NOVEL VACCINE TECHNOLOGIES IN ANIMAL HEALTH

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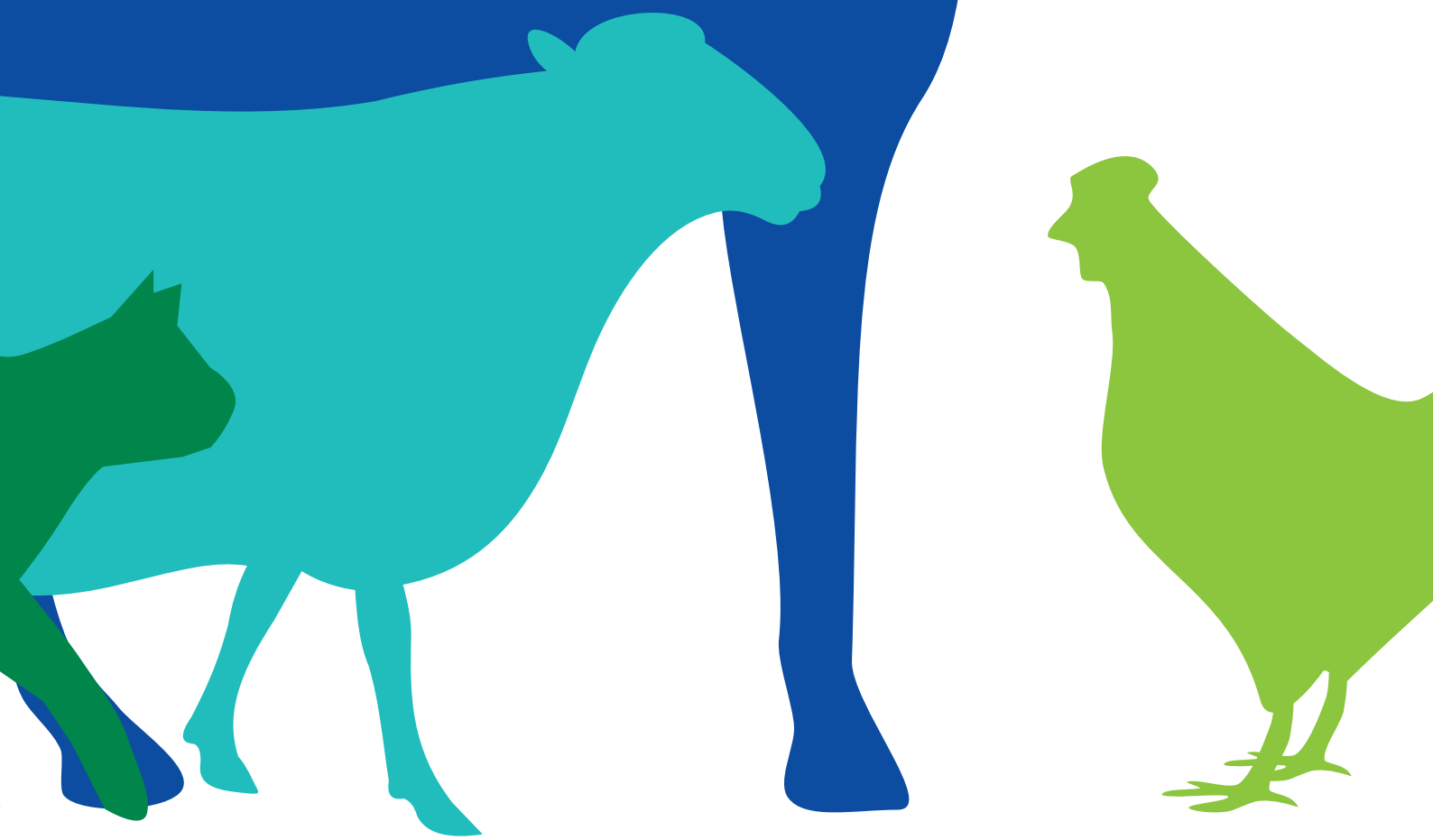

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NOVEL VACCINE TECHNOLOGIES IN ANIMAL HEALTH

Topic Editors:
Constantinos S. Kyriakis, Auburn University, United States
George C. Fthenakis, University of Thessaly, Greece

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EDITORIAL
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Editorial: Novel Vaccine Technologies in Animal Health

Vasilis C. Pliasas[1], *George C. Fthenakis*[2] *and Constantinos S. Kyriakis*[1,3]*

[1] *Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL, United States,* [2] *Veterinary Faculty, University of Thessaly, Karditsa, Greece,* [3] *Center for Vaccines and Immunology, University of Georgia, Athens, GA, United States*

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Editorial on the Research Topic

Novel Vaccine Technologies in Animal Health

Conventional vaccine technologies, such as inactivated and attenuated live vaccines, have saved millions of lives during the past century, yet the unmitigated spread of SARS-CoV-2 revealed a blind spot in our capacity to respond to emerging infectious diseases (1). Suboptimal performance issues, high costs and limitations in regards to scale-up production, illustrate the major caveats of traditional vaccine approaches for a time-sensitive response to emergent pathogens with pandemic potential (2, 3). Despite significant advances in vaccine research and development, human vaccinology was arguably kept in a relative stalemate in the pre-pandemic era with limited innovative vaccine approaches receiving licensure (4). On the other hand, veterinary science is a fertile ground for the development and commercialization of novel vaccine technologies. Direct evaluation of vaccine efficacy in target species capitalizes on the growing interest in livestock and companion animal health driving the progress and innovation of veterinary vaccines (3, 5).

The objective of this Research Topic was to bring attention on the state-of-the-art research conducted in veterinary vaccinology and highlight innovative vaccine technologies that are being explored and exploited for the improvement of animal health.

Aida et al. gave a comprehensive overview of the current advances in the field of veterinary vaccinology and reviewed commercially available novel vaccine technologies utilized in animal health, including recombinant protein/subunit vaccines, DNA constructs, viral vector technologies, and DIVA vaccines. This study reported that 52% of licensed novel vaccines in animal health were viral vector technologies, while subunit-recombinant protein vaccines were the second most available platform with 27%. Additionally, the vast majority of innovative veterinary vaccines are commercialized in food animals, with swine constituting approximately one third of the overall licensed novel vaccines.

Inactivated and attenuated live vaccines which represent first-generation vaccine technologies, are often reported to be less effective in inducing sufficient protection against a plethora of pathogens, including the porcine epidemic diarrhea virus (PEDV), a swine enteric coronavirus. Singh et al. developed a novel PEDV vaccine that utilized elements of both inactivated and attenuated live vaccines for the generation of an immunogenic construct that shows diminished, yet not abolished virus replication, which is a requirement for the elicitation of mucosal immunity and protection from PEDV.

A different coronavirus that causes significant economic losses to the poultry industry is the Infectious Bronchitis virus (IBV). While vaccines are the most effective countermeasure for disease prevention, limitations in vaccine effectiveness against heterologous IBV strains, pose a great threat in disease control. Improvement on current serological assays such as ELISA is pivotal for the development of more potent IBV vaccines. In this regard,

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Michael Kogut, Agricultural Research Service (USDA), United States

***Correspondence:**
Constantinos S. Kyriakis
csk@auburn.edu

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Yin et al. developed and validated an ELISA that utilized a peptide comprised by a conserved, across variant IBV strains, epitope of the S2 subunit of the spike protein. The high sensitivity and specificity of this novel diagnostic assay facilitates early identification of anti-IBV antibodies from day 7 post-immunization and detection of antibodies against multiple IBV genotypes, and could prove to be a valuable tool for the generation of effective IBV vaccines.

Second-generation vaccine technologies, constituted by subunit and recombinant protein vaccines evoke antigen-specific immune targeting and represent an attractive alternative to the safety and production cost concerns of first-generation vaccines (6). Kalaiyarasu et al. recently optimized a recombinant M2-HA2 fusion protein, comprised by conserved regions of the corresponding M2 and Hemagglutinin (HA) proteins, across highly-pathogenic avian influenza virus (HP-AIV) strains, as a broadly protective HP-AIV vaccine strategy. However, a relative disadvantage of second-generation vaccine platforms, is that they often require the presence of adjuvants (7). Thus, an important aspect for the generation of effective vaccines is the enhancement of their immunogenicity by including a potent adjuvant. Lee et al. evaluated a novel adjuvant that was able to protect vaccinated mice from lethal AIV challenge and elicit comparable, to the commercial adjuvant, humoral responses against AIV and Newcastle disease virus in vaccinated chickens.

In addition to poultry, swine are a natural host of influenza A virus (IAV), which causes substantial economic impact to the pork industry. A review by Gracia et al. addressed the implications of utilizing commercial inactivated vaccines for disease control as a result of the complex epidemiology of IAV worldwide. Additionally, the authors provide an overview of the innovative IAV vaccine approaches currently explored in swine. First-generation IAV vaccines predominately target anti-HA specific epitopes and show limited efficacy against heterologous strains. Neuraminidase (NA), is an attractive IAV immunogen due to the limitations of HA-targeting constructs. Anti-NA antibodies inhibit the enzymatic activity of NA thus rendering Neuraminidase Inhibition (NI) assays as the golden standard for assessing NA-targeting humoral responses (8, 9). Skarlupka and Ross reported that raw sera may have non-specific NA inhibitory activities. Innate NA inhibitory properties can skew the NI assay results, if sera is not properly treated, which could prove detrimental especially when assessing novel NA-based vaccine technologies.

Similar to viruses, intracellular bacteria (ICB) require the induction of both humoral and cell mediated responses for effective clearance, and oftentimes ICB infections are characterized by the evasion of the former (10). On this account, Kim et al. measured the differential antibody responses induced by different antigenic forms of Salmonella Gallinarum (SG) in vaccinated chickens, with the intention of detecting antigenic epitopes that could be utilized in highly immunogenic SG vaccines. Another ICB infection that poses a significant zoonotic threat is Brucellosis. Huy et al. developed and evaluated the efficacy of a novel vaccine consisting of four recombinant Brucella abortus proteins. This combined subunit vaccine (CSV) enhanced the expression of innate bactericidal factors and conferred comparable protection in mice against disease and bacterial replication, to the commercial vaccine, by inducing a robust Th1 phenotype immune response.

In summary, this Research Topic highlighted some of the latest developments and innovations in the dynamic field of veterinary vaccinology research. It is essential to keep exploring and investigating novel vaccine approaches if we aim to effectively control infectious diseases in public and animal health.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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A Minimally Replicative Vaccine Protects Vaccinated Piglets Against Challenge With the Porcine Epidemic Diarrhea Virus

*Gagandeep Singh[1], Pankaj Singh[1], Angela Pillatzki[2], Eric Nelson[2], Brett Webb[3], Steven Dillberger-Lawson[2] and Sheela Ramamoorthy[1]**

[1] Department of Microbiological Sciences, North Dakota State University, Fargo, ND, United States, [2] Animal Disease Research and Diagnostic Laboratory, South Dakota State University, Brookings, SD, United States, [3] Veterinary Diagnostic Laboratory, North Dakota State University, Fargo, ND, United States

Porcine epidemic diarrhea virus (PEDV), is an economically important enteric coronavirus, with over a 90% mortality rate in neonatal piglets. The virus emerged in the US in 2013, resulting in severe production losses. Effective vaccine development against PEDV is a challenge. Inactivated vaccines are of questionable efficacy. Attenuated vaccines, while more effective, require a relatively long lead development time, are associated with safety concerns and are also unable to prevent new field outbreaks. To combine the safety and efficacy advantages of inactivated and attenuated PEDV vaccines, respectively, in this study, we tested the hypothesis that subjecting PEDV virions to heat treatment at 44°C for 10 min to reversibly unfold structural proteins, followed by exposure to RNAse to fragment the genome, would result in a vaccine preparation with intact viral structure/antigenicity but highly diminished replicative abilities. We expected the vaccine to be both safe and effective in a piglet challenge model. Following the heat and RNAse treatment, PEDV virions had an intact electron microscopic ultrastructure and were amplified only in the 3rd passage in Vero cells, indicating that diminished replication was achieved *in vitro*. Strong PEDV spike-protein specific and virus neutralizing antibody responses were elicited in vaccinated piglets. Upon challenge, all vaccinated pigs were protected against fecal viral shedding and intestinal pathology, while the unvaccinated controls were not. The vaccine virus was not detected in the fecal matter of vaccinated pigs prior to challenge; nor did they develop intestinal lesions. Thus, the described approach has significant promise in improving current approaches for PEDV immunization.

Keywords: vaccine, porcine epidemic diarrhea virus, PEDV, antibody, spike

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Edited by:
Zhenhai Chen,
Yangzhou University, China

Reviewed by:
Luis Enjuanes,
Centro Nacional de Biotecnología (CNB), Spain
Dongbo Sun,
Heilongjiang Bayi Agricultural University, China

***Correspondence:**
Sheela Ramamoorthy
sheela.ramamoorthy@ndsu.edu

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INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is an enteric coronavirus which causes diarrhea, vomiting, severe dehydration, and death in pigs. Neonatal pigs are particularly susceptible, with mortality rates that can be as high as 90–100%. In older pigs, manifestation of the disease is milder but growth and production parameters are affected (1, 2). Classical strains of PEDV (G1 strains) were first detected in the UK in 1971, and spread to Asia and Europe. More recently, highly virulent strains

(G2 strains) which emerged in China have spread to other countries, with the first case in the US being recorded in 2013 (1, 3, 4). It is estimated that the outbreak resulted in the losses of $0.9–1.8 billion and the death of 7 million pigs (5, 6). The availability of effective vaccines and the practice of stringent biosecurity measures are critical for the prevention of PEDV. However, the development of effective vaccines has been complicated by frequent viral evolution and the fact that PED is most severe in immunologically naïve neonates. Effective and safe vaccines development was also challenging because active vaccine replication in the gut is required to induce good and lasting mucosal immunity.

