



## Characterization of a novel oil-in-water emulsion adjuvant for swine influenza virus and *Mycoplasma hyopneumoniae* vaccines



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### ABSTRACT

Vaccines consisting of subunit or inactivated bacteria/virus and potent adjuvants are widely used to control and prevent infectious diseases. Because inactivated and subunit antigens are often less antigenic than live microbes, a growing need exists for the development of new and improved vaccine adjuvants that can elicit rapid and long-lasting immunity. Here we describe the development and characterization of a novel oil-in-water emulsion, OW-14. OW-14 contains low-cost plant-based emulsifiers and was added to antigen at a ratio of 1:3 with simple hand mixing. OW-14 was stable for prolonged periods of time at temperatures ranging from 4 to 40 °C and could be sterilized by autoclaving. Our results showed that OW-14 adjuvanted inactivated swine influenza viruses (SIV; H3N2 and H1N1) and *Mycoplasma hyopneumoniae* (*M. hyo*) vaccines could be safely administered to piglets in two doses, three weeks apart. Injection sites were monitored and no adverse reactions were observed. Vaccinated pigs developed high and prolonged antibody titers to both SIV and *M. hyo*. Interestingly, antibody titers were either comparable or greater than those produced by commercially available FluSure (SIV) or RespiSure (*M. hyo*) vaccines. We also found that OW-14 can induce high antibody responses in pigs that were vaccinated with a decreased antigen dose. This study provides direct evidence that we have developed an easy-to-use and low-cost emulsion that can act as a powerful adjuvant in two common types of swine vaccines.

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### 1. Introduction

Although attenuated live organisms are frequently used as vaccines to control infectious diseases, these modified live vaccines (MLV) may pose potential safety risks when administered to immune-compromised animals or the virus/bacteria is capable of reverting to a virulent form. Another disadvantage of using modified live vaccines in disease management is that it is difficult to differentiate infected from vaccinated animals. Inactivated virus/bacteria, purified proteins, and synthetic peptides are considered to be much safer compared to live pathogens, but they are often less immunogenic [1,2]. Consequently, these vaccines

rely on adjuvants to stimulate the innate immune response which in turn facilitates a strong adaptive response [3]. The incorporation of an adjuvant into a vaccine can achieve qualitative and quantitative alteration of the immune protection and provide functionally appropriate types of immune responses. Adjuvants also act to reduce the antigen dose required to generate a protective response and extend the duration of effective immunity [4]. New vaccine candidates have been developed recently against not only infectious agents but also allergic and autoimmune diseases, cancer, and infertility [5–10]. All these applications require adjuvants with desirable functions and performance in order to successfully achieve vaccination-induced immune protection and therapeutic effects.

Adjuvants can be broadly divided into two categories, the first being antigen vehicles, such as emulsions and liposomes, which act to present vaccine antigens to the immune system in a more efficient way and prolong the release of antigens to increase the

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specific immune responses [11]. The second category of adjuvants are immuno-stimulants, such as Toll-like receptor (TLR) agonists, aluminum hydroxide, saponins and cytokines [12,13]. One major limiting factor of adjuvants is that many of them have unacceptable side effects and lack of biocompatibility. The most commonly used adjuvant in the U.S. is aluminum hydroxide (ALOH). ALOH is considered safe and is currently a component of several veterinary and human vaccines [14]. However, recent reports suggest that ALOH adjuvants lack efficacy for several pathogens [15]. Conventional oil-in-water emulsions use various chemical emulsifiers (i.e. Tween 80 and Span 80), but the safety of these chemicals when injected intramuscularly remains controversial [16]. Here, we use food-grade plant-derived surfactants commonly used in human food processing as emulsifiers to stabilize a novel oil-in-water emulsion, referred to as OW-14. OW-14 uses inexpensive, readily available materials, is stable at temperatures up to 40 °C, and can be autoclaved for sterilization. Furthermore, OW-14, when mixed with whole inactivated swine influenza and mycoplasma antigens can elicit higher and prolonged antibody responses than commercial vaccines for the same pathogens. Here, we provide evidence that OW-14 emulsion is a low-cost, easy-to-use alternative adjuvant for use in swine vaccines.

