Ability of adjuvants to enhance the systemic antibody responses against H1N1 and H3N2 swine influenza viruses

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Introduction

Swine influenza (SI) is an acute infectious disease in pigs caused by swine influenza virus (SIV). During the past decade, SI has become a widespread and endemic disease in pig populations worldwide. The currently available method for the control of SI in a pig herd is the vaccination of young pigs with an inactivated whole virus vaccine containing an adjuvant. A monovalent vaccine containing SIV subtype H1N1 and a bivalent vaccine containing subtypes H1N1 and H3N2 are available commercially. These vaccines are well accepted. It is also well accepted that a hemagglutination inhibition (HI) titer of >1:40 is protective.[1] However, it has been reported that inactivated SIV vaccines do not consistently provide complete protection to virus challenges in vaccinated pigs.[2] One possible way to improve protection provided by SIV vaccines is to add more potent adjuvants that stimulate higher immune responses.

During the past few years, novel adjuvants, such as virosomes[3], muramyl peptides[4], MF59[5], and ISCOMS[6] have been tested with influenza vaccines in both animal and human models with variable efficacy. In response to market requirements, a variety of promising new adjuvants have been developed. These include EMULSIGEN® -D, EMULSIGEN®/Rehydragel-LV, EMULSIGEN®-BCL, and POLYGEN™ (MVP Laboratories, Inc.).

This study was conducted in order to evaluate some of these adjuvants with SIV antigens, comparing the capability of each adjuvant to enhance immune responses in young pigs vaccinated with currently available inactivated SIV antigens. A commercial vaccine containing inactivated freeze-dried H1N1 and H3N2 swine influenza antigens was used to supply the antigen mass. Five test vaccines were prepared by adding the recommended amount of either the manufacturer's adjuvant (ADJUVANT A) or one of the four new adjuvants to the inactivated freeze-dried bivalent antigens. With test vaccines containing a constant antigen, any variability in immune response would be related to the effect of the adjuvant. Immune responses stimulated in pigs by each adjuvant were evaluated using HI and ELISA antibody specific for H1N1 and H3N2.

Materials and methods

Adjuvants

The adjuvants used in this study were EMULSIGEN®-D, EMULSIGEN®/Rehydragel-LV, EMULSIGEN®-BCL and POLYGEN™ (MVP Laboratories, Inc.), plus the adjuvant that was supplied by the manufacturer of the freeze-dried SIV antigens used in this study. EMULSIGEN®-D is an oil-in-water emulsion containing dimethyldioctadecylammonium bromide (DDA) for added immune stimulation. EMULSIGEN®/Rehydragel-LV contains a controlled particle size emulsion plus aluminum hydroxide gel. EMULSIGEN®-BCL is a novel oil-in-water emulsion containing an immune-stimulant proprietary to the company supplying the adjuvant. POLYGEN™ is a low molecular weight, non-particulate copolymer adjuvant that has been demonstrated to stimulate excellent gamma interferon responses in cattle.[7]

Preparation of experimental vaccines

A commercial vaccine that contained inactivated freeze-dried H1N1 and H3N2 SI viruses with the manufacturer's adjuvant (ADJUVANT A) was purchased from a veterinary distributor. Just prior to vaccination, each experimental vaccine was prepared by adding and mixing the calculated amount of ADJUVANT A or one of the other four adjuvants to rehydrate the freeze-dried vaccine according to the manufacturer's recommendations.

Vaccination protocol

Prior to the study, approximately 20 sows were bled and their sera were evaluated for HI titer. Six pigs from

each of the 6 sows having the lowest HI titers for SIV were selected and identified as to litter by ear tags. Pigs were transported to the research facilities at the University of Nebraska for testing. When the pigs were 21 days of age, they were assigned randomly to 6 vaccine groups with one pig from each litter being assigned to each vaccine group. Pigs of group 1 through 5 were given vaccines containing EMULSIGEN®-D, EMULSIGEN®/Rehydragel-LV, EMULSIGEN®-BCL, POLYGEN™, and ADJUVANT A, respectively. Pigs in group 6 were given PBS only. The latter group of pigs served as the negative control group. On Day 0, all of the vaccinated pigs were injected intramuscularly with a 2.0 ml dose. On Day 21, all pigs were given a second dose of vaccine. All pigs were bled on Days 0, 21, and 42. The sera were processed and frozen.