Both attenuated and inactivated PEDV vaccines have been routinely used in Asian countries for several years. Vaccination of sows prior to farrowing induces lactogenic immunity which is transferred to neonatal piglets via colostrum. Inactivated vaccines are very safe but have a low duration of immunity and appear to produce a predominantly Th2 type immune response (7). Attenuated vaccines, produced by serially passaging field strains between 83 and 100 passages, are more effective against homologous strains but have a long lead development time and have been associated with safety concerns of recombination with field strains (2). Regardless of the type of vaccine used, viremia and transmission of PEDV is not prevented in vaccinated animals. Outbreaks in vaccinated herds and the periodical emergence of new, highly pathogenic strains are not uncommon in countries were vaccines have been routinely used for many years (2, 7–9).

In North America, a S-protein based subunit vaccine (iPED plus, Harris Vaccines Inc.) and inactivated vaccines produced by Zoetis and VIDO-Intervac were conditionally licensed. However, their efficacy has also been questioned by independent studies, as vaccination of PEDV naïve sows did not result in strong protection in neonatal piglets (8, 10, 11). As the strong need for effective PEDV vaccines remains unmet, the practice of feeding back minced intestines from infected piglets to sows, in an attempt to induce more effective immunity against PEDV, is common in the field (8, 10, 11). The use of autogenous vaccines, where a custom inactivated vaccine tailored to each herd is prepared using a sample provided from the production unit, is also practiced (8, 12, 13). Both the feedback and autogenous vaccine approaches are, once again, associated with significant safety and efficacy issues but natural or intentional exposure of pigs of all ages to PEDV provides stronger homologous and partial heterologous protection (2, 8). Further, vaccination of naïve animals is less effective than vaccination of previously exposed pigs, indicating that current vaccines are less effective than natural infection at priming the immune response but can effectively boost the memory response (14). It is established that the viral spike protein is a critical protective antigen, as anti-spike protein-specific serum IgG levels correlate well with protection against PEDV and virus neutralizing responses (15). However, the S-protein based subunit vaccine (iPED plus, Harris Vaccines Inc.) is of questionable efficacy, indicating that other viral components could contribute to protection.

Based on the above, we hypothesized that development of a process whereby the structural integrity of the virus was maintained but viral replication was highly diminished but not abrogated, would result in a vaccine with the combined advantages of inactivated and attenuated vaccines, namely, high safety and efficacy margins. Previously published data shows that the SARS coronavirus capsid is metastable and can be reversibly denatured by changes in temperature or pH, with unfolding commencing at 35°C and complete denaturation occurring at 55°C (16). Hence, in this study, our vaccine development approach consisted of exposing PEDV virions to 44°C to unfold the capsid, followed by fragmentation or digestion of the genome with RNAse to diminish viral replication and subsequent refolding of the capsid at 25°C. Gamma-irradiated PEDV virions were used as an inactivated control vaccine (17). The objective of this study was to evaluate the heat and RNAse treated PEDV vaccine for its safety, immunogenicity and ability to reduce viremia in a weanling piglet model, with the ultimate goal of developing a process which can potentially reduce lead vaccine development time, is safe and be easily applied to newly emerging strains.

MATERIALS AND METHODS

Cells and Viruses

Porcine epidemic diarrhea virus (PEDV) strain PEDV CO2013 [National Veterinary Services Laboratory (NVSL), Ames, IA] was cultured at a multiplicity index (MOI) of 0.1 using Vero cells in the presence of trypsin as previously described (18, 19). The stock virus was titrated three times to obtain the mean 50% tissue culture infectious dose [$TCID_{50}$] using the Spearman and Karber formula (20) and stored in aliquots at −80°C until further use.

Vaccine Preparation

To optimize the temperature, time of incubation, and dose of RNAse treatment, the virus stock was resuspended to 1×10^5 $TCID_{50}$/ml in media (pH 7.2). Diluted virus culture was exposed to temperatures ranging from 37 to 60°C for 10 min for unfolding, followed by incubation at 25°C for 30 min for refolding, and then moved to 4°C for 1 h, as previously described for the SARS coronavirus (16). Cultures were visualized by electron microscopy to ensure structural integrity. A temperature of 44°C for 10 min was selected for unfolding. Similarly, to fragment the genomic RNA, varying combinations of concentrations of RNAse A (Ameresco) and RNAse T (Thermo Scientific) were tested by adding them to the unfolded virus cultures, followed by incubation for 5, 4, 3, or 2 h at 44°C. Treated cultures were then exposed to 25°C for 30 min for refolding and cooled down on ice for 1 h. The final optimized protocol consisted of exposing the virus culture, resuspended to 10^5 $TCID_{50}$/ml, to 44°C for 10 min, followed by 0.1 mg/ml of RNase A and 1 μl/ml of RNase T1 (equivalent to 10 units/ml RNase A or 1,000 units/ml of RNAse T1), incubation at 44°C for 4 h, exposure to 25°C for 30 min and cooling down on ice for 1 h before storage at −80°C for further testing. The final process was tested 3 times to ensure reproducibility.

To prepare the inactivated control vaccine, 1×10^5 $TCID_{50}$/ml of PEDV was irradiated in a Cesium-137 source gamma (γ) irradiator at time points of 8 h to 24 h at 753 rad/min.

An effective dose of 24 h (1,084,320 rad), was used to prepare the irradiated vaccine, after validation as described above.

Viral Amplification Test

To determine the effect of the treatment on viability, the treated virus and an untreated control were serially passaged 3 times in Vero cells as described above. After each passage, flasks were subject to three freeze-thaw cycles. The culture obtained was centrifuged at 10,000 × g for 10 min 4°C to remove debris. One ml of the supernatant was used to infect Vero cell monolayers and also infect 8 well chamber slides (Nunc) to visualize viral replication by an indirect immunofluorescence assay (IFA) as described below.

Immunofluorescence Assay

Visualization of viral replication in treated and untreated cultures was achieved using an indirect immunofluorescence assay (IFA), performed essentially as described previously (18, 19). Cultured and fixed cells were stained with polyclonal swine anti-PEDV sera (NVSL) and examined with a fluorescent microscope for green cytoplasmic fluorescence characteristic of RNA viral replication.

Electron Microscopy

To visualize structure, treated, and untreated viral cultures were negatively stained by standard methods (21). Stained grids were examined with a JEOL JEM-100CX II transmission electron microscope (**Figure 1**).

Deep Sequencing of Treated PEDV Virions

Possible genetic differences between untreated and treated vaccine virions were assessed by deep sequencing. Heat and RNAse treated and untreated viral particles were purified from infected cells by ultra-centrifugation at 100,000 × g for 2.50 h and re-suspended in PBS. Unpackaged RNA and DNA were removed by a RNase and DNase cocktail containing 20 units of RNase One (Promega), 20 units Benzonase (Novagen), and 14 units of turbo DNase (Ambion) incubated in 1X buffer (Ambion) for 37°C for 1.5 h. Viral RNA was then isolated by using the Qiamp Viral RNA isolation kit (Qiagen) according to the manufacturer's protocol.

Purified viral RNA was deep sequenced by a commercial vendor (BGI Genomic). The cDNA library was prepared using TruSeq library construction kit (Illumina Inc., USA) with random hexamer primers. The prepared cDNA library was then sequenced using HiSeq 4000 PE100 platform (Illumina Inc., USA) and raw reads (100 bp) were obtained. The resultant sequences reads were analyzed by BGI Genomic, Philadelphia, PA. The raw reads were filtered out using SOAPnuke to get "Clean reads" by removing the reads with adaptors, reads with more than 5% of unknown bases (N), and low-quality reads (22). Clean reads were mapped to reference PEDV genome (GenBank: KF267450.1) using HISAT (Hierarchical Indexing for Spliced Alignment of Transcripts) (23). The genome mapping results further analyzed using the Genome Analysis Toolkit (GATK) to call single nucleotide polymorphism (SNP) and INDEL (insertion and deletion of bases) (24). Only SNPs with a quality score above the threshold (Qpred > 20) and with a SNP frequency of over 85% were included in assembling the consensus sequences. The consensus sequences of the treated and untreated samples were compared by alignment with Clustal Omega (25) to obtain changes which could be attributed to the treatment. Detected changes were annotated to include the locations and proteins affected (**Table 1**). Clean reads were mapped to the reference genome using BOWTIE2 to detect differentially expressed genes. Gene expression levels were calculated with RSEM version 1.2.12 (26). Differentially expressed genes were identified by the possionDis, EBSeq software for samples without replicates (27).

Ethics Statement

All animal experimentation was approved by the Institutional Animal Care and Use Committee (IACUC) of S. Dakota State Universities (SDSU) (Protocol number: 15-013A). No other specific permissions were required for these activities. This study did not involve endangered or protected species.

Swine Vaccine and Challenge

Twenty-four, 2 to 3-week-old piglets which were negative for PEDV by PCR and serology were divided into 3 groups; Group 1—unvaccinated control group ($N = 8$) (2 ml of PBS intramuscular and oral route each), Group 2—RNase and Heat treated PEDV vaccine group (PEDV-VAC) group ($N = 8$) (2 ml of 10^5 $TCID_{50}$/ml, intramuscular and oral route each) and Group 3—irradiated PEDV vaccine group ($N = 8$) (2 ml of 10^5 $TCID_{50}$/ml, intramuscular and oral route each). Piglets were boosted by the same route and dose at DPV 14 and 28. On DPV 43, small intestine, heart, liver, and spleen were collected 2 piglets from each group ($N = 2$/group) to assess vaccine safety. The remaining piglets ($N = 6$/group) were challenged orally with 10^5 $TCID_{50}$/ml of PEDV CO2013, as previously described (28, 29). Post-challenge, the piglets were observed daily for clinical signs of PED. All piglets were euthanized 1-week post challenge (DPC) or at DPV 49 and three sections of the small intestine (duodenum, jejunum, and ileum) were collected for histopathological (HP) and immunohistochemical (IHC) analysis. Serum was collected from all piglets on DPV 0, 14, 28, 43, and 49 to measure binding and neutralizing Ab responses. Fecal swabs were collected at DPV 7, 21, 38, and 42 from all piglets to measure shedding of the vaccine virus by RT-qPCR. Fecal swabs were collected on DPV 45 and 49 (DPC day 3 and 7) from all piglets to measure protection against shedding of the challenge virus by RT-qPCR.

Antibody Responses to the PEDV Spike and Nucleoproteins

Spike protein-specific IgG responses in pigs were measured in duplicate by an indirect ELISA as previously described, using the PEDV S antigen or NP antigen for capture (18). The assay format was pre-validated at the Animal Disease Research and Diagnostic Laboratory (ADRDL), SDSU, using serum samples from animals of known serological status. A standardized operating procedure was followed in sample analysis. The results were calculated as sample to positive (S/P) ratios as follows: S/P = optical density (OD) of the sample—OD of buffer/OD of positive control—OD of the buffer.

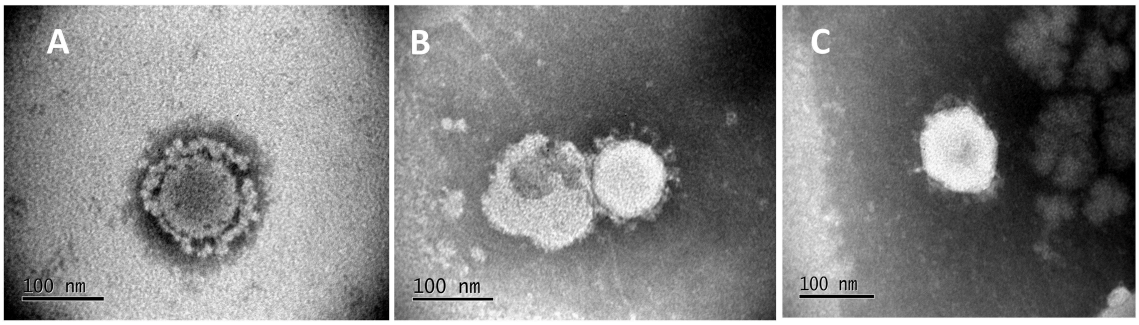

FIGURE 1 | Electron Micrographs of untreated and treated PEDV. Micrographs show the characteristic corona-like structure formed by the immunogenic spike protein embedded in the virus envelop of the icosahedral virus particle. **(A)** Untreated PEDV, **(B)** Heat and RNAse treated PEDV, **(C)** Irradiated PEDV.

TABLE 1 | Microscopic lesion scores.