## 2. Materials and methods

### 2.1. Materials

Whole killed swine influenza virus (H3N2, 1.6 mg/ml) and whole killed *Mycoplasma hyopneumoniae* (*M. hyo*) bacterin (20 mg/ml) were purchased from Newport Labs (Worthington, MN). Commercial SIV antigen from FluSure® (H1N1 and H3N2) without adjuvant, FluSure® vaccine, and RespiSure® (*M. hyo*) vaccines were purchased from Zoetis (Florham Park, NJ). Ticamulsion A-2010 was purchased from Tic Gums (White Marsh, MD). Penreco Drakeol 5 oil was purchased from Penreco (Karns City, PA).

### 2.2. Emulsion and vaccine formulation

To make the OW-14 emulsion adjuvant, Ticamulsion A-2010, a Gum arabic emulsifier was dissolved in deionized water with a 7.5% (W/V) final concentration. Mineral oil (Penreco Drakeol 5) was added to the water phase (15%, w/v) and mixed on a Silverson Lab mixer (L5M-A, Silverson, East Longmeadow, MA) for 15 min at 10,000 rpm. The emulsion was then passed five times through a Microfluidizer (M-110P, Microfluidics, Newton, MA) at ~10,000 psi. Emulsions were sterilized by autoclaving for 20 min at 117 °C and stored at 4 °C, room temperature, or 40 °C. Vaccines were prepared by simple hand mixing OW-14 with antigens to produce a final 5% w/v of mineral oil and 2.5% w/v of the Ticamulsion A-2010. The viscosity of OW-14 was measured using a Barnant falling ball viscometer (Fisher Sci., Waltham, MA).

### 2.3. Transmission electron microscopy

OW-14 emulsion was visualized using a Transmission Electron Microscope housed within the Department of Biology at Kansas State University. Undiluted OW-14 was placed on a 200 mesh formvar-carbon filter (Electron Microscopy Sciences, Hatfield, PA) for 5 min at room temperature. Samples were then counterstained with uranyl acetate for 5 min at room temperature. Grids were air-dried and imaged on FEI CM100 Transmission Electron Microscope (TEM) equipped with an AMT digital image capturing system using a magnification of 130,000 and an accelerating voltage of 100 kV.

### 2.4. Particle size and zeta potential characterization

Adjuvants were diluted in deionized water before being analyzed on a Malvern Zetasizer Nano ZS 90 instrument (Malvern Instruments, Westborough, MA). The particle size distribution was measured by dynamic light scattering (DLS), where the distribution of diffusion coefficients was determined through the measurement and correlation of the statistical fluctuations in the light scattered from a system of particle diffusion under the influence of Brownian motion. Adjuvants were then assayed for zeta potential using automatic software determination. Measurements were taken in triplicate to assess repeatability of the results and highlight any sample changes such as agglomeration or sedimentation during measurements.

