) were i.m. injected 0.3 mL saline as control. Two birds from each immunized group and 1 bird from control group were randomly euthanized to check the injection sites at 1, 2, 3, 4, 5, and 6 wk after immunization. The places with azure arrows were observed to have the residues of vaccine emulsion. Abbreviations: i.m., intramuscularly; NDV, Newcastle disease virus; s.c., subcutaneously.

provide higher protection against NDV than that of Marcol 52 and #10 white oil. Several ELISA kits based on whole or part of the virus antigen have been developed for rapid diagnosis of ND (Berinstein et al., 2005; Bello et al., 2018). Many of these kits are commercially available in the form of sandwich, competitive, or indirect ELISA (De Oliveira et al., 2013; Desingu et al., 2014). They are highly sensitive and produce results that pretty well correlate with HI test results. The ELISA kit was used to test antibody titers against all proteins in NDV particle in this study. Figures 3A and 3B shows that the highest antibody titers among all groups were detected by the ELISA method at 6 wk after vaccination. Serum NDV-neutralizing antibody is a marker for efficient protection against NDV replication and complete protection against NDV disease and death (Nayak et al., 2012). As shown in Figures 3C and 3D, E515-D significantly improved the serum NDV-neutralizing antibody response when compared with Marcol 52 and #10 white oil, which paralleled data of HI and antibody. These observations suggested that E515-D could induce potent humoral immune responses against the NDV vaccine.

Absorption of the vaccine at the injection sites is another point that should be considered when immunization is administered in food animals. Domestic animals grow much faster than their ancestors owing to modern genetic and breeding technology. For example, only 49 D are needed for broilers growing up to be consumed (Broomhead et al., 2002). However, Yamanaka et al. found that granulomatous reaction and cyst formulation still occurred in the injection sites of birds at 12 wk after

the injection of mineral oil–adjuvanted NDV vaccine (Yamanaka et al., 1993). Similarly, Stone (1997) reported that immunization with mineral oil–adjuvanted vaccines resulted in undesirable tissue reaction that could persist for mo. Similar results were observed in our study when broilers were immunized with vaccines adjuvanted with mineral oils. We found that at least 5 or 6 wk were required for the absorption of vaccine emulsion at the injection sites (Figure 4), whereas only 4 wk were needed when vaccine was adjuvanted with E515-D. Therefore, the vaccine with E515-D was absorbed faster than the vaccine with mineral oil and is suitable to be used in vaccines for food animals.

In conclusion, the present study demonstrated that a vegetable origin oil E515-D had lower PAH and higher flash point than the commercial products Marcol 52 and #10 white oil. E515-D could be mixed with an aqueous phase containing NDV antigen to form a stable W/O vaccine emulsion and exhibited more potent adjuvant effect on the immune response than Marcol 52 and #10 white oil. Moreover, the absorption of E515-D–adjuvanted vaccine was faster than Marcol 52– and #10 white oil–adjuvanted vaccines when NDV vaccines were injected in broilers. Therefore, E515-D was safe and could be a suitable adjuvant used in vaccines for food animals. In addition, E515-D is not easy to be flammable during shipping and storage owing to its higher flash point.

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