HI assay

The HI assays were performed at the Veterinary Diagnostic Laboratory at Iowa State University (Swine Influenza Virus H1N1 HI Pfizer Test, and Swine Influenza Virus H3N2 HI). All sera were coded so that the evaluations were blinded.

ELISA for detection of antibodies to H1N1 and H3N2 swine influenza viruses

ELISA antibody responses were evaluated using the IDEXX HerdChek Swine Influenza Antibody Test Kit-H1N1 and Test Kit-H3N2. The test procedures and interpretation of results were performed according to the manufacturer's instructions. The sera from vaccinated pigs were diluted 1:40 with Sample Diluent and the positive and negative control sera were tested undiluted. One hundred microliters of the prepared serum samples were added to each well of ELISA plates precoated with SIV antigen specific for either H1N1 or H3N2. Positive and negative control sera were added to appropriate wells and were used to determine the S/P ratio for calculation of seroconversion. All samples were run in duplicate. Plates were incubated at room temperature for 30 minutes after which the liquid from each well was aspirated off and discarded. Each well was washed with 350 ul of Wash Solution 3 to 5 times. After removing the Wash Solution, 100 ul of Anti-Porcine: HRPO Conjugate was dispensed into each well. Plates were incubated again for 30 minutes at room temperature. Washing as described above was repeated and then 100 ul of TMS Substrate Solution was dispensed into each well. Plates were incubated for 15 minutes at room temperature after which 100 ul of Stop Solution was dispensed into each well to stop color reaction. The absorbance at 650 nm was measured and recorded. Seroconversion to SIV positive was determined by calculating the sample/positive (S/P) ratio for each sample. If the S/P ratio was less than 0.4, the sample was classified as negative for SI antibody. If the S/P ratio was greater than or equal to 0.4, the sample was classified as positive for SI antibody.

Statistical methods

A one-tailed Student's T Test was used for analysis of significance between groups.

Results

Hemagglutination inhibition (HI) responses

All pig sera from Days 0, 21 and 42 were tested for the presence of HI antibody titers against either H1N1 or H3N2 SI viruses. On Day 0, all 36 pigs were seronegative to both subtypes with HI titers <1:10. On Days 21 and 42, the HI titers of Group 6 pigs (negative control group) remained seronegative at <1:10 except for one pig that developed a titer to H1N1of 1:20 by Day 42. Pigs vaccinated with experimental vaccines formulated with either EMULSIGEN®-D or EMULSIGEN®-BCL gave enhanced HI responses when compared to all other adjuvants in this study (Tables 1, 2 and Figures 1, 2). The H1N1 Geometric Mean Titer (GMT) of the EMULSIGEN®-D group was 1437 as compared with GMTs of 640, 320, 127 and 320 for groups receiving EMULSIGEN®-BCL, EMULSIGEN®/Rehydragel-LV, POLYGEN(tm) and ADJUVANT A, respectively. The H3N2 GMT of the EMULSIGEN®-D group was 1613 as compared with GMTs of 453, 180, 57 and 226 for groups receiving EMULSIGEN®-BCL, EMULSIGEN®/Rehydragel-LV, POLYGEN™ and ADJUVANT A, respectively.

HI seroconversion

Tables 3 and 4 show that EMULSIGEN®-D and EMULSIGEN®-BCL produced the best results with H1N1 seroconversions of 50% and 33% respectively by Day 21, whereas none of the pigs in the other groups seroconverted by Day 21. EMULSIGEN®-D and EMULSIGEN®-BCL were again the only adjuvants that seroconverted pigs to H3N2 by Day 21 (83% for EMULSIGEN®-D and 33% for EMULSIGEN®-BCL). All pigs in all vaccinate groups seroconverted by Day 42.

Table 1: Systemic antibody responses against swine influenza virus, subtype H1N1, in young pigs vaccinated with one of the five experimental SIV vaccines.