### 2.5. Swine vaccination experiments

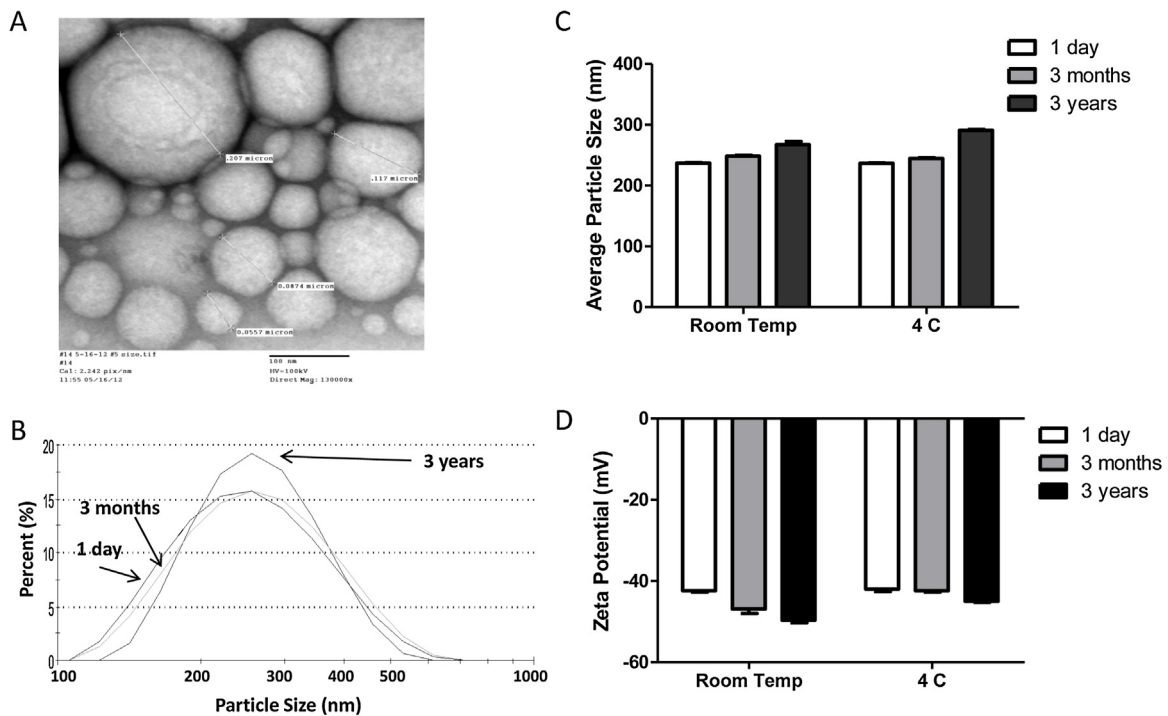
All animal experiments were approved by the Institutional Animal Care and Use Committee at Kansas State University. Conventional large White-Duroc crossbred weaned specific-pathogen free piglets (3 weeks of age) were used in all studies. The adjuvant/vaccine efficacy evaluation and adjuvant thermal stability/efficacy studies were conducted at the Swine Research Unit, Kansas State University. The antigen sparing experiment was conducted at Large Animal Research Center (LARC) facility, Kansas State University. All piglets were confirmed sera-negative for antibodies to swine influenza virus (by hemagglutination inhibition test) and *M. hyo* on day 0. For each experiment, pigs were immunized intramuscularly using a 20 gauge needle on day post-vaccination (DPV) 0 and 21 with experimental or commercial vaccines. During the adjuvant/vaccine efficacy evaluation experiment, blood samples were collected on day 0, 3 weeks, 5 weeks, 2 months, 4 months, and 5 months post initial vaccination. During adjuvant thermal stability/efficacy studies, blood samples were collected on DPV 0, 21, and 35. During the antigen sparing experiment blood samples were collected at DPV 0 and 42. Serum was separated from clotted blood and preserved at –20 °C. Vaccine injection sites were examined by trained veterinary pathologists on the day of slaughter (5 months post initial vaccination) for any pathological changes to the muscle or surrounding tissues. Pigs vaccinated with experimental adjuvant/vaccines were slaughtered/euthanized and disposed at the end of the experiments.

### 2.6. Antibody response

Serum was sent to Iowa State University Veterinary Diagnostic Laboratory (Ames, IA) for the evaluation of *M. hyo* antibody production using IDEXX *M. hyo* Ab Test (IDEXX Laboratories, Westbrook, ME) according to their standard protocols.

### 2.7. Hemagglutination inhibition titer

The hemagglutination inhibition (HAI) test is the most accurate serologic method of determining a swine herd's immune status [17]. Serum was heat inactivated for 30 min at 56 °C then diluted 10 fold and incubated with chicken red blood cells (RBC's) overnight at 4 °C. Samples were serially diluted (1:2), mixed with 4 HA units (HAU) of whole killed H3N2 swine influenza virus, and incubated for 30 min at room temperature. Chicken RBC's (0.5%) were added to the samples and incubated 3 h at room temperature. The HAI titer was defined as the highest serum dilution that completely inhibited hemagglutination.