Group	Mean microscopic lesion score[%] (No of positive animals/total animals)	Mean IHC score [&](No. of positive animals/total animals)	Total mean histology score (No. of positive animals/total animals)	Mean fecal score*	Total necropsy score[#]
VACCINE EFFICACY					
Unvaccinated	2.67 ± 1.89 (4/6)	1.5 ± 1.11 (4/6)	4.16 ± 3.25 (4/6)	2.50 ± 1.22 (5/6)	6.66 ± 3.14 (6/6)
RNase + Heat treated PEDV/Challenged	0 (0/6)	0 (0/6)	0 (0/6) ($p = 0.03^{@}$)	0.50 ± 1.22 (1/6) ($p = 0.03^{@}$)	0.50 ± 1.22 (1/6) ($p = 0.004^{@}$)
Irradiated PEDV/Challenged	4.33 ± 3.35 (4/6)	3.0 ± 1.90 (5/6)	7.33 ± 5.49 (4/6) ($p = 0.168$)	0.50 ± 1.22 (1/6) ($p = 0.03^{@}$)	7.83 ± 6.50 (5/6) ($p = 0.37$)
VACCINE SAFETY					
RNase + Heat treated PEDV/ Unchallenged	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)
Irradiated PEDV/Unchallenged	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)	0 (0/2)

Total number of pigs = 8, No. of pigs sacrificed for vaccine safety assessment prior to challenge = 2, No. of pigs sacrificed at day 7 post challenge = 6.
[%] Total atrophic enteritis score for the ileum, jejunum, duodenum where 0, negative; 2, mild; 4, moderate; 6, severe; 2, sections with crypt hypertrophy.
[&] Total immunohistochemistry (IHC) for the Ileum, jejunum, duodenum where 0, negative; 2, positive; ≤10%, 4, positive, 11–50%; 6, positive, >50%.
[*] Fecal score at necropsy-Formed Feces = 0, Semi-formed feces = 3, Liquid feces = 6.
[#] Sum of the microscopic and fecal scores.
[@] $p < 0.05$ as determined by the Mann–Whitney U-test, compared to the unvaccinated group.

Fluorescent Focus Neutralization Assay

To assess the neutralizing antibody responses elicited by vaccination, a pre-validated fluorescent focus neutralization (FFN) assay was used as previously described (18), following the standard operating procedures of the ADRDL, SDSU. Briefly, doubling dilutions of heat inactivated sera were incubated with 100 foci forming units, incubated for 1 h and cultured on Vero cell monolayers. Plates were stained with a PEDV-specific fluorescein-labeled monoclonal antibody (SD6-29) to visualize the end point, which was defined as a 90% reduction of foci compared to the controls.

RT-qPCR for Vaccine and Challenge Virus Shedding

Virus shedding through fecal route was assessed by a RT-qPCR performed by the NDSU Veterinary Diagnostic Laboratory, using pre-validated standard operating procedures, and a commercial PCR kit called the Swine Enteric PCR Panel (Thermo Fisher) following the manufacturer's instructions. Each pig was considered a biological replicate ($N = 6$, as 2 pigs/ group were sacrificed to assess vaccine safety prior to challenge), and each sample was assessed in duplicate. The obtained Ct-values were converted to viral copy numbers using a standard curve and log transformed for representation.

Histology

Tissue samples, collected as described above, were fixed in neutral buffered formalin for 48 h, trimmed, processed, and embedded in paraffin. Tissues were cut into 5 μm thick sections and stained with hematoxylin and eosin (HE) or a PEDV N protein-specific monoclonal antibody (SD6-29) for immunohistochemistry (IHC) following the standard operating procedures of the ADRDL, SDSU. Scores were recorded in a blinded fashion by a board-certified veterinary pathologist. Scores to measure atrophic enteritis characteristic of PED were assigned as follows: 0 = negative, 2 = mild, 4 = moderate,

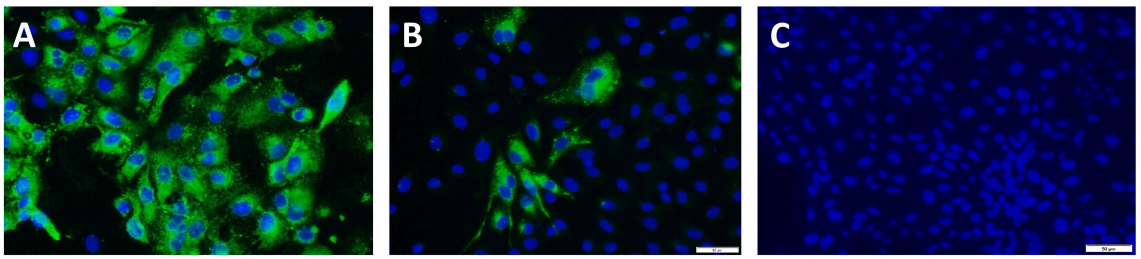

FIGURE 2 | Amplification test for viral inactivation: Immunofluorescence images of vaccine viruses at the 3rd passage. Green cytoplasmic fluorescence is indicative of viral replication and blue fluorescence localizes to the nucleus of the infected Vero cells. Images were obtained by staining with a PEDV-specific polyclonal antibody. **(A)** Untreated PEDV, **(B)** Heat and RNAse treated. PEDV at the 3rd passage showing minimal replication, **(C)** Irradiated PEDV at the 3rd passage.

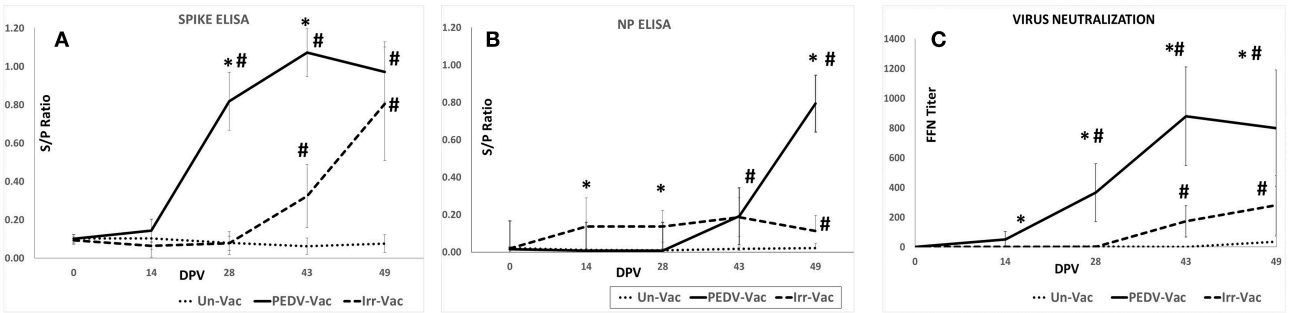

FIGURE 3 | Serological responses to vaccination: **(A)** Antibody responses to the PEDV spike protein as assessed by ELISA **(B)**. Antibody responses to the PEDV nucleoprotein as assessed by ELISA **(C)**. Virus neutralizing antibody responses as assessed by a fluorescent focus neutralization (FFN) assay. X axis, Days post vaccination; Y axis, ELISA OD value expressed as a signal to positive control ratio; Line with dots, Unvaccinated controls; Solid line, Heat and RNAse treated vaccine; Dashed line, Irradiated vaccine. Mean duplicate values for 8 pigs and standard deviations are presented. *Significantly different from the unvaccinated group, #Significantly different from the other vaccine group. *$p < 0.05$ by the Student's *T*-test.

6 = severe. Sections with crypt hypertrophy were assigned an additional 2 points. Antigen detection in enterocytes by IHC was semi-quantitatively scored based on the following criteria: 0 = negative, 2 = positive, ≤10%, 4 = positive, 11–50%, 6 = positive, >50%. The consistency of fecal matter during necropsy was assigned scores as follows: Formed Feces = 0, Semi-formed feces = 3, Liquid feces = 6. Total scores were calculated as the mean sum of the histology and fecal scores (**Table 1**).

Statistical Analysis

Significant differences between treatments were assessed by ANOVA and when significant ($p < 0.05$) *post-hoc* analysis was used to determine differences between groups. The Student's *T*-test was used for the serology and RT-qPCR data and the Mann–Whitney *U*-test for the pathology lesion scores. The mean values of replicates, standard deviation and statistical significance are represented in the Figures and tables.

RESULTS

Treatment With Heat and RNAse Diminishes Viral Replication While Maintaining Structural Integrity

To achieve the targeted outcomes of maintaining structural integrity while achieving diminished viral replication, rather than complete inactivation, PEDV virus cultures were first exposed to temperatures ranging from 37 to 60°C for 10 min and visualized by electron microscopy. Intact structures were detected at all temperatures tested. However, increasing numbers of misshapen and fragmented virions were detected at 50°C and above. Cultures treated at 37 and 45°C remained viable as viral replication was visible by immunofluorescence (IFA) in infected Vero cells using a PEDV-specific antibody, without any amplification by serial passaging. Virus was detected after the 1st passage in the cultures treated at 50°C. Virus cultures treated at 55 and 60°C were not amplified even after four serial passages in Vero cells, indicating that complete inactivation occurred at these temperatures. Hence a temperature of 44°C for 10 min was chosen for reversible unfolding of the viral capsid (**Figure 1B**) without completely inactivating the virus. Untreated control virus culture remained structurally intact as expected (**Figure 1A**). Similarly, while RNAse treatment alone did not affect viability, the reduction in viral replication was proportional to the dose and time of exposure to RNase in the heat-treated virions. A dose of 10 units of RNase A and 1,000 units of RNase T with an exposure time of 4 h was chosen as optimal for the final vaccine preparation. While the untreated virus control showed robust replication (**Figure 2A**), following the heat and RNAse treatment protocol, viral replication was detected only in the 3rd passage in Vero cells (**Figure 2B**).

For the gamma (γ) irradiated, inactivated control vaccine, typical icosahedral structures were seen in electron microscopy after 23 h of exposure to radiation. However, the corona-like layer containing the protective spike antigens appeared to be damaged (**Figure 1C**). At this dose of radiation, the virus was not detected by the IFA with a PEDV-specific Ab at the third serial passage in cell culture (**Figure 2C**). Hence, a final dose of 24 h (1,084,320 rad) was selected to prepare the inactivated control vaccine.

Vaccination of Pigs With the Heat and RNAse Treated Virions Elicits a Strong Protective Antibody (Ab) Response

Measurement of Ab responses against the PEDV spike and nucleocapsid proteins (NP) by ELISA (18) showed that animals vaccinated with the heat and RNAse treated virions mounted strong Ab responses against the protective PEDV spike antigen following the booster vaccinations on DPV 14 and 28 (**Figures 3A,B**). However, Ab responses to non-structural nucleocapsid protein (NP) remained low prior to the challenge. In pigs immunized with the irradiated vaccine, Ab responses to both viral antigens were low. The mean optical density values for the ELISAs were significantly different between the groups (**Figures 3A,B**).

Measurement of virus neutralizing antibodies by a fluorescent focus inhibition test (FFN) (18) showed a trend which was similar to that of the spike protein-specific Abs. Strong virus neutralizing Ab responses, were detected in animals vaccinated with the heat and RNAse treated virions but not in the pigs which received the irradiated viral vaccine. The differences between the groups was statistically significant (**Figure 3C**). The spike protein-specific Ab and virus neutralizing Ab levels were strongly correlated in the heat and RNAse treated PEDV vaccinated pigs, with a correlation coefficient of 95.11%. As expected, the unvaccinated control pigs remained sero-negative for the duration of the study.

Vaccination Protects Against Fecal Viral Shedding

To assess the efficacy of the vaccine in protecting against challenge, shedding of the challenge viral RNA in fecal matter was assessed by a PEDV-specific RT-qPCR on days 0, 3, and 7 post-challenge. All experimental animals were RT-qPCR negative on day 0 post-challenge (DPC). At DPC 3 and 7, challenge viral RNA was not detected in any of the pigs vaccinated with the heat and RNAse treated PEDV vaccine (**Figure 4**), while 4 of the 6 pigs administered the irradiated vaccine were positive by RT-qPCR on DPC3. All 6 pigs in the irradiated vaccine group turned positive by DPC7 (**Figure 4**). As expected, viral RNA was detected in the fecal matter of all unvaccinated pigs on both sample collection days with titers increasing between DPC 3 and 7. While the viral RNA loads were significantly different between the two vaccine groups at both time points, there were no significant differences between the unvaccinated controls and pigs administered the irradiated vaccine at both the time points tested, indicating that the irradiated vaccine did not provide protection against viral replication and shedding in the host.

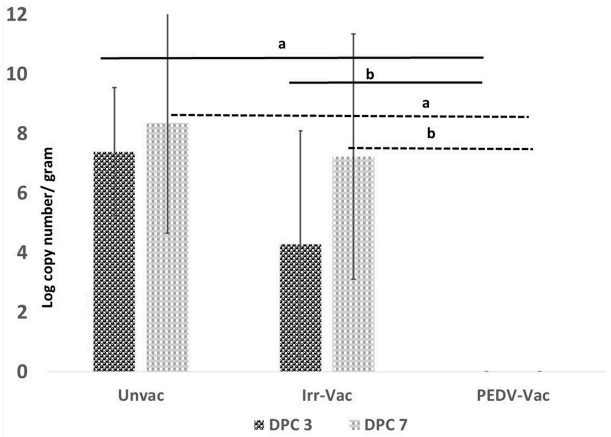

FIGURE 4 | Post-challenge fecal viral loads: Viral RNA detected by a PEDV-specific RT-qPCR on day 3 and day 7 post-challenge. X axis, experimental groups $N = 6$ pigs/group (2 pigs/group were sacrificed prior to challenge to assess vaccine safety); Y axis, mean of duplicate values of viral RNA copy number per gram of fecal matter; Dark bar, Day 3 post-challenge; Light bar, Day 7 post-challenge; a, Significantly different from the unvaccinated group; b, Significantly different from the other vaccine group. $p < 0.05$ by the Student's *T*-test. Differences between the unvaccinated and irradiated vaccine group were not significant.