Vaccine adjuvant	Day 0		Day 21		Day 42	
and pig ID	ELISA*	HI	ELISA	HI	ELISA	HI
1. EMULSIGEN® -D						
#1	0.053 (--)	<10	0.059 (--)	20	0.560 (+)	1280
#14	0.055 (--)	<10	0.057 (--)	20	0.192 (+)	2560
#27	0.053 (--)	<10	0.084 (--)	80	0.330 (+)	320
#40	0.053 (--)	<10	0.073 (--)	40	0.381 (+)	1280
#11	0.052 (--)	<10	0.057 (--)	10	0.244 (+)	1280
#24	0.052 (--)	<10	0.058 (--)	40	0.289 (+)	5120
GMT	0.053	<10	0.064	28	0.314	1437
2. EMULSIGEN® /Rehydragel-LV						
#2	0.052 (--)	<10	0.055 (--)	20	0.184 (+)	320
#15	0.055 (--)	<10	0.059 (--)	10	0.096 (--)	320
#28	0.052 (--)	<10	0.053 (--)	10	0.161 (--)	160
#41	0.055 (--)	<10	0.058 (--)	20	0.048 (--)	320
#12	0.051 (--)	<10	0.066 (--)	10	0.125 (--)	320
#31	0.049 (--)	<10	0.058 (--)	20	0.276 (+)	640
GMT	0.052	<10	0.059	14	0.121	320
3. EMULSIGEN® -BCL						
#3	0.050 (--)	<10	0.052 (--)	20	0.353 (+)	1280
#16	0.056 (--)	<10	0.055 (--)	10	0.350 (+)	2560
#29	0.049 (--)	<10	0.065 (--)	40	0.195 (+)	160
#42	0.048 (--)	<10	0.073 (--)	20	0.238 (+)	320
#19	0.056 (--)	<10	0.058 (--)	20	0.170 (+)	320
#32	0.050 (--)	<10	0.071 (--)	80	0.201 (+)	1280
GMT	0.051	<10	0.062	25	0.241	640
4. POLYGEN™						
#4	0.050 (--)	<10	0.053 (--)	10	0.098 (--)	160
#17	0.050 (--)	<10	0.054 (--)	<10	0.072 (--)	160
#30	0.051 (--)	<10	0.056 (--)	<10	0.082 (--)	80
#7	0.053 (--)	<10	0.054 (--)	<10	0.086 (--)	80
#20	0.050 (--)	<10	0.056 (--)	<10	0.082 (--)	80
#33	0.051 (--)	<10	0.053 (--)	20	0.126 (--)	320
GMT	0.051	<10	0.054	0.5	0.090	127
5. ADJUVANT A (from commercial vaccine)						
#5	0.049 (--)	<10	0.054 (--)	20	0.094 (--)	160
#18	0.057 (--)	<10	0.052 (--)	10	0.089 (--)	160
#37	0.049 (--)	<10	0.050 (--)	10	0.275 (+)	1280
#8	0.045 (--)	<10	0.059 (--)	10	0.171 (+)	320
#21	0.049 (--)	<10	0.054 (--)	10	0.218 (+)	320
#34	0.047 (--)	<10	0.053 (--)	10	0.168 (--)	320
GMT	0.049	<10	0.054	11	0.156	320
6. PBS (negative control group)						
#13	0.052 (--)	<10	0.056 (--)	<10	0.065 (--)	<10
#26	0.054 (--)	<10	0.060 (--)	<10	0.062 (--)	<10
#39	0.052 (--)	<10	0.054 (--)	<10	0.063 (--)	<10
#10	0.047 (--)	<10	0.053 (--)	<10	0.084 (--)	20
#23	0.046 (--)	<10	0.051 (--)	<10	0.057 (--)	<10
#36	0.045 (--)	<10	0.056 (--)	<10	0.092 (--)	<10
GMT	0.049	<10	0.055	<10	0.069	<10

* According to the ELISA Kit instructions, the presence or absence of antibody to SIV subtype is determined by calculating the S/P ratio for each sample. If the S/P ratio is greater than or equal to 0.4, then the sample is positive for SIV antibody. In this study, the Positive Control mean is 0.488 for H1N1and 0.490 for H3N2, and the Negative Control is 0.059 for H1N1 and 0.059 for H3N2.

Table 2: Systemic antibody responses against swine influenza virus, subtype H3N2, in young pigs vaccinated with one of the five experimental SIV vaccines.