**Fig. 1.** Oil-in-water emulsion (OW-14) formulated with a cost-effective edible plant derived emulsifier is highly stable at room temperature and 4 °C. A plant-based emulsifier was dissolved in water before adding oil and mixed for 15 min at 10,000 rpm. Emulsion was then passed five times through a microfluidizer and sterilized by autoclaving. Vaccines were prepared by simple hand mixing OW-14 with antigens to produce a final 5% w/v of oil. (A) Oil droplets were visualized by Transmission Electron Microscopy (TEM). (B and C) OW-14 was diluted in deionized water before being analyzed on a Malvern Zetasizer Nano ZS 90 instrument. The particle size distribution was measured using the dynamic light scattering technique (DLS). (D) Emulsions were then assayed for zeta potential using automatic software determination. All measurements were taken in triplicate.

## 2.8. Statistical analysis

Data from pig experiments are the mean  $\pm$  SEM values from groups of five or six pigs. The differences among each treatment group were determined by the one-way analysis of variance (ANOVA) followed by post-hoc Tukey's test using GraphPad Prism 5 software (San Diego, CA).

## 3. Results

### 3.1. Creation of a stable oil-in-water emulsion (OW-14) using a plant-based, food-grade emulsifier.

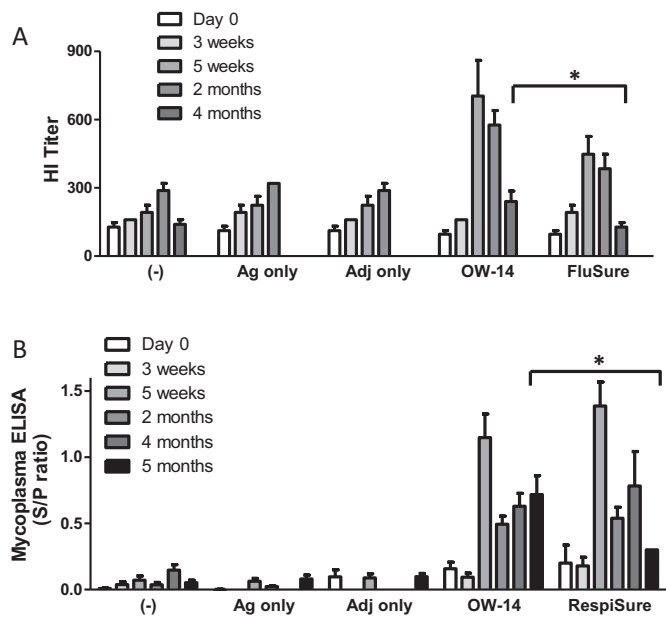
The OW-14 emulsion was made in two steps. First, 7.5% Tica-mulsion A-2010 powder was made with deionized water in a high speed mixer. After the Tica-mulsion A-2010 was dissolved in water, 15% w/v of mineral oil was added and mixed at 10,000 RPM for 10 min. Secondly, the above oil-water mixture was then passed through a microfluidizer five times at a pressure of  $\sim$ 10,000 PSI. The resulting milky like emulsion was named OW-14. OW-14 was sterilized by autoclaving using a 20 min liquid cycle. The density of OW-14 was 1.01 g and viscosity was 13.61 cP. Emulsions were imaged under an electron microscope and Fig. 1A shows that OW-14 formed oil droplets over a range of sizes. Therefore, a particle analyzer was used to determine the average size of the emulsified oil droplets. OW-14 emulsions were stored at room temperature or 4 °C for 3 days, 3 months, or 3 years. Oil droplets were uniform and mono-dispersed and between 100 and 700 nm in diameter (Fig. 1B). In freshly made OW-14, the average hydrodynamic diameter of the oil droplets was  $236 \pm 1.6$  nm (Mean  $\pm$  SD, Fig. 1C). The average oil droplet size did not significantly change with time, temperatures, or autoclaving, indicating that the oil droplets were stable. Emulsion droplets had an average zeta potential of less than  $-40$  V (Fig. 1D),

which also suggests that OW-14 is a highly stable oil-in-water emulsion.