Vaccination Protects Against Intestinal Pathology

Examination of the intestinal tissue of the experimental animals by histology and immunohistochemistry (IHC) showed that the heat and RNAse treated PEDV vaccine completely protected vaccinated pigs against the development of microscopic lesions following challenge. Characteristic microscopic intestinal lesions of atrophic enteropathy and crypt hyperplasia were detected in the duodenum, jejunum, and ileum of animals in the control groups (**Figures 5D–G**). Viral antigen was also detected in the enterocytes in all three sections using a PEDV-specific monoclonal Ab-based immunohistochemistry assay (**Figures 6A–E**). There were no significant differences between the 3 sections, indicating the entire small intestine was affected. The total microscopic score, including the histopathology and immunohistochemistry scores was 4.16 for the unvaccinated animals and 7.33 for the pigs immunized with the irradiated vaccine and 0 for pigs administered the heat and RNAse treated vaccine. While the difference between the unvaccinated group and irradiated vaccine group was not statistically significant, the irradiated vaccine appeared to enhance intestinal pathology (**Table 1**). Similarly, the total necropsy scores, a sum of both the fecal and histology scores, were significantly different ($p = 0.04$) between the two vaccine groups but not between the unvaccinated group and the irradiated vaccine group ($p = 0.37$) (**Table 1**).

The Experimental Vaccines Are Safe

No side effects or clinical signs of PED were observed in vaccinated pigs after either the primary or booster vaccines. Vaccine viral RNA was not detected by RT-qPCR in the fecal

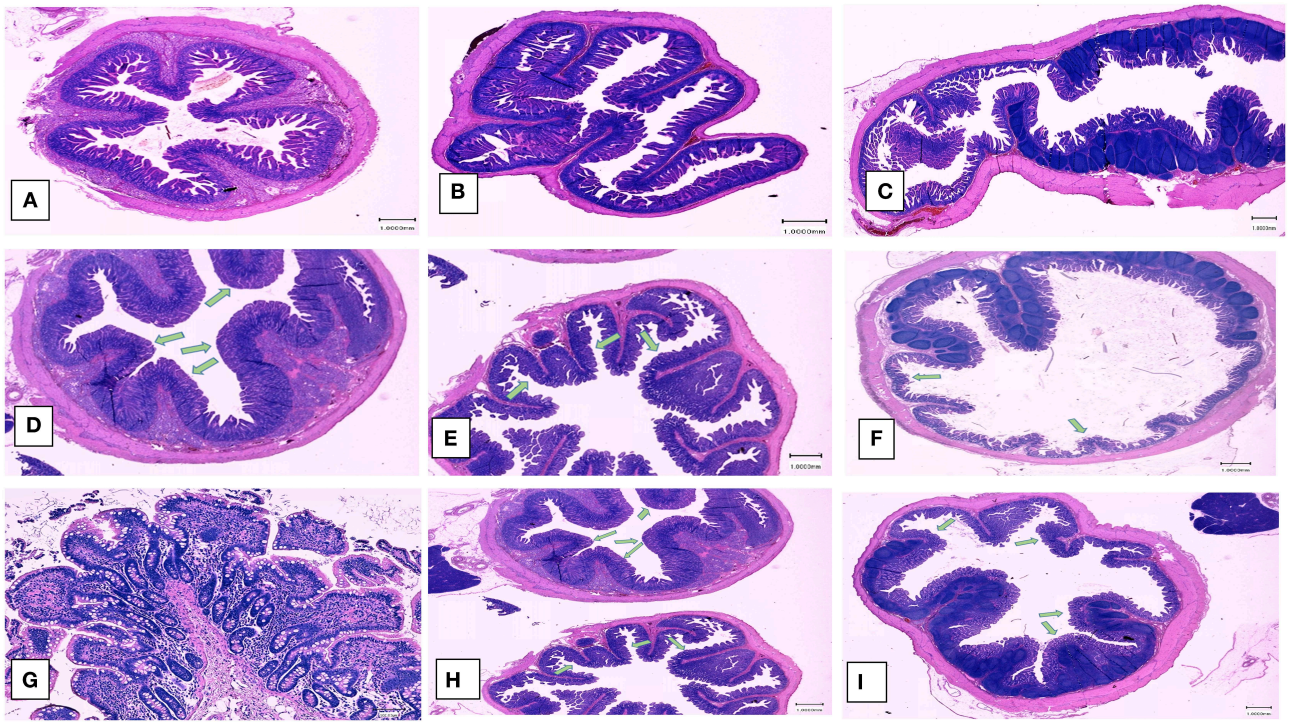

FIGURE 5 | Post-challenge histopathology of small intestines. Left panel **(A,D,G)** Hematoxylin and eosin stained sections showing representative microscopic lesions (10X magnification). **(A–C)** Healthy pigs. **(A)** Duodenum, **(B)** Jejunum, **(C)** Ileum, **(D–G)** Unvaccinated, PEDV challenged pigs, **(D)** Duodenum, **(E)** Jejunum, **(F)** Ileum, **(G)** Ileum (100x). **(H,I)** Pigs vaccinated with the irradiated vaccine and challenged **(D)** Duodenum and Jejunum **(F)** Ileum. Green arrows indicate areas of villus atrophy and crypt hyperplasia.

matter of any of the vaccinated pigs from both groups at 7 days after the primary vaccination or at 1 week after the boosters. All animals remained PCR negative until the day of challenge. Therefore, although the heat and RNase treated PEDV virions were detected by amplification after 3 serial passages in Vero cells, replication of the vaccine virus in the host appeared to be curtailed by its immune system. In the 2 pigs euthanized from each group prior to challenge, stools were fully formed at necropsy (**Table 1**). No microscopic lesions or viral antigen were detected in the small intestine sections, heart, spleen, and liver of the 2 animals necropsied from each group prior to challenge (**Table 1**). Representative images of the duodenum, jejunum, and ileum are depicted in **Figure 5**.

Heat and RNAse Treatment Results in Genetic Changes

To identify possible mutations that could explain the highly effective attenuation observed, deep sequencing of heat and RNAse treated virions from infected vero cells resulted in a total of 59.42 and 24.44 MB of raw reads were obtained by RNA seq for the treated and untreated samples, respectively. Clean reads obtained after trimming were 26.94 and 19.53 GB, respectively. The Qphred20 values for the clean reads were 96.69 and 98.49 for the untreated and tread samples, respectively, indicating satisfactory quality of the data obtained. As listed in **Table 2** SNPs and insertions or deletions (INDELS) were detected in the polyprotein, spike and envelope proteins (**Table 2**, **S1 Sequence File** and **Supplementary Figures 1–3**) of heat and RNAse treated virions, when compared to the untreated virions. In addition, insertions and deletions were detected in the S1 region for the spike protein. The N terminal signal peptide region of the spike protein had a 2 amino acid deletion and one non-synonymous change at position 355, changing the sequence from IGEN to K—N. A conservative in-frame insertion was detected at position 355 in the S1 region, changing the amino acid sequence from L----AT to LKKKGAT (**Table 2** and **Supplementary Figure 2**).

DISCUSSION

Chemical methods for inactivation of viruses have long been in use for vaccine development. While they are rapid and convenient, commonly used inactivation agents may not only affect nucleic acids but also protein structures and hence antigen presentation and vaccine efficacy. Gamma irradiation has been traditionally used to inactivate viruses. The mechanisms involved include nucleic acid degradation, destruction of covalent bonds, and release of free radicals (30). As commercial inactivated vaccines were not available at the time of testing gamma irradiation was selected as the method of choice to prepare an inactivated control vaccine for this study. Moreover, similar to the heat and RNAse treated vaccine, the virus-like-particulate

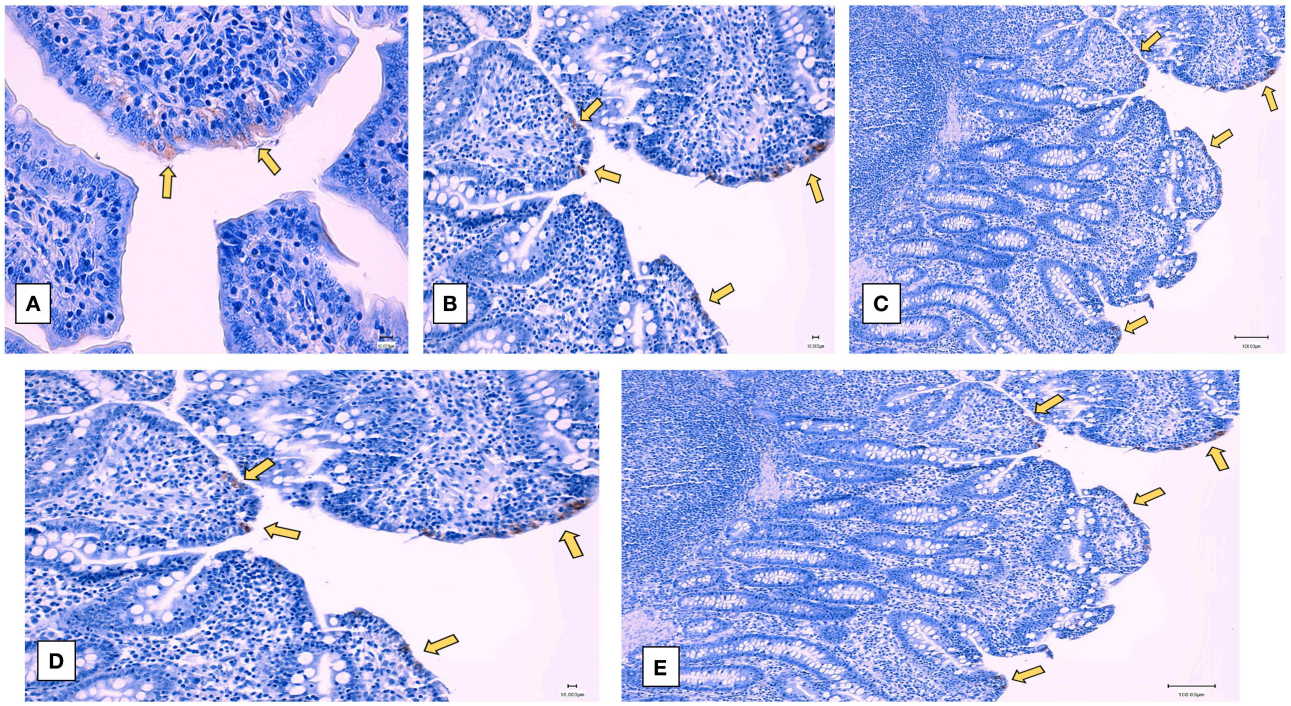

FIGURE 6 | Post-challenge immunohistochemistry of small intestines—Representative immunohistochemistry images of sections stained by a PEDV-specific antibody. **(A–C)** unvaccinated challenged pigs. **(A)** Jejunum (400X), **(B)** ileum (200X), **(C)** Duodenum (100X), **(D,E)** Pigs vaccinated with the irradiated vaccine and challenged **(D)** ileum (200X) **(E)** Duodenum (100X). Yellow arrows indicate viral antigen localized to enterocytes. Pigs vaccinated with the heat and RNase treated vaccine and challenged did not show microscopic or immunohistochemistry changes (data not represented).

structure was more likely to be maintained by gamma irradiation, while achieving complete inactivation.

Gamma irradiation had been previously used for vaccine development with varying success, depending on the pathogen (17). For example, we have previously demonstrated that a gamma irradiated vaccine against *Neospora caninum* was effective in mice (31). However, a gamma irradiated, Lassa virus vaccine failed to protect vaccinated mice (32). Although both approaches tested in this study targeted nucleic acids and preservation of structure, the protective outcomes varied significantly between the two vaccines tested. It is possible that release of free radicals during the irradiation process could have a deleterious effect on integrity of antigenic structures and antigen presentation *in vivo*. A more detailed characterization of these parameters will be the focus of future studies. Similar results for the gamma irradiated vaccine in this study, it has been shown that a dendritic cell targeted spike protein-based subunit vaccine against PEDV exacerbated intestinal pathology in vaccinated pigs, despite stimulating strong $CD4^+/CD8^+$ T cell responses (33).

While characterizing the exact physical interactions involved in the heat and RNAse treatment is not within the scope of this study, our finding that exposure of PEDV to temperatures below 50°C did not affect structure was similar to other studies showing that the SARS coronavirus structure is metastable and can be reversibly denatured by exposure to varying physical conditions such pH and temperature (16, 34). Although the heat and RNAse treated virus culture was amplified after 3 passages in cell culture (**Figure 2**), the absence its detection by RT-qPCR (**Figure 4**), or immunohistochemistry (**Table 1** and **Figure 5**) and the lack of strong Ab responses to the non-structural NP (**Figure 3**), in vaccinated pigs prior to challenge indicates that active vaccine viral replication was absent in the host or was undetectable by the techniques used. Therefore, unlike other attenuated PEDV vaccines or vaccination strategies that rely on prior exposure to field strains, it is highly improbable that reversion to virulence or recombination with field strains could occur with the heat and RNAse treated vaccine.

Viral genomes that were identical to the untreated parental virus were not detected by deep sequencing of the heat and RNAse treated virus from infected Vero cells. Insertions and deletions in the spike protein, especially the S1 region, influence pathogenicity, and immunogenicity of PEDV. The core neutralizing epitope of the PEDV spike protein has been localized to amino acid positions 503–568 (35, 36). The SNPs identified in the spike protein of the vaccine virions (**Table 2**) did not map to these residues. While a limitation of the described method is that genetic changes induced by treatment and repair are unpredictable, repair of mutations (37) or complementation in trans of the fragmented genome could have led to detection of a fluorescent signal in the 3rd passage after treatment. Indeed, it has been shown that replication deficient genomes with deletions or mutations are produced during serial passaging of foot and mouth disease virus (FMDV) for attenuation. They are not

TABLE 2 | SNPs and INDELS.