Vaccine adjuvant	Day 0		Day 21		Day 42	
and pig ID	ELISA*	HI	ELISA	HI	ELISA	HI
1. EMULSIGEN® -D						
#1	0.053 (--)	<10	0.318 (+)	40	0.832 (+)	5120
#14	0.055 (--)	<10	0.278 (+)	40	0.588 (+)	2560
#27	0.052 (--)	<10	0.394 (+)	40	0.631 (+)	640
#40	0.053 (--)	<10	0.263 (+)	40	0.685 (+)	2560
#11	0.056 (--)	<10	0.113 (--)	<10	0.633 (+)	640
#24	0.059 (--)	<10	0.259 (+)	40	0.667 (+)	1280
GMT	0.055	<10	0.254	22	0.669	1613
2. EMULSIGEN® /Rehydragel-LV						
#2	0.057 (--)	<10	0.157 (--)	10	0.731 (+)	320
#15	0.057 (--)	<10	0.140 (--)	20	0.564 (+)	80
#28	0.056 (--)	<10	0.099 (--)	20	0.601 (+)	160
#41	0.073 (--)	<10	0.054 (--)	<10	0.476 (+)	160
#12	0.055 (--)	<10	0.131 (--)	10	0.644 (+)	160
#31	0.055 (--)	<10	0.199 (+)	20	0.653 (+)	320
GMT	0.058	<10	0.121	10	0.606	180
3. EMULSIGEN® -BCL						
#3	0.057 (--)	<10	0.129 (--)	10	0.778 (+)	640
#16	0.059 (--)	<10	0.117 (--)	10	0.727 (+)	640
#29	0.053 (--)	<10	0.480 (+)	160	0.628 (+)	320
#42	0.051 (--)	<10	0.138 (--)	20	0.544 (+)	320
#19	0.060 (--)	<10	0.157 (--)	10	0.635 (+)	320
#32	0.056 (--)	<10	0.432 (+)	160	0.678 (+)	640
GMT	0.056	<10	0.202	62	0.661	453
4. POLYGEN™						
#4	0.055 (--)	<10	0.071 (--)	<10	0.516 (+)	80
#17	0.053 (--)	<10	0.066 (--)	<10	0.571 (+)	40
#30	0.055 (--)	<10	0.084 (--)	<10	0.614 (+)	40
#7	0.057 (--)	<10	0.101 (--)	<10	0.506 (+)	40
#20	0.057 (--)	<10	0.067 (--)	<10	0.463 (+)	40
#33	0.053 (--)	<10	0.078 (--)	<10	0.640 (+)	160
GMT	0.055	<10	0.077	<10	0.548	57
5. ADJUVANT A (from commercial vaccine)						
#5	0.060 (--)	<10	0.213 (+)	20	0.694 (+)	160
#18	0.065 (--)	<10	0.107 (--)	10	0.566 (+)	80
#37	0.055 (--)	<10	0.099 (--)	<10	0.696 (+)	640
#8	0.053 (--)	<10	0.115 (--)	20	0.730 (+)	160
#21	0.052 (--)	<10	0.146 (--)	10	0.674 (+)	320
#34	0.051 (--)	<10	0.132 (--)	20	0.566 (+)	320
GMT	0.056	<10	0.131	10	0.651	226
6. PBS (negative control group)						
#13	0.073 (--)	<10	0.065 (--)	<10	0.061 (--)	<10
#26	0.058 (--)	<10	0.059 (--)	<10	0.060 (--)	<10
#39	0.059 (--)	<10	0.056 (--)	<10	0.058 (--)	<10
#10	0.058 (--)	<10	0.061 (--)	<10	0.160 (--)	20
#23	0.054 (--)	<10	0.060 (--)	<10	0.063 (--)	<10
#36	0.054 (--)	<10	0.062 (--)	<10	0.070 (--)	<10
GMT	0.059	<10	0.060	<10	0.073	<10

Figure 1: Adjuvant stimulation of HI antibody to SIV H1N1 in swine receiving bivalent SIV vaccines containing a constant antigen mass.