### 3.2. OW-14 emulsion acts as a potent adjuvant for swine influenza and *M. hyo* antigens and promotes prolonged antibody responses in pigs

To assess the adjuvanticity of our OW-14 emulsion formulation, we mixed it with killed H3N2 and H1N1 swine influenza virus (from FluSure) and killed *M. hyo*. One part OW-14 was added to two parts antigen and the tubes were inverted three times to mix. The antigen/adjuvant mixture was stable for greater than 3 months when stored at 40 °C, greater than 6 months when stored at 25 °C, and over 1 year when stored at 4 °C (data not shown). OW-14/antigen formulation or commercial vaccines FluSure (positive control for SIV vaccination) and RespiSure (positive control for *M. hyo* vaccination) were intramuscularly administered into pigs twice at an interval of 3 weeks. OW-14 formulations as well as commercial vaccines were easily administered to pigs with a standard 5 cm<sup>3</sup> syringe coupled to a 20 ga needle. In order to track the antibody response induced by the vaccines, blood samples were collected and serum was isolated throughout the study until the pigs reached to market age and were slaughtered. Analysis of antigen-specific serum IgG revealed that 35 days post vaccination, pigs vaccinated with OW-14-based vaccines and pigs vaccinated with commercial vaccines both developed high HAI titer to H3N2 (Fig. 2A) and high titers of *M. hyo*-specific antibodies (Fig. 2B). Furthermore, H3N2 and *M. hyo*-specific antibody titers remained high in pigs administered with OW-14 formulated vaccines until market age (five months post vaccination) while titers in pigs receiving RespiSure or FluSure vaccines dropped by four months post vaccination.

The injection sites of all animals were monitored weekly throughout the study. Pigs vaccinated with FluSure, RespiSure, and

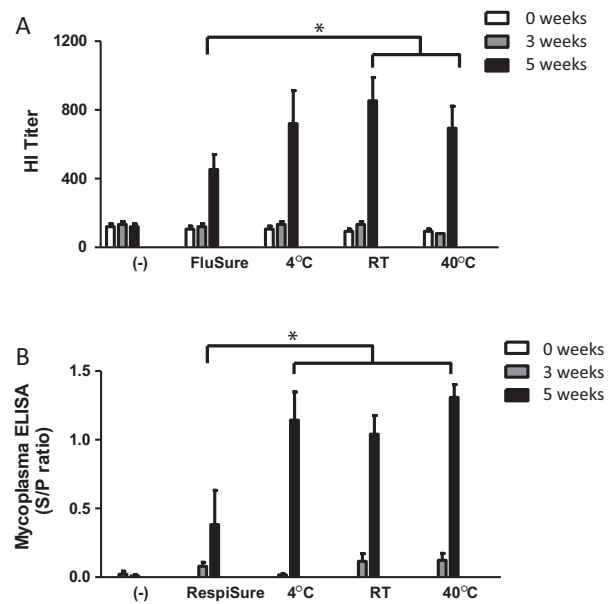


**Fig. 2.** OW-14 adjuvanted vaccines induce long lasting and high level antibody response against swine influenza virus and mycoplasma antigens in pigs. Pigs (3 weeks old, 5 pigs/group) were vaccinated on Day 0 & 21 with swine influenza virus and *Mycoplasma hyopneumoniae* antigens mixed with or without adjuvant. The antibody response was followed until pigs reached to market age (5 months). Pathology evaluation at necropsy (5 months) did not find any injection site damage in any animals. (A) Hemagglutination inhibition titers against whole killed H3N2 swine influenza virus. The threshold for seroconversion was set at 320, which was 2 fold over negative pig serum. (B) *Mycoplasma hyopneumoniae* antibody production using IDEXX *M. hyo.* Ab kit. The threshold for seroconversion was set at a sample-to-positive (s/p) ratio of 0.4 according to manufacturers' instructions. (–): unvaccinated pigs (negative control); Ag only: pigs administered with antigens only; Adj only: pigs administered with adjuvant OW-14 only; OW-14: pigs administered with vaccines adjuvanted with OW-14; RespiSure and FluSure: Pigs vaccinated with commercial vaccines RespiSure and FluSure, respectively. \*  $p < 0.05$ .