Pos	R	Un-Trt	Trt	Con-sequence	Residue change	Gene	AF	Type
POLY-PROTEIN								
4,982	C	C	T	NS	S1564F	PP-NSP3	1.0	Ti
12,156	TC	TC	CG	NS	R3956G	PP-NSP9	0.99	Ti
20,203	A	A	–	Frame-shift	P6640- VGTWWYCSY. to LALGGTVAIK.	PP-NSP13	0.99	
SPIKE PROTEIN								
20,796	TTGGTG	TTGGTG	–	NS & Del	P55- IGEN to K–N	S-N term	1.0	
21,307	T	T	C	S	_	S-S1	1.0	Ti
21,698	–	–	AAGAAGAAAGGT	In-frame insertion, conservative	P355 L----AT to LKKKGAT	S-S1	0.86	
21,761	C	C	T	NS	L377F	S-S1	1.0	Ti
22,541	T	C	C	NS	F637L	S-S1	1.0	Ti
23,300	G	G	C	NS	G890R	S-S2	1.0	Tv
24,395	G	G	T	NS	D1211Y	S-S2	1.0	Tv
24,796	G	T	T	NS	Q1388H	S-S2	1.0	Tv
ENVELOP PROTEIN								
25,638	C	C	T	NS	S62F	Envelop	1.0	Ti

Pos, position on the consensus sequence of the treated vaccine virus; R, nucleotide in the reference genome; Un-Trt, SNP in the un-treated PEDV; Trt, SNP on the treated PEDV; NS, Non-Synonymous; S, Synonymous; PP, Polyprotein; S, Spike; AF, allele frequency; Ti, transition mutation; Tv, transversion mutation.

infective by themselves, but when present in the same cell, the mutations in the genomes can complement each other in trans to produce plaques *in vitro*. When the defective-complementing virus system was used as a vaccine by Rodriguez-Calvo et al. vaccine virus replication was not detected but strong protection was elicited. This observation can be explained by vaccine virus replication in the host being limited by the requirement of coinfection of the same cell. Even if such an unlikely coinfection event were to happen despite active host innate immunity, the recombined progeny viruses were more likely to be highly attenuated than acquire virulence, thus providing an additional vaccine safety barrier *in vivo* (38). *In vivo*, the presence of the host innate immune system was likely able to effectively curtail replication, despite exposure to 10^5 $TCID_{50}$ of the heat and RNAse treated virus culture. More detailed studies are required to confirm these hypotheses, but they are not within the scope of this manuscript.

The importance of spike protein-specific antibodies for protection against PEDV is well-established (15). Several studies describing experimental subunit and vectored vaccines or commercial attenuated and inactivated vaccines against PEDV establish a strong correlation between spike protein-specific antibodies, virus neutralization titers and protection against infection (9, 29, 37, 39–43). Similar to these studies, strong spike-protein specific Ab responses and virus neutralizing responses were noted in the pigs immunized with the heat and RNAse treated vaccine. A commercial inactivated vaccine was able to reduce challenge viral shedding by 3–4 logs but an attenuated vaccine induced IgA responses but did not affect viral shedding (43). Testing of two attenuated PEDV strains produced by serial passage in weanling pigs showed that the passaged viruses were attenuated but were not protected against challenge viral shedding or clinical signs (29). While direct comparisons are not possible due to differences in experimental conditions, unlike the other cited studies, intestinal lesions, or challenge virus was not detected by qPCR in the heat and RNAse treated vaccine group in this study. Although boosters were incorporated in the study design to minimize risk, it is likely that they were not required to achieve adequate protection as strong spike protein specific antibody responses and virus neutralizing responses were detected after the first dose of the heat and RNAse treated vaccine, at DPV 14 (**Figure 3**). While cell mediated immunity was not assessed due to difficulties with transportation of cells, it is very likely that it was not compromised by the process used as the heat and RNAse treated vaccine was very effective in preventing challenge viral replication in vaccinated pigs.

While ideal for PEDV, studying vaccine efficacy in pregnant sows and neonatal pigs is expensive and procedurally tedious. Although clinical signs are less severe in older piglets (28) and virulence can vary between isolates used for challenge (44, 45), PEDV can infect and replicate well in pigs of all ages (14, 46). Hence several researchers have used weanling piglets to screen vaccine candidates for efficacy and safety (9, 13, 29, 43, 47–52). This approach can help reduce animal use and cost if the candidates fall short of expectations. Several swine bioassay studies in growing piglets have reported that peak PEDV replication occurs between DPI 3 and DPI 7 after which viral loads decrease (28, 43, 47, 48). Similar patterns of infectivity were observed in this study, as the uninfected control pigs had a mean fecal viral RNA load of 8.35 log copy numbers at DPI 7 (**Figure 4**) developed microscopic lesions, but not severe clinical signs. In comparison to the untreated control and irradiated vaccine groups, no fecal viral shedding or intestinal pathology was detected in the pigs immunized with the heat and RNase treated vaccine, indicating that vaccine induced immunity was highly effective against PEDV challenge, within the limits of this weanling pig study model.

The primary advantages of this innovative approach are safety, efficacy, convenience and a short development time. As the method can be easily adapted to newly evolving strains, provided they are readily cultured, this approach is very relevant to current field immunization

practices of feedback exposure and autogenous vaccination. Our future goals include testing the heat and RNase treated vaccine in pregnant sows, and improving oral and respiratory mucosal vaccine delivery systems to target improved protection.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of S. Dakota State Universities (SDSU) (Protocol number- 15-013A).

AUTHOR CONTRIBUTIONS

GS, PS, and SD-L: data collection, analysis, and manuscript editing. AP, EN, and BW: data collection and manuscript editing. SR: conception, funding, and manuscript preparation and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2019.00347/full#supplementary-material

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Influenza A Virus in Swine: Epidemiology, Challenges and Vaccination Strategies

José Carlos Mancera Gracia[1]*, *Douglas S. Pearce*[2], *Aleksandar Masic*[2] and *Monica Balasch*[3]

[1] Zoetis Belgium S.A., Zaventem, Belgium, [2] Zoetis Inc., Veterinary Medicine Research and Development, Kalamazoo, MI, United States, [3] Zoetis Manufacturing & Research Spain S.L. Ctra., Girona, Spain

Influenza A viruses cause acute respiratory infections in swine that result in significant economic losses for global pig production. Currently, three different subtypes of influenza A viruses of swine (IAV-S) co-circulate worldwide: H1N1, H3N2, and H1N2. However, the origin, genetic background and antigenic properties of those IAV-S vary considerably from region to region. Pigs could also have a role in the adaptation of avian influenza A viruses to humans and other mammalian hosts, either as intermediate hosts in which avian influenza viruses may adapt to humans, or as a "mixing vessel" in which influenza viruses from various origins may reassort, generating novel progeny viruses capable of replicating and spreading among humans. These potential roles highlight the importance of controlling influenza A viruses in pigs. Vaccination is currently the main tool to control IAV-S. Vaccines containing whole inactivated virus (WIV) with adjuvant have been traditionally used to generate highly specific antibodies against hemagglutinin (HA), the main antigenic protein. WIV vaccines are safe and protect against antigenically identical or very similar strains in the absence of maternally derived antibodies (MDAs). Yet, their efficacy is reduced against heterologous strains, or in presence of MDAs. Moreover, vaccine-associated enhanced respiratory disease (VAERD) has been described in pigs vaccinated with WIV vaccines and challenged with heterologous strains in the US. This, together with the increasingly complex epidemiology of SIVs, illustrates the need to explore new vaccination technologies and strategies. Currently, there are two different non-inactivated vaccines commercialized for swine in the US: an RNA vector vaccine expressing the HA of a H3N2 cluster IV, and a bivalent modified live vaccine (MLV) containing H1N2 γ-clade and H3N2 cluster IV. In addition, recombinant-protein vaccines, DNA vector vaccines and alternative attenuation technologies are being explored, but none of these new technologies has yet reached the market. The aim of this article is to provide a thorough review of the current epidemiological scenario of IAV-S, the challenges faced in the control of IAV-S infection and the tools being explored to overcome those challenges.

Keywords: influenza A, swine, epidemology, vaccines, role of pig

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***Correspondence:**
José Carlos Mancera Gracia
pepe.mancera@zoetis.com

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NATURE OF INFLUENZA A VIRUSES

Influenza A viruses belong to the family *Orthomyxoviridae* and their genome is composed of eight, negative-sense, single-stranded RNA segments (**Figure 1**). Two of those segments encode the two main surface proteins: the hemagglutinin (HA) and the neuraminidase (NA). These two viral proteins are major determinants of virus pathogenicity that play a crucial role in virus binding and release. In addition, HA and NA are used to classify the virus into subtypes according to their antigenic properties (1).

Because of their RNA viral genome, influenza viruses carry their own polymerase genes, which lack exonuclease proofreading capability. Therefore, influenza A viruses exist as dynamic populations with high mutation rates (2). Mutations that change amino acids in the antigenic sites of those proteins may allow influenza viruses to escape from pre-existing immunity. Such selective mutations produced in the antigenic domains of these surface proteins are responsible for a phenomenon known as "antigenic drift."

Due to the presence of eight independent segments in the virus genome, simultaneous co-infection of a host cell with two or more different viruses can result in progeny viruses that contain novel combinations of gene segments from both parental viruses. This phenomenon is known as genetic "reassortment." When genetic reassortment results in the emergence of a virus that contains a novel HA and/or NA protein, this is called "antigenic shift" (4). The combination of antigenic drift and shift poses a continuous threat to animal and human health increasing the challenge of developing efficacious vaccines (5).

INFLUENZA A VIRUSES IN SWINE

Influenza A viruses are an important cause of acute respiratory disease in pigs and contribute to Porcine Respiratory Disease Complex along with Porcine Reproductive and Respiratory Syndrome (PRRS), Porcine Circovirus Type 2 (PCV2), *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae*. Influenza A viruses of swine (IAV-S) target epithelial cells of the entire respiratory tract, replicating primarily in the lungs. As virus replication is restricted to the respiratory tract, virus transmission occurs only via the respiratory route. In pigs, influenza A infection lasts for 6–7 days and clinical signs such as fever, respiratory distress and weakness are resolved within a few days. Infection is usually mild and rarely causes death (1). However, this disease can cause a significant economic impact due to reproductive failure in sows due to the fever and weight loss in growing pigs.

Three different influenza A virus subtypes (H1N1, H3N2, and H1N2) are currently circulating in swine worldwide (6). However, the origins and the antigenic characteristics of these subtypes differ from region to region throughout the world.

Figure 2 summarizes the IAV-S epidemiology in Europe. Briefly, the first significant influenza A virus outbreaks occurred in 1979 when an avian H1N1 virus jumped from wild ducks to pigs in Germany and Belgium (7). This virus is referred as European "avian-like" H1N1 (H1avN1), 1C clade based on the 2016 HA nomenclature for H1 subtype (8). H1avN1 viruses rapidly spread and became the predominant subtype throughout Europe (9, 10). During the mid-1980s, H3N2 strains spread and became the second endemic virus subtype in Europe. Those were reassortant H3N2 viruses containing the HA and NA from a descendant of the human 1968 "Hong Kong pandemic" H3N2 and the remaining genes from H1avN1 (9). In the mid-1990s, those H3N2 viruses reassorted with a human-seasonal H1N1 virus HA generating the H1huN2 virus lineage (11, 12). These viruses also became established throughout Europe and are classified as clade 1B (8). For many years, those three lineages co-circulated in the different European countries keeping the epidemiological situation rather stable (9, 13). However, this situation dramatically changed with the emergence of the 2009 pandemic H1N1 virus (H1N1pdm09) (13). This virus was the result of reassortment between a North American "triple-reassortant" swine influenza virus and a European H1avN1 (14). After its introduction in Europe, this H1N1pdm09 became established and widely reassorted with pre-existing H1N1, H3N2, and H1N2 subtypes, further complicating swine influenza epidemiology (6, 10, 15–19). Moreover, the H1N1pdm09 internal gene cassette extensively reassorted with domestic viruses in the UK and became the dominant backbone there (13).