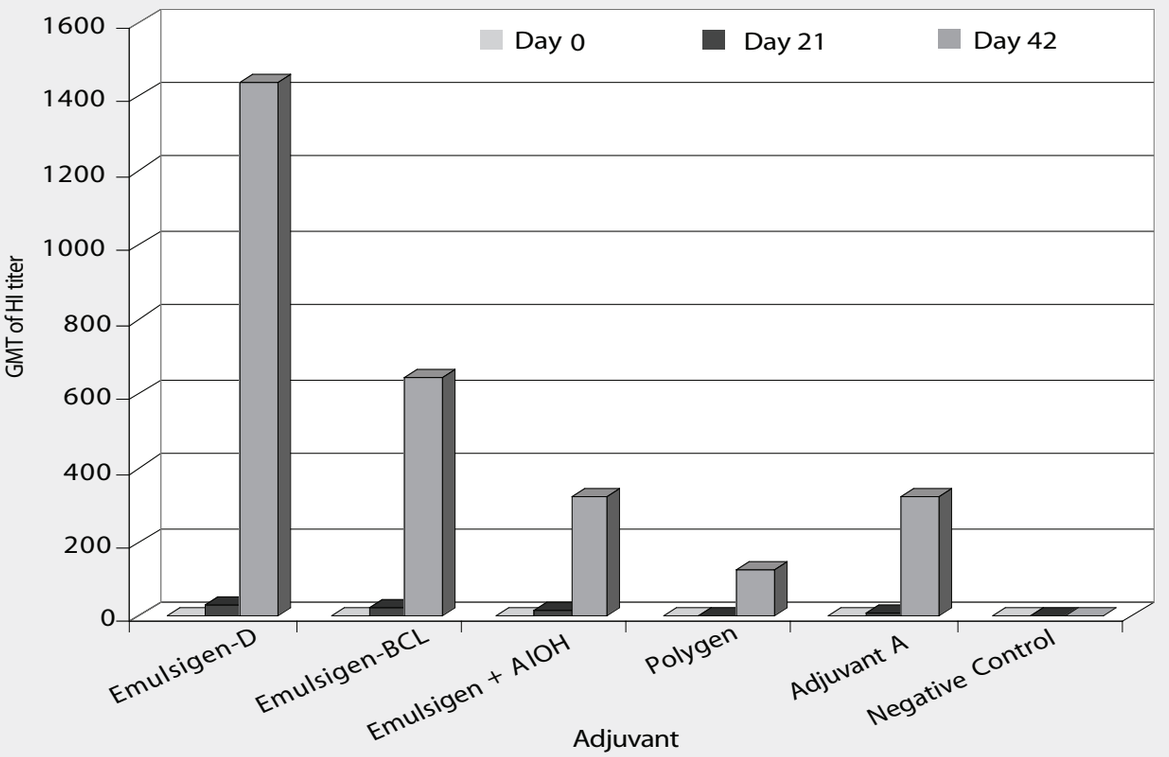


Figure 2: Adjuvant stimulation of HI antibody response to SIV H3N2 in swine receiving vaccines containing a constant antigen mass.

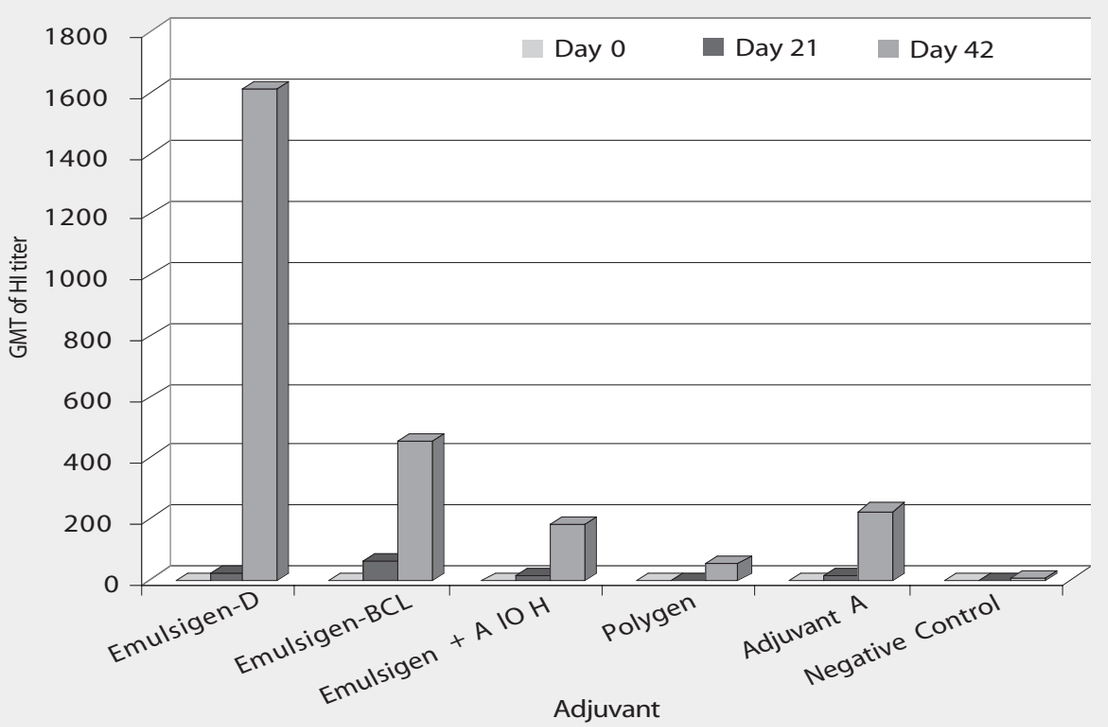


Figure 3: Adjuvant stimulation of ELISA antibody response to SIV H1N1 in swine receiving vaccines containing a constant antigen mass.