OW-14/antigen had small (<2 cm) lumps in the neck muscle 1 week post vaccination. The lumps were not inflamed or readily visible and resolved by 2 weeks post injection. At slaughter, injection sites and all surrounding tissues were blindly examined by a veterinary pathologist. None of the animals had pathological changes to the tissue (data not shown).

### 3.3. OW-14 exposed to high temperatures is still an effective adjuvant for swine influenza and mycoplasma vaccines

Due to limited cold-chain availability at most farms, an ideal swine vaccine would not require refrigeration and could be subjected to transient warm environments. In order to determine if our OW-14 formulation was stable at transient high temperature, OW-14 was prepared as described above. OW-14 was then stored at 4°C, room temperature (RT), or 40°C for two weeks prior to being mixed with SIV and *M. hyo* antigens. OW-14 formulated or commercial vaccines were administered to pigs via intramuscular injection twice, three weeks between doses. Blood samples were collected and serum was isolated to monitor the development of antigen-specific IgG antibodies. It was found that HAI titers to H3N2 antigens were higher in OW-14 formulated vaccines than commercial FluSure vaccines at 35 days post vaccination (Fig. 3A). Fig. 3B shows that all OW-14 formulated vaccines, regardless of storage temperature, elicited an *M. hyo* antibody response greater than commercially available RespiSure vaccine 35 days post vaccination.



**Fig. 3.** OW-14 exposed to high temperatures is still an effective adjuvant for swine influenza virus (SIV) and mycoplasma vaccines. OW-14 adjuvant was stored at 4°C, room temperature (RT), or 40°C for 2 weeks prior to being mixed with SIV and *M. hyo* antigens. Pigs (3 weeks old, 6 pigs/group) were vaccinated on Day 0 & 21. (A) Hemagglutination titers against whole killed H3N2 swine influenza virus. The threshold for seroconversion was set at 320, which was 2 fold over negative pig serum. (B) *Mycoplasma hyopneumoniae* antibody production using IDEXX *M. hyo.* Ab kit. The threshold for seroconversion was set at a sample-to-positive (s/p) ratio of 0.4 according to manufacturers' instructions. \*  $p < 0.05$ .

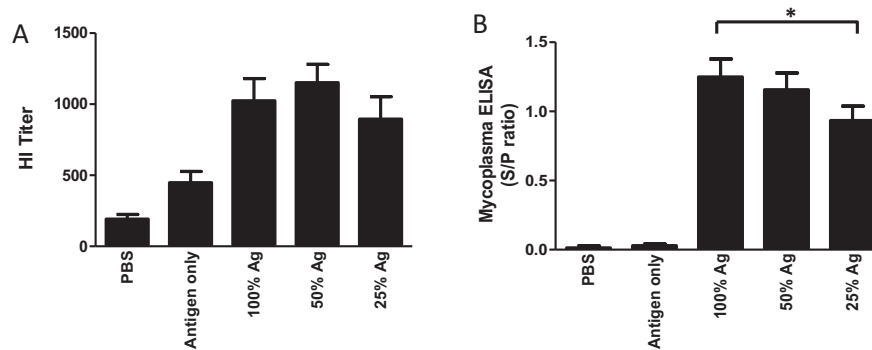
### 3.4. OW-14 adjuvant induces robust antibody production even at lower antigen doses

We mixed the OW-14 adjuvant with the standard antigen dose (1 mg H3N2 and 13.34 mg *M. hyo*), or diluted the antigens with PBS to create a vaccine containing a 50% antigen dose (0.5 mg H3N2 and 6.67 mg *M. hyo*), or 25% antigen dose (0.25 mg H3N2 and 3.34 mg *M. hyo*). Pigs were vaccinated with one of the three vaccines formulations on day 0 and 21 of the study. On day 42, serum was collected and assayed for H3N2- and *M. hyo*-specific antibodies. Pigs immunized with the 50% antigen dose had similar levels of antigen-specific antibody titers as that in pigs vaccinated with the full dose antigens (Fig. 4).