Figure 3 summarizes the IAV-S epidemiology in North America (Canada, Mexico, and the United States). In brief, in that region the epidemiological situation was stable until late 1990s. The "classical swine (cs)" H1N1, clade 1A (8), derived from the 1918 H1N1 pandemic (also known as "Spanish flu"), was the dominant subtype. Then, sometime in 1998, a novel H3N2 subtype emerged from the reassortment between csH1N1 virus genes (NP, M, and NS), human-seasonal H3N2 virus genes (PB1, HA and NA) and avian influenza virus genes (PB2 and PA) (20, 21). Due to the combination of swine, human and avian origin genes these viruses were designated "triple-reassortant" H3N2. This H3N2 subtype became established and further evolved into defined phylogenetic clades over time from Cluster-I to Cluster IV, which is the dominant cluster at the present day (22). The "triple-reassortant" H3N2 viruses further reassorted with csH1N1 leading to the generation and spread of novel "triple-reassortant" H1N1 or H1N2 viruses (23–25). These H1N1 and H1N2 lineage viruses related to the csH1N1 ancestor were designated as α, β, and γ clades (26). In addition, a minor clade (γ2-H1) was identified in 2013 and reported to be circulating in US herds since 1995 as a minor virus population (27). During the early 2000s, human-seasonal H1 and N2 genes were introduced into the US swine population by reassortment with the established "triple-reassortant" viruses. Those H1 viruses were antigenically different to those of the "classical swine" lineage and were classified as clades δ-1 and δ-2 (6). In 2009, the novel H1N1pdm09 emerged in Mexico. This was the first pandemic virus in the twenty-first century and was a reassortant containing M and NA genes derived from the European H1avN1 subtype and the remaining genes from a US "triple reassortant" H1 subtype (14, 28). The H1N1pdm09 efficiently spread in the human population but also spread in the North America swine population. Like Europe, the introduction and circulation of the H1N1pdm09 together with its reassortment with the endemic

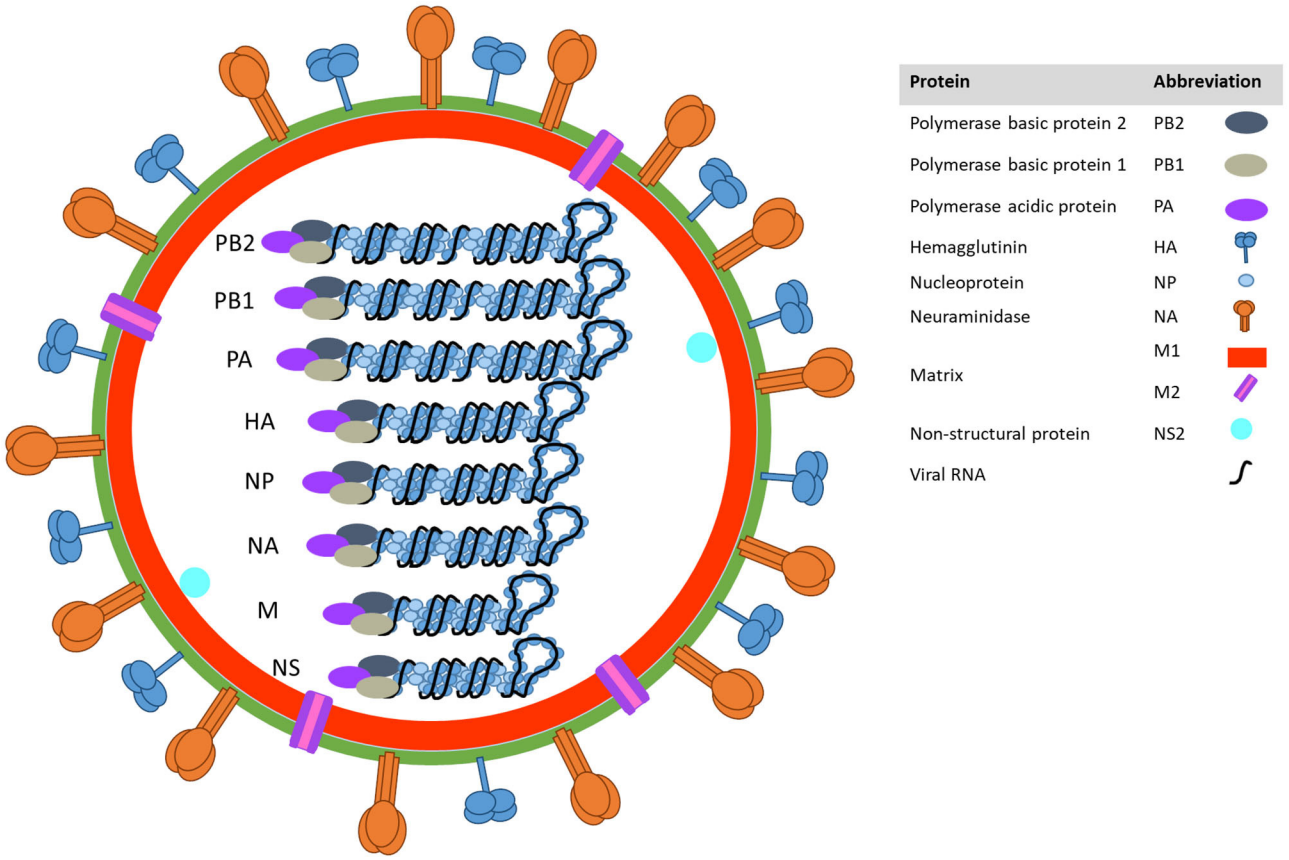

FIGURE 1 | Influenza A virion structure.

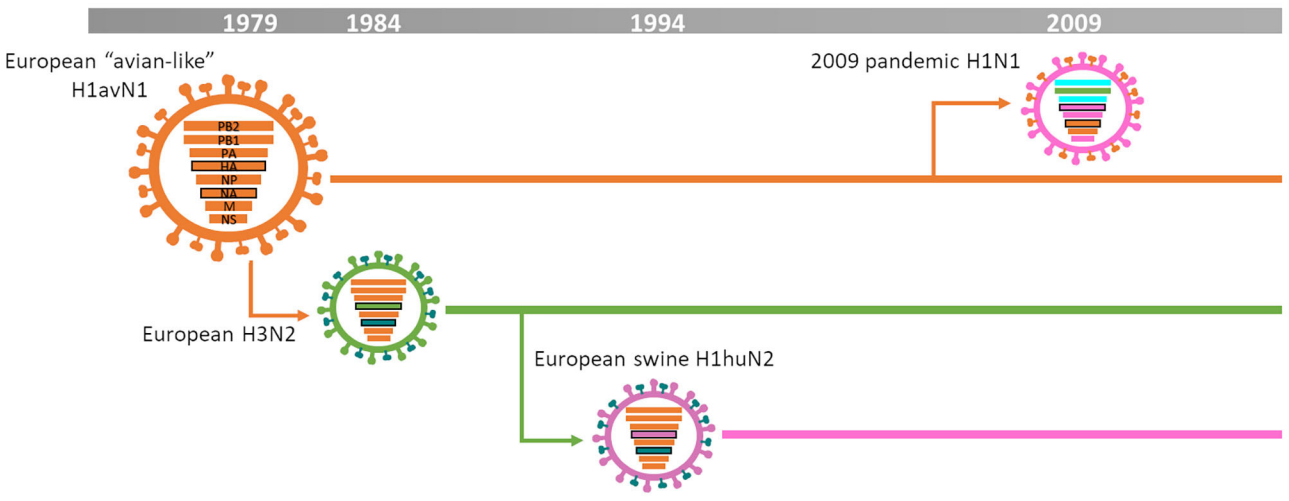

FIGURE 2 | IAV-S epidemiology in Europe [based on (3)].

strains has deeply modified the scenario in North America (29, 30). During the 2010–2011 season, a novel human H3 virus lineage adapted to swine (31). This H3 was genetically and antigenically different from the cluster-IV lineage and currently coexists with them (32). In addition, H3N2, H1N1, and H1N2 viruses containing the M gene derived from the H1N1pdm09

spread throughout the US swine population and have been recurrently isolated from humans since 2011, raising public health concerns. These viruses were called "variant" viruses because of their ability to infect humans (29, 33).

In South America many different lineages likely circulated undetected for many years due to the lack of surveillance and reporting. Genetically and antigenically different lineages have been reported in the different countries. In Brazil, together with H1N1pdm09, various lineages containing human-seasonal H1N1 and H3N2 viruses surface genes and H1N1pdm09 internal genes were reported (34, 35). In Chile and Argentina multiple human-derived H1N1, H1N2, and H3N2 lineages have been reported, in addition to reassortants containing H1N1pdm09 internal genes (36–38).

Asia and the Pacific also show significant regional differences in swine influenza epidemiology. In China and Southeast Asia, which house more than 50% of the worldwide swine population, csH1N1 viruses were endemic until the 1990s. Later, due to pig imports from other continents, European H1avN1 and H3N2, and North American "triple reassortant" lineage viruses were introduced. The H1N1pdm09 also became endemic in the region after 2010. Those viruses widely spread and reassorted with endemic strains leading to a very complex collection of viruses (39, 40). In addition, other subtype viruses such as H3N8, H4N8, H5N1, H6N6, and H9N2 have been repeatedly reported in China. However, stable endemic status was never reached (41). In Australia, the introduction of specific lineages derived from human seasonal viruses such as H1N1 subtypes from 1977 and 1995 and H3N2 subtypes from 1968 and 2003 were detected in addition to H1N1pdm09. Three specific HA lineages derived from H3 (1995) and H1 (1977 and 1995) in combination with other human seasonal genes from the 1960s and the 2000s are the currently dominant subtypes (42, 43).

POSSIBLE ROLE OF THE PIG IN PANDEMIC GENERATION

According to the classical dogma, pigs may play a role in the adaptation of animal influenza viruses to humans. This hypothesis was first supported by Scholtissek and colleagues in 1985. They examined the rescue of temperature-sensitive (ts) nucleoprotein-gene mutants of an avian H7N1 virus by co-infecting chicken embryo fibroblasts with either avian, human or swine H3N2 isolates. They found that the ts mutants could be rescued by all avian viruses, by none of the human viruses and by two out of 10 swine viruses. In consequence, they proposed that the nucleoprotein of swine influenza viruses may have a broader host range when compared to human or avian viruses and that pigs were a potential "mixing vessel" for the generation of those reassortant viruses (44). This "mixing vessel" hypothesis was supported by two findings. First, subtype similarities between circulating human and swine influenza A viruses. Second, pigs could be simultaneously infected with avian, human and swine influenza viruses, which led to the generation and isolation of reassortants (45). In 1998, Ito and colleagues gave molecular support to this hypothesis by demonstrating the presence of the main sialic acid receptors for avian and human influenza viruses (Siaα2,3Gal and Siaα2,6Gal, respectively) in the pig trachea. Furthermore, they demonstrated that some "avian-like" swine influenza viruses acquired molecular traits of human adaptation by continuous replication in pig tracheal explants (46). This led to the hypothesis that the pig may also act as an intermediate host in which avian influenza viruses might gain mammalian adaptation traits. Still, later studies demonstrated that the presence of both sialic acid receptors in swine mimics that of human and ferrets (47–50). This sialic acid receptor distribution along with similar clinical manifestations and pathogenicity between swine and humans suggest that pigs could be an optimal model to evaluate influenza A virus infection and immunity with results that could have implications for human health (51, 52). More recent studies demonstrated that four serial passages of an avian H9N2 virus in pigs enhanced virus replication and transmission. However, efficient adaptation to reach endemic IAV-S replication and transmissibility parameters will likely need more adaptation (53).

In 2009, the emergence of the H1N1pdm09 virus from swine again caused concern that pigs act as a source of pandemics and stimulated additional research (14, 28, 54). Some groups suggested that also the previous pandemic viruses from twentieth century may have been generated by reassortment in a mammalian host, possibly swine (55, 56). An additional role for the pig in the generation of pandemic influenza viruses was then suggested. While the pig population may act as a reservoir for human-derived viruses circulating with lower drift rates, the human population is likely generating protective immunity only against recent seasonal strains with higher drift rates. Therefore, the human population is provided protection against recent seasonal strains but remains naive against old strains that are only circulating in swine (57, 58). A serological study demonstrated that infection immunity to recent human H3N2 viruses confers minimal cross-protection against European human-derived H3N2 viruses circulating in swine (59).

Today, the exact role of the pig in the interspecies transmission and the exact mechanisms under cross-species transmission remain unknown. Recent studies showed that human-to-swine transmission is key to understanding the evolution of influenza diversity in pigs and that more information exists on human to swine transmission than swine to human transmission (60). Therefore, this question should be taken as a One Health approach to avoid implicating swine as a source of human viruses.

STRATEGIES TO CONTROL INFLUENZA A VIRUS IN SWINE

The most effective strategy to control and prevent IAV-S infection is vaccination. In contrast to other species such as humans or horses, there is no formal strain recommendation system for swine. This review will summarize approved and tested vaccine technologies for swine by dividing them in two main blocks: non-replicative vaccines, which are considered a safer approach as the lack of replication eliminates the risk of reassortment, and replicative vaccines, which can reassort with circulating field strains.

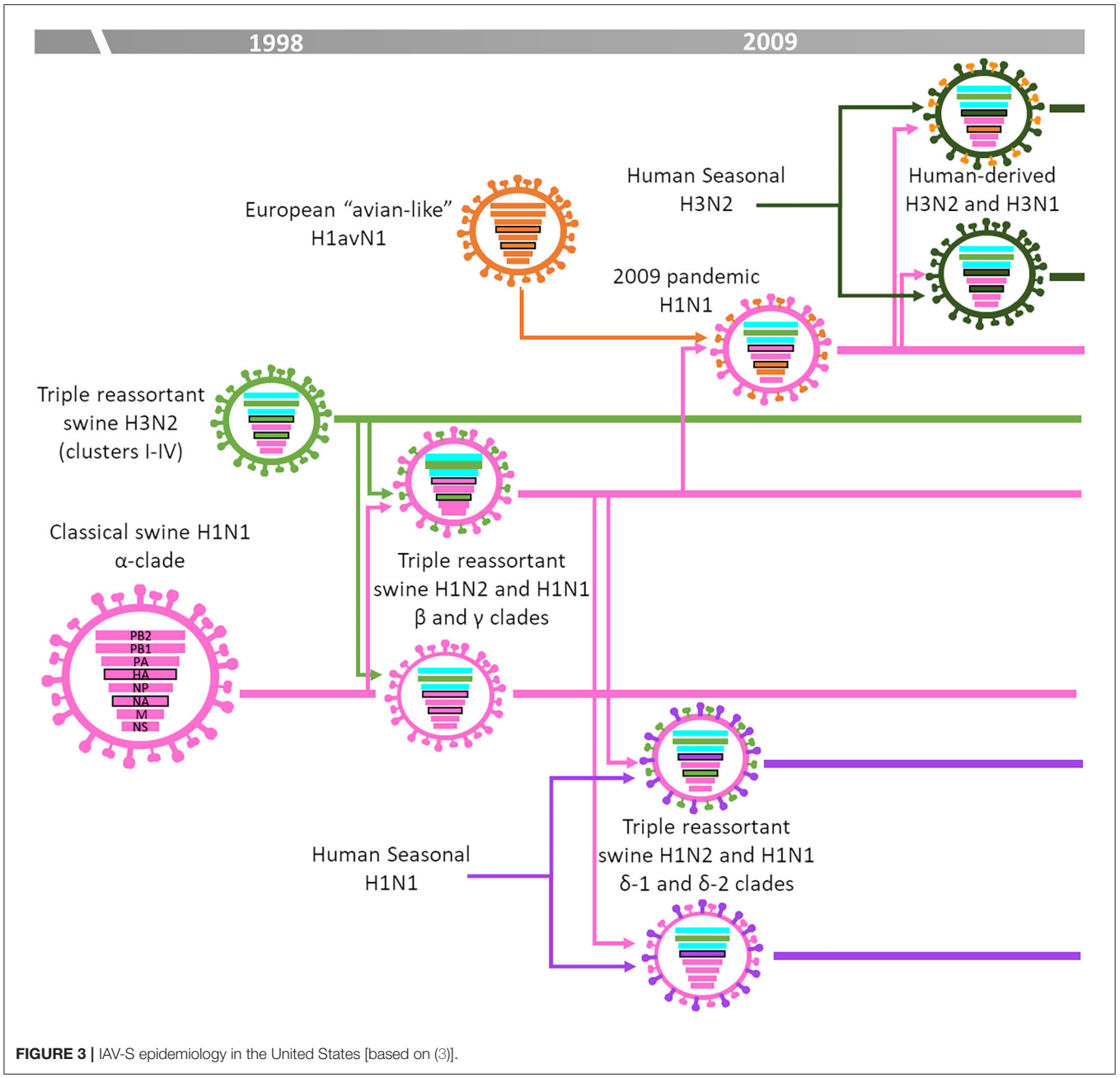

FIGURE 3 | IAV-S epidemiology in the United States [based on (3)].