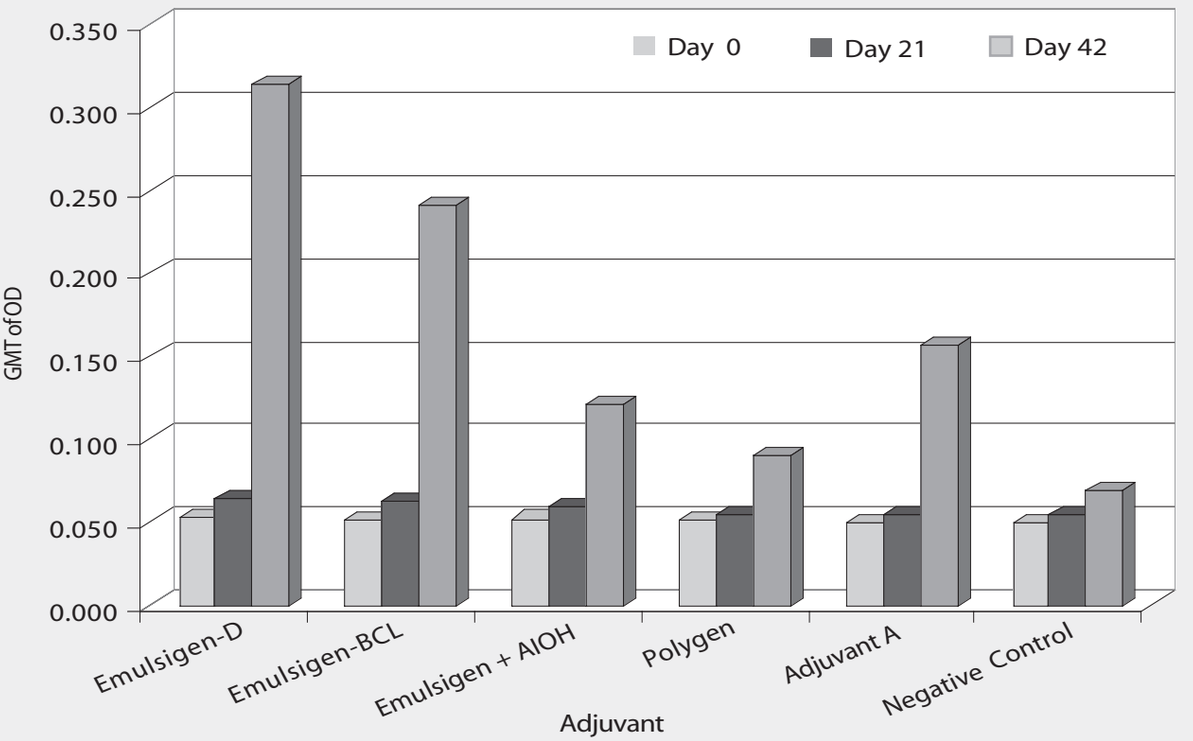
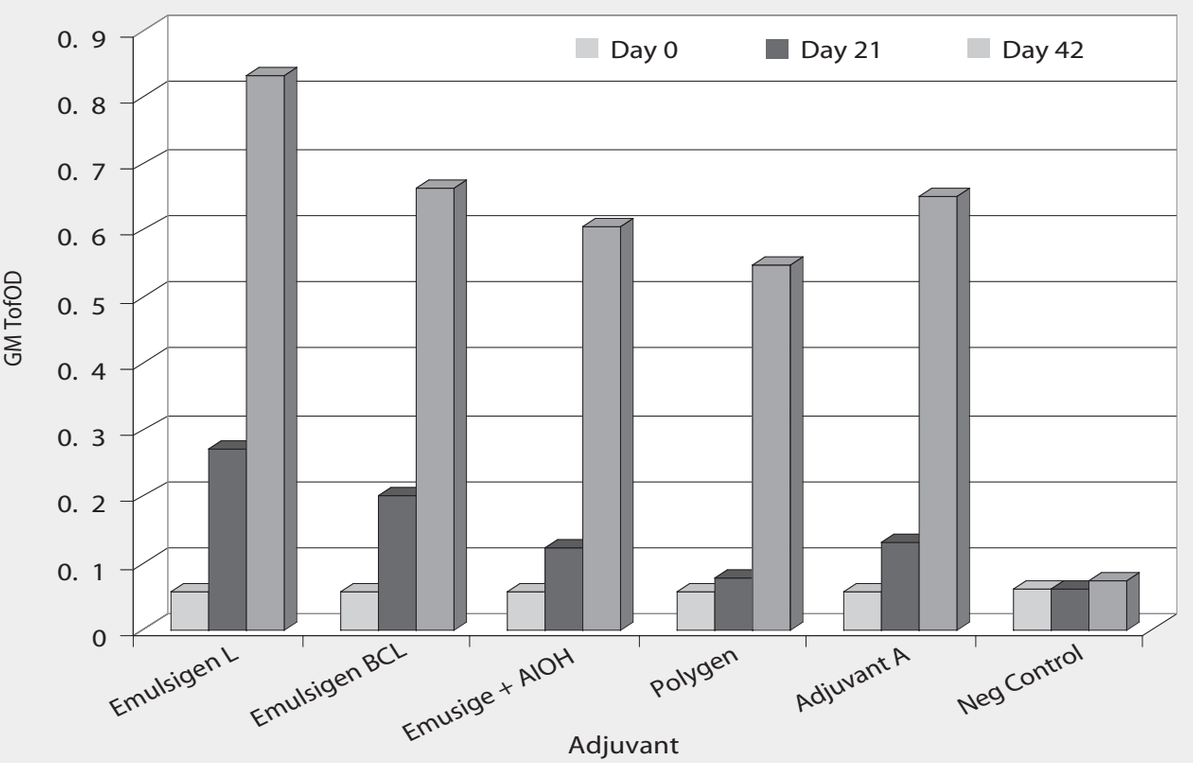