## 4. Discussion

One type of the cost-effective vaccine adjuvants is oil-in-water emulsions and they have been used in animal vaccines for over 50 years. We show here the development and characterization of a novel oil-in-water adjuvant formulation using a modified starch-based plant emulsifier (Ticamulsion A-2010). This emulsion is made with food-grade emulsifier and therefore could alleviate safety concerns with current detergent-emulsified formulations [16]. We have demonstrated that this formulation, OW-14, is stable for up to 3 years when stored at <25°C (Fig. 1) and oil droplets were determined to be between 50 and 700 nm with an average diameter of 240 nm, which has been determined to be the optimal size for stimulating the immune system [18–20]. Furthermore, OW-14 was stable, had a long shelf-life, and could easily be sterilized by autoclaving (Fig. 1).

We compared OW-14 formulated vaccines to commercially available FluSure and RespiSure vaccines due to their proven ability to induce high antibody responses and protection against microbial challenges [21–23]. Our results show that vaccines adjuvanted with OW-14 induce antibody responses equal to or better than that from



**Fig. 4.** OW-14 adjuvant induces robust antibody production even at lower antigen doses. OW-14 adjuvant was mixed with SIV and *M. hyo.* antigens. 100% antigen was equal to 1 mg of whole inactivated H3N2 virus and 13.34 mg of whole inactivated *M. hyo.* Pigs (3 weeks old, 5 pigs/group) were vaccinated on Day 0 & 21. (A) Hemagglutination inhibition titers against whole killed H3N2 swine influenza virus. The threshold for seroconversion was set at 320, which was 2 fold over negative pig serum. (B) *Mycoplasma hyopneumoniae* antibody production using IDEXX *M. hyo.* Ab kit. The threshold for seroconversion was set at a sample-to-positive (s/p) ratio of 0.4 according to manufacturers' instructions. \*  $p < 0.05$ .

commercial products (Figs. 2 and 3). In light of the fact that high antibody titers do not always correlate with protection, a future study in which vaccinated pigs are challenged with swine influenza virus or *M. hyo* will be essential in order to determine the efficacy of vaccines adjuvanted with OW-14.

The HI titers in pigs vaccinated with OW-14-experimental vaccine or the commercial vaccine at five weeks and two months post vaccination were significantly higher than that in control pigs (pigs administered with PBS, antigen alone, or adjuvant alone). However, a HI titer of ~300 in these control pigs at two months after the experiment started indicates that there might be a subclinical SIV infection in the herd. Furthermore, although the antibody responses in pigs vaccinated with OW-14-adjuvanted mycoplasma vaccine were comparable among different studies, we did observe a discrepancy on the anti-mycoplasma antibody titers in pigs vaccinated with RespiSure at five weeks post vaccination between two different experiments: ~1.5 in Fig. 2B and ~0.5 in Fig. 3B. There was no difference in the amount of RespiSure antigen or adjuvant used for the work reported in Figs. 2B and 3B. The difference in anti-mycoplasma antibody titer between the two experiments could be due to the variation of pigs used in these two studies or the product lot difference of RespiSure which was obtained from a commercial source on two different dates.

Mineral oil such as Penreco Drakeol 5 is routinely used to make oil-in-water emulsion adjuvants for animal vaccines. The use of Ticamulsion A-2010, a food grade plant-based emulsifier and a simple mixing protocol allow us to produce OW-14 at only a fraction of a penny per dose of vaccine. Additionally, we show in Fig. 4 that pigs vaccinated with vaccines containing half or a quarter of the amount of antigen produced high antibody titers at 42 days post vaccination. These results suggest that the amount of antigen within OW-14-formulated vaccines can be reduced and the vaccine will still be efficacious. Therefore, lowering the amount of antigen will further cut the overall cost of administering SIV and/or mycoplasma vaccines in pigs.

## 5. Conclusion

We have created a stable oil-in-water emulsion (OW-14) using cost-effective emulsifiers and oils. Animals vaccinated with OW-14-formulated vaccines had high antigen-specific antibodies titers, which were higher and lasted longer than that in pigs administered with commercial vaccines. We also found that vaccines formulated with lower antigen doses also produced a high antibody response. Therefore, OW-14 could be a cheaper and more effective alternate adjuvant for use in animal vaccines.

## Conflict of interest statement

The authors report no potential conflict of interests.

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