Non-replicative Vaccines

Inactivated Vaccines

Inactivated vaccines are the traditional method to control IAV-S. Most current IAV-S vaccines contain whole inactivated viruses (WIV) with adjuvant for intramuscular injection and are either used in sows to protect them during gestation and their piglets during the suckling period or in growing pigs to decrease clinical disease (61). The goal of those vaccines is to induce serum neutralizing antibodies that target the viral HA (62). Antibodies are transferred to the mucosae of the respiratory tract to neutralize influenza viruses. Inactivated vaccines are locally produced, and they contain different strains in line with different antigenic and genetic virus strains circulating within each region.

In Europe, WIV vaccines are generally administered only to sows, yet only 10–20% of the sow population is vaccinated (61). As illustrated in **Table 1**, bi-valent vaccines containing H1avN1 and H3N2 subtypes were commercialized during the late 1980s. Nowadays, some of those vaccines are still commercialized in different European countries including Italy or Spain. Later, in 2010, a trivalent vaccine also containing H1huN2 was licensed, and this is still the main vaccine in most European countries. The most recent vaccine available in Europe was a monovalent H1N1pdm09 licensed in 2017.

TABLE 1 | IAV-S vaccines commercialized in Europe from 1980s until 2020.

Product name (manufacturer)	IAV-S strains	Type of adjuvant	Comments
Gripovac (Merial[a])	A/New Jersey/8/1976 (csH1N1) A/Port Chalmers/1/1973 (H3N2)	Oil	Production stopped
Suvaxyn Flu (Fort Dodge[b])	A/swine/Netherlands/25/1980 (H1avN1) A/Port Chalmers/1/1973 (H3N2)	Oil	Production stopped
Respiporc Flu (IDT Biologika[c])	A/swine/Belgium/230/1992 (H1avN1) A/swine/Belgium/220/1992 (H3N2)	Aluminum hydroxide-oil	Production stopped
Gripork (Hipra)	A/swine/Olot/1984 (H1avN1) A/Port Chalmers/1/1973 (H3N2)	Oil	Commercialized in Spain, Portugal, Ukraine, Greece, Russia, and Romania
Respiporc Flu 3 (IDT Biologika[c])	A/swine/Hasselunne/2617/2003 (H1avN1) A/swine/Bakum/1769/2003 (H3N2) A/swine/Bakum/1832/2000 (H1huN2)	Carbomer	Commercialized in most European countries and the United Kingdom
Respiporc Flu pan (IDT Biologika[c])	A/Jena/VI5258/2009 (H1N1pdm2009)	Carbomer	Commercialized in most European countries and the United Kingdom

[a] *Currently Boehringer Ingelheim.*
[b] *Currently Zoetis.*
[c] *Currently CEVA.*

Initial efficacy studies using inactivated vaccines in pigs were conducted using bi-valent formulations containing human-derived A/New Jersey/1976 (H1N1) and A/Port Chalmers/1973 (H3N2) strains. Interestingly, these vaccines were protective against non-related H1avN1 and more recent H3N2 IAV-S isolates. In fact, a Port Chalmers-based vaccine induced considerable antibody titers against H3N2 IAV-S strains isolated between 2008 and 2012 and significantly reduced clinical signs, replication in respiratory tissues and shedding after heterologous challenge with A/swine/Gent/172/2008 (H3N2) (63). Although A/New Jersey/1976-based-vaccine did not provide protection against A/swine/Gent/172/2007 (H1N1), other bivalent vaccines containing H1N1 isolates from the early 1980s and early 1990s showed a significant reduction of viral replication in the lungs. The most recent tri-valent vaccine did not show complete efficacy against the 2007 isolate despite containing a more recent H1N1 isolate (64). As expected and contrary to the tri-valent vaccine, none of the bi-valent vaccines conferred full protection against H1N2 (65). Finally, none of the commercial bi-valent or tri-valent vaccines were efficacious against H1N1pdm09. This gap was supposed to be filled by the commercialization of a monovalent vaccine containing H1N1pdm09. Nevertheless, a recent study demonstrated that this monovalent H1N1pdm09 vaccine does not confer full protection against antigenically distant H1N1pdm09 challenge (66). Studies investigating interference between European inactivated vaccines and pre-existing immunity are scarce. One serological study evaluated the antibody response induced in intranasally inoculated pigs (67). In this study, pigs were inoculated with one to three IAV-S belonging to the European endemic subtypes and later vaccinated with a commercial inactivated H1N1- and H3N2- based vaccine. Single vaccination of pigs previously infected resulted in a dramatic rise in hemagglutinating and neutralizing antibody titers to any of the viruses to which they were previously exposed. This suggests that a close antigenic relationship between vaccine and field strains is less important to provide heterologous protection in pigs previously infected with field strains. In addition, a more recent study demonstrated that heterologous prime and boost vaccination with European and North American (cluster IV) H3N2 subtype strains induced broadly cross-reactive antibodies that protected against homologous infection with both strains (68). The mechanisms behind that and whether those results can be extrapolated to the H1 subtype have yet to be elucidated.

In North America, vaccination against IAV-S is used more than in the EU with ∼70% of the pig population being vaccinated (25). **Table 2** summarizes the IAV-S inactivated vaccines commercialized in the US. The development and launch of inactivated vaccines in the US market coincided with the identification of novel circulating subtypes or clades. Thus, the first vaccine available was a monovalent vaccine developed against an α-H1N1 virus. Later, with the emergence of triple-reassortant H3N2 viruses, monovalent H3N2 and bivalent H1N1/H3N2 vaccines were released. Finally, due to the emergence of antigenically different H1 and H3 clusters, novel multivalent vaccines were launched. Also, in December 2009, a monovalent vaccine based on H1N1pdm09 was licensed (69). In addition to the commercial vaccines, around 50% of the inactivated vaccines used in the USA are autogenous, formulated to contain herd-specific strains.

In Latin America, IAV-S vaccines are primarily used in Argentina and Brazil. In Brazil the only vaccine commercialized is Flusure Pandemic, while in the remaining countries the same commercial vaccines as in the US are used.

Pigs enrolled in initial US-based efficacy trials were vaccinated twice with commercial monovalent csH1N1 vaccine and then challenged with a heterologous α-H1N1 (70, 71). Vaccinated pigs showed reduced clinical signs and lung lesions and nasal virus shed was either reduced or abolished. After the emergence of the H3N2 subtype, pigs vaccinated with commercial bivalent vaccines showed reduced clinical signs, pneumonia and viral excretion when challenged with a heterologous H1N1 (72). In contrast, although the same bivalent vaccines containing cluster I H3N2 IAV-s reduced clinical signs and lung lesions after challenge with a heterologous cluster III H3N2 virus,

TABLE 2 | IAV-S inactivated vaccines commercialized in North America from 1994 until 2020.

Product name (manufacturer)	IAV-S strains	Type of adjuvant	Comments
MaxiVac FLU (Syntro Vet[a])	α-H1N1	Oil	Production stopped
FluSure Legacy (Pfizer Animal Health[b])	α-H1N1 Cluster I H3N2	Amphigen®	Production stopped in 2002
MaxiVac Excell 3.0 (Schering-Plow Animal Health[a])	α-H1N1 β-H1N1 Cluster I H3N2	EMUNADE®	Production stopped
PneumoSTAR SIV (Novartis Animal Health)	α-H1N1 Cluster I H3N2	ImmunSTAR®	
FluSure XP (Pfizer Animal Health[b])	A/swine/Iowa/110600/2000 (γ-H1N1) A/swine/Oklahoma/0726H/2008 (δ1-H1N2) A/swine/Missouri/069/2005 Cluster IV H3N2	Amphigen®	Formulation used in the United States 2008. Also in Canada, Mexico.
FluSure XP (Pfizer Animal Health[b])	A/swine/Iowa/110600/2000 (γ-H1N1) A/swine/Oklahoma/0726H/2008 (δ1-H1N2) A/swine/North Carolina/031/2005 (δ2-H1N1) A/swine/Missouri/069/2005 Cluster IV H3N2	Amphigen®	Formulation used in the United States only (addition of δ2-H1N1 strain). Production stopped in 2016
FluSure XP (Zoetis)	γ-H1N1 δ1-H1N2 Cluster IVA H3N2 Cluster IVB H3N2	Amphigen®	Updated version of FluSureXP, commercialized from 2016, in US only
FluSure Pandemic (Zoetis)	A/California/04/2009 H1N1pdm09	Amphigen®	In US since 2009, final license in 2010
MaxiVac Excell 5.0 (Merck Animal Health)	β-H1N1 γ-H1N1 δ-H1N1 Cluster I H3N2 Cluster IV H3N2	EMUNADE®	

[a] *Currently Merck Animal Health.*
[b] *Currently Zoetis.*

they failed to significantly reduce virus shedding (73). This lack of efficient protection was explained due to the genetic divergence between cluster I vaccine strain and cluster III challenge strain, which showed ∼93% homology at the amino acid level (74). From early 2000's, both endemic H1 and H3 subtypes showed increased genetic and antigenic diversity which made controlling the disease with inactivated vaccines more challenging. For instance, pigs vaccinated with an experimental vaccine containing A/swine/Iowa/1930 (α-H1N1) strain were not fully protected against challenge with a heterologous A/swine/Minnesota/00194/2003 (γ-H1N2) strain (75). Moreover, in the heterologous challenged group, three out of nine pigs had significantly higher percentages of lung lesions when compared to the other groups. This phenomenon called vaccine-associated enhanced respiratory disease (VAERD) and was repeatedly reported with other H1N1 clade combinations, such as 2009H1N1pdm and δ1-H1N1, with both viruses used either as vaccination or challenge (76, 77). Later studies demonstrated that VAERD was related to the use of whole inactivated vaccines containing divergent HA and NA strains to those of the challenge viruses but also by the type of adjuvant used (78, 79). Interestingly, this phenomenon was never described in European vaccine studies. These results suggested that prediction of protection based on HA similarity was unreliable. Vaccination with the first version of the multivalent FluSure XP significantly reduced and delayed the level of β-H1N1 virus transmission virus from shedders to vaccinated animals compared to non-vaccinated animals but to a lesser extent than animals vaccinated with an homologous vaccine, which prevented this transmission completely (80). Another study performed with the same vaccine using A/swine/Illinois/02450/2008 (α-H1N1) as challenge showed partial protection demonstrated by significant reduction of virus present in bronchoalveolar lavages (BALF), nasal secretions and lungs, but no reduction in lung lesions (81). With the spread of 2009H1N1pdm in the US, three commercial vaccines were evaluated for their ability to induce protection. Although the 2009H1N1pdm HA belongs to γ-H1N1 clade and the three tested vaccines contained γ-H1N1 strains, none was able to confer complete protection and high levels of cross-reactive antibody titers (82). Challenge studies were also performed to evaluate the degree of heterologous protection against H3N2 provided by the multivalent vaccines. The conclusions achieved from those studies were that vaccines containing cluster IV H3N2 provided significantly better protection to circulating cluster IV H3N2 viruses when compared to older vaccines containing cluster I H3N2 strains (29, 83). Few studies demonstrated that the presence of maternally derived antibodies (MDAs) does not confer protection against heterologous challenge strains (84). To understand the different vaccine scenarios in the US and Europe, it is important to understand the different regulatory framework needed to approve new vaccines. In Europe, the European Medicine Agency (EMA), requires demonstration of vaccine efficacy through experimental vaccination-challenge studies (using heterologous challenge) against each vaccine subtype following the requirements of the European Pharmacopeia. In contrast, in the USA, the United States Department of Agriculture (USDA) allows the evaluation of the immunogenicity of additional or updated strains by serology only (69). This gives US manufacturers the opportunity to address vaccine updates in a more flexible manner when compared with their European counterparts.