Figure 4: Adjuvant stimulation of ELISA antibody response to SIV H3N2 in swine receiving vaccines containing a constant antigen mass.



ELISA responses

All pigs were seronegative to both H1N1 and H3N2 on Day 0, confirming the HI results. ELISA antibody could not be detected in any of the Group 6 pigs throughout the study. ELISA antibody titers as indicated by optical densities showed the same general pattern as the HI titers (Figures 1, 3, and figures 2, 4). For both H1N1 and H3N2, the highest ELISA ODs were produced by the vaccine containing EMULSIGEN®-D, with the next highest results being produced by EMULSIGEN®-BCL. On Day 42, H1N1 geometric mean OD values were 0.314, 0.121, 0.241, 0.090, 0.156 and 0.069 for EMULSIGEN®-D, EMULSIGEN® /Rehydragen-LV, EMULSIGEN®-BCL, POLYGEN™, ADJUVANT A and the Negative Control Group, respectively. All pigs remained seronegative on Day 21 to H1N1. H3N2 geometric mean OD values were higher. Day 42 values were 0.669, 0.606, 0.661, 0.548, 0.651 and 0.073, respectively for the groups listed above. Day 21 values were 0.254, 0.121, 0.202, 0.077, 0.131 and 0.060, respectively for the above listed groups. This indicates that pigs were responding to H3N2 by Day 21, especially pigs in the EMULSIGEN®-D and EMULSIGEN®-BCL groups.

ELISA seroconversion

Tables 3 and 4 illustrate the results of this testing. The H1N1 and H3N2 Positive Control optical density vaules were 0.488 and 0.490, respectively. These values were used for calculation of the S/P ratio. All pigs remained seronegative to H1N1 on Day 21. By Day 42 (three weeks after the second vaccination), 100% of the EMULSIGEN®-D and EMULSIGEN®-BCL pigs seroconverted to H1N1, whereas 33% of the EMULSIGEN®/Rehydragel-LV, 0% of the POLYGEN™ and 50% of the ADJUVANT A group seroconverted. Pigs vaccinated with H3N2 antigen began to seroconvert by Day 21. The seroconversion percentage by Day 21

Table 3: Comparison of ELISA and HI seroconversion to SIV H1N1 antigen in pigs receiving vaccines containing various adjuvants.