Literature regarding availability of influenza vaccines in Asia is scarce and reports vary from country to country. In China, at least four inactivated adjuvanted licensed vaccines are available. Those vaccines are manufactured by local companies and are

either H1N1 monovalent or H1N1, H3N2 bivalent products. Inactivated vaccines based on local strains are also mainly used in Japan and South Korea. In Japan, the main commercialized bivalent vaccine contains H1N1 and H3N2 strains isolated in the late 1960s and 1970s. In South Korea, there are three inactivated vaccines available, two of which are trivalent containing strains from 2004 to 2005. In both Asian countries, SIV vaccines contain mainly non-oil-based adjuvants.

Viral Vector Vaccines

In the late 2000s, the emergence of H1N1pdm09 both in pigs and humans and the isolation of variant H3N2 IAV-S from humans highlighted the need of a rapid response immunization strategy for pandemic influenza outbreaks. Alphavirus replicon particles containing IAV-S structural gene segments were included in that strategy because they allow for quick strain updates. Alphavirus replicon particles are propagation-defective, single-cycle vectors which deliver genetic material into the cytoplasm of the cell but cannot spread from cell to cell (85). The first recombinant product approved for IAV-S vaccination in the USA was an alphavirus-derived replicon particle vaccine licensed by Harrisvaccines (currently Merck Animal Health) in the early 2010s ("Swine Influenza Vaccine RNA," Harrisvaccines, Inc. Ames, IA, USA) (86). This product consisted of an attenuated Venezuelan-equine encephalitis virus, which was replication-defective due to the substitution of structural genes by the HA of a North American cluster IV H3N2 IAV-S. This product was administered intramuscularly in a priming-boost schedule with a 2–3 weeks interval between each vaccination. After homologous challenge, vaccinated pigs showed reduced amount of viral RNA in nasal swabs and BALF, reduction of clinical signs, gross and histological lung damage. However, protection was not efficacious in the presence of MDAs (87). The homologous protection of this technology was confirmed by Vander Veen et al., which also demonstrated protection using the same platform expressing recombinant H1N1pdm09 HA (88). In the same study, a replicon-particle vaccine expressing a cluster IV H3N2 derived-NP gene was able to decrease nasal shedding and viral load in pigs after heterosubtypic challenge with H1N1pdm09. Later another study aimed to test the efficacy of a monovalent and bivalent combination of the vaccine expressing two different H3N2 HA genes against homologous and heterologous challenge (89). One of the monovalent vaccines provided good protection against homologous and heterologous challenge while the other monovalent vaccine conferred significant protection only against the homologous challenge. In contrast, pigs vaccinated with the bivalent vaccine showed minimal lung lesions and low or undetectable virus in lungs and nasal swabs after challenge. Another advantage of this vaccine platform is that it could be paired with diagnostic strategies of differentiating infected from vaccinated animals (DIVA).

An additional viral vector strategy explored in swine was the use of replication-defective human adenovirus serotype 5 (Ad5) as vector. This technology is based on the deletion of two segments of the Ad5 virus genome creating a replication defective phenotype and space to insert the desired extraneous genes. The HA and the NP genes of a cluster I H3N2 IAV-S were inserted into Ad5 and tested for vaccine efficacy in pigs (90). A single intramuscular dose of Ad5-HA alone or combined with Ad5-NP induced high levels of hemagglutination inhibition (HI) antibodies. Pigs vaccinated with the combination were completely protected against heterologous challenge as shown by lack of virus shedding and lung lesions. On the other hand, pigs vaccinated with Ad5-HA or Ad5-NP alone showed partial or no protection, respectively. This Ad5-HA + Ad5-NP combination could also be delivered with a needle-free device, but results were similar when compared to those of IM injection (91). The efficacy of the Ad5-HA + Ad5-NP combination in the presence of MDAs was also tested (92). A prime-boost vaccination strategy with the Ad5-HA + Ad5-NP combination followed by a commercial bivalent vaccine conferred protection against a heterologous H3N2 challenge in presence of H3N2-specific MDAs. In addition, a recombinant Ad5 encoding H1N1pdm09 HA gene was used to vaccinate pigs with a single intranasal (IN) dose (93). The vaccine induced mucosal antibodies and conferred solid protection against homologous challenge. However, immune response generated was only partially cross-protective against a heterologous challenge with a δ-H1N2 virus.

Other viral vectors have been experimentally tested in swine. Vaccination with recombinant equine herpes virus-1 or swinepox vectors expressing the HA genes of IAV-S protected against homologous challenge (94). However, those studies did not evaluate protection against a heterologous challenge or the impact of MDAs on vaccine performance. In a more recent study, pigs were vaccinated either with vesicular stomatitis virus- or with classical swine fever-derived replicon particles expressing the NP of a European H1N1 IAVs (95). Both vector vaccines elicited a potent antibody and T-cell response and were efficacious against homologous challenge. However, although antibodies and T-cells were cross-reactive, they did not provide protection against heterologous H1N2 infection.

Other Non-replicative Vaccine Technologies Tested in Swine

Exploration of DNA plasmid vaccines against influenza began in the 1990s as an alternative to avoid many issues associated with egg-based vaccine production, which was the main production method for inactivated influenza vaccines at the time (96). DNA vaccines consist of an antigen-encoding gene cloned into a non-replicative expression plasmid that is delivered into the host. This platform offers the advantage that several antigens can be combined in a single plasmid and that they are expected to generate cell-mediated and humoral immunity even in presence of MDAs. Several studies evaluated the immune response generated and protection conferred by DNA vaccines in pigs. DNA vaccines based on different gene combinations (mainly HA) demonstrated good degrees of protection against homologous challenge (70, 71, 97–99). Needle-free and IM delivery methods were tested to be successful, but recent studies evaluated needle-free delivery as it was claimed to be safer and easier to administer for large scale vaccination (97–99). The combination of priming with a DNA vaccine and boosting with an inactivated vaccine conferred significantly better protection than only two doses of

DNA vaccine (71). However, heterologous cross protection was demonstrated even in presence of MDAs after two doses of DNA vaccine (99). The major handicap of those vaccines is that large doses of DNA and several vaccination doses were required to confer protection.

Another technology explored is the vaccination with HA trimers. In the context of the 2009 pandemic, another research group in the Netherlands evaluated the immune response and the protection generated against H1N1pdm09 in pigs after vaccination with recombinant H1N1pdm09 HA trimers (100). Upon double vaccination, pigs vaccinated with HA trimers were almost completely protected against challenge virus. Only low levels of virus replication were detected in the pig's respiratory tract. This finding was in line with the high levels of HI and virus neutralizing antibodies found against the homologous strain. Although heterologous challenge was not performed, HI cross-reactive antibody levels against H1avN1 and H1N2 were lower when compared to those raised against the homologous strain. Therefore, lower levels of protection may be expected.

The primary function of the influenza A virus M2 protein is to act as an ion channel for disassembly of the viral core, but also as a secondary conserved antigenic site in contrast to HA and NA antigenic sites, which are less conserved. Therefore, recombinant vaccines based on the M2 protein were proposed as universal influenza A vaccine candidates (101). This strategy showed promising results in mice (102, 103), but M2 based vaccines alone were not able to confer significant protection (104, 105).

Replicative Vaccines

Live-Attenuated Virus Vaccines

Live-attenuated influenza virus (LAIV) vaccines consist of viruses produced by reverse genetics genetically modified to reduce viral replication. LAIV vaccines are administered directly to the respiratory mucosa by intranasal (IN) administration, which mimics natural infection and activates both mucosal and systemic immune responses. Mucosal antibodies, such as IgAs, are important to control IAV-S, and the cell mediated immune response induced by the replicating LAIVs is essential for broader cross-protection against natural infection as T cells mostly recognize conserved epitopes. Three different LAIV strategies have been tested in swine.

Attenuation by Non-Structural NS1 Protein Truncation

The goal of this technology is to hijack the ability of the virus to evade host cell type I interferon (IFN)-mediated antiviral response and to restrict virus replication. This is achieved by the deletion of 126 amino acids from non-structural NS1 protein, which is only expressed in virus-infected cells. This deletion was applied to a North American cluster I H3N2 (A/swine/Texas/4199-2/1998) strain that resulted in the absence of or minimal lung lesions and significantly lower virus titers in BALF when compared to the wild-type inoculated group. Interestingly, the attenuated virus had strong immunogenic properties in spite of its lower levels of replication (106). This immune response, generated after IN inoculation, was composed of high levels of mucosal IgAs and systemic cell mediated immune responses, as well as modest levels of systemic neutralizing antibodies (107–111). The LAIV vaccine conferred strong protection against homologous challenge in influenza-naïve pigs and nearly complete protection against the heterologous cluster II H3N2 (A/swine/Colorado/23619/1999), which is antigenically different (108). In contrast, after challenge with a heterosubtypic H1N1, vaccinated animals showed no reduction in lung lesions and a slight reduction of virus titers in BALF and nasal swabs at 5 days post challenge (107, 108). In addition, the NS1 LAIV vaccine showed partial protection in piglets with MDAs without inducing VAERD (112, 113). Since 2017, a NS1 LAIV vaccine became commercially available in the USA for use in pigs from 1 day of age. Ingelvac Provenza (Boehringer Ingelheim, St. Joseph, MO, USA), which is a bivalent product containing two reverse genetic generated LAIVs: one cluster I H3N2 virus based on A/swine/Texas/4199-2/1998 with the NS1 truncation and one virus containing the same attenuated internal gene cassette derived from the H3N2 strain but with the HA and NA derived from a γ2 beta-like H1N1 strain (A/swine/Minnesota/37866/1999). This vaccine was efficacious in reducing virus nasal shedding after challenge with heterologous strains, either H1N1 or H3N2, with and without presence of MDAs (113, 114). However, a recent phylogenetic study done in the US with samples collected in 2018 found reassortant strains containing LAIV vaccine strain genes in combination with US endemic field strain genes (115). These data indicate that viral reassortment is possible with LAIV vaccines. Further research will be required to evaluate its impact to the IAV-S epidemiology.

Attenuation by Polymerase Genes Mutations

Influenza virus polymerase complex is composed of polymerase basic 2 (PB2), polymerase basic 1 (PB1) and polymerase acidic (PA). Those three subunits working together are responsible for virus replication in the host cell (116). Previous studies in humans and horses identified that certain mutations in the viral polymerase PB1 and PB2 genes caused impaired polymerase activity and reduced replication at the temperature of the lower respiratory tract (117, 118). These cold-adapted and temperature sensitive (ts) mutations were also evaluated in a cluster I H3N2 IAV-S confirming the restricted virus growth in the respiratory epithelium of pigs (119, 120). Viruses were generated by reverse genetics to contain the ts internal gene cassette and several different HA and NA combinations. For instance, a single IN vaccination with the ts internal gene cassette combined with H1N1pdm2009 conferred sterilizing immunity against homologous challenge (120). Another study compared the efficacy in pigs of three US commercial vaccines each containing different H3N2 strains against two different LAIV vaccines, one with a NS1-truncated cluster I H3N2 strain and the other with a ts cluster IV H3N2 (83). After two doses all vaccines conferred significant protection against a heterologous cluster IV H3N2 challenge strain. However, only the ts-LAIV vaccine prevented aerosol transmission to indirect contact pigs. Another study compared the effects of heterologous challenge with a δ2-H1N2 strain after vaccination with two different H1N1pdm09

vaccines, a recombinant HA subunit vaccine or a ts-LAIV (121). The ts-LAIV partially protected pigs, as demonstrated by reduced virus shedding and faster viral clearance. In contrast, pigs vaccinated with the subunit vaccine developed more severe lung lesions right after challenge, which was consistent with VAERD. This absence of VAERD in pigs vaccinated with ts-LAIVs was further confirmed by another study (122). Although there are no specific studies that evaluate the efficacy of ts-LAIV vaccines in the presence of MDAs, the nature of the immune response generated by LAIV vaccines, which is mainly composed by mucosal antibodies and a cell-mediated component, suggests low levels of interference compared to the interference observed against inactivated vaccines. Additionally, there are no reports available that evaluate the potential of this vaccine to reassort with endemic field strains.

Other Technologies of Attenuation

Influenza A virus HA protein is synthesized as a precursor (HA0), which in order to become infectious must be cleaved by host proteases, usually trypsin, into HA1 and HA2. In IAV-S this process is usually mediated by trypsin-like proteins and it is essential for the virus to efficiently bind and replicate in the host cells (123). The modification of the HA cleavage site to be activated by elastase enzyme instead of trypsin resulted in virus attenuation due to the scarce presence of elastase in the host tissues when compare to the trypsin (124). Elastase-dependent mutants viruses based on a Canadian avian-like H1N1 strain (A/swine/Saskatchewan/18789/2002) (125), did not induce clinical signs or virus shedding in inoculated pigs. Those elastase-dependent strains generated robust cell-mediated and mucosal antibody responses after two IN or IT doses (126). After challenge, those H1N1 based LAIVs conferred robust protection against homologous and heterologous challenge strains (126, 127). However, only partial protection was described when challenged with an heterosubtypic H3N2 subtype (126). The same group generated one novel virus containing two HAs, an H1 and H3 in the genetic context of the previous H1N1 LAIV (128). This mutant was generated by fusing the H3 HA ectodomain of a triple reassortant H3N2 to the N1 NA transmembrane and cytoplasmic tail of the A/swine/Saskatchewan/18789/2002 to replace NA ectodomain and ultimately attenuate the virus. Like the previously described elastase-sensitive construct, the new chimeric H1–H3 vaccine candidate was highly dependent on presence of exogenous neuraminidase to