Vaccine adjuvant	Percentage of swine converting to positive H1N1					
	Day 0		Day 21		Day 42	
	ELISA	HI	ELISA	HI	ELISA	HI
EMULSIGEN® -D	0	0	0	50	100	100
EMULSIGEN® /Rehydragel-LV	0	0	0	0	33	100
EMULSIGEN® -BCL	0	0	0	33	100	100
POLYGEN™	0	0	0	0	0	100
ADJUVANT-A	0	0	0	0	50	100
Negative control	0	0	0	0	0	0

HI seroconversion is considered to be a titer of >1:40

Table 4: Comparison of ELISA and HI seroconversion to SIV H3N2 antigen in pigs receiving vaccines containing various adjuvants.

Vaccine adjuvant	Percentage of swine converting to positive H3N2					
	Day 0		Day 21		Day 42	
	ELISA	HI	ELISA	HI	ELISA	HI
EMULSIGEN® -D	0	0	83	83	100	100
EMULSIGEN® /Rehydragel-LV	0	0	017	0	100	100
EMULSIGEN® -BCL	0	0	33	33	100	100
POLYGEN™	0	0	0	0	100	100
ADJUVANT-A	0	0	0	0	100	100
Negative control	0	0	0	0	0	0

HI seroconversion is considered to be a titer of >1:40

was 83% for the EMULSIGEN®-D group, 17% for the EMULSIGEN®/Rehydragel-LV group, 33% for the EMULSIGEN®-BCL group, 0% for the POLYGEN(tm) group and 17% for the ADJUVANT A group. All of the vaccinated pigs in all five vaccine groups seroconverted to H3N2 by Day 42.

Discussion

Vaccination of pigs against swine influenza is generally carried out by administering two intra-muscular injections of an inactivated whole virus vaccine containing an adjuvant. The purpose of the present study was to determine the comparative adjuvant effects of five different adjuvants, when added to a constant antigenic mass of bivalent, freeze-dried H1N1 and H3N2 SIV antigens from a commercial source. Adjuvants evaluated in this study were EMULSIGEN®-D, EMULSIGEN®-BCL, EMULSIGEN®/Rehydragel-LV, POLYGEN™ and the adjuvant supplied by the manufacturer of the vaccine (ADJUVANT A). All of the adjuvants except POLYGEN™ contained an oil and water base. Therefore, this study evaluated the enhancement of the immune responses when immunostimulants were added to the oil and water base. The study also provided a comparison of oil and water-based adjuvants with a copolymer base adjuvant (POLYGEN™) for use with SIV vaccines.

Each of the adjuvants was used as a diluent for a bottle of the freeze-dried combination antigens after which they were used to vaccinate groups of young pigs (6 pigs per group). An additional group of pigs was injected with PBS and served as a negative control group. Serum samples from all of the pigs were evaluated for specific H1N1 and H3N2 HI titers and ELISA antibody at Days 0, 21 and 42.

All vaccine/adjuvant groups stimulated protective HI titers to both H1N1 and H3N2 in all pigs by Day 42 post vaccination. Additionally, all adjuvants stimulated a significant increase in HI and ELISA antibody responses when compared with the negative control group at the $P = < 0.05$ level. EMULSIGEN®-D was shown to be the most effective adjuvant for use with SIV antigens. The SIV vaccine containing EMULSIGEN®-D produced significantly higher HI titers against both H1N1 and H3N2 on Days 21 and 42 than the positive control group containing ADJUVANT A ($P = < 0.05$ for all values). Further, the ELISA antibody stimulated by EMULSIGEN®-D was significantly higher than that stimulated by ADJUVANT A for both H1N1 and H3N2 on Day 21 and for H1N1 on Day 42 ($P = < 0.05$ for all values). Finally, the vaccine containing EMULSIGEN®-D produced a higher seroconversion rate to both H1N1 and H3N2 than the vaccine containing ADJUVANT A. EMULSIGEN®-BCL was the second best adjuvant for use with SIV antigens. It produced Day 42 antibody responses that were significantly higher than those produced by ADJUVANT A using the H1N1 ELISA ($P = < 0.05$) and enhancement of all other antibody responses when compared to ADJUVANT A. Also, vaccine containing EMULSIGEN®-BCL produced the second best rate of seroconversion by both HI and ELISA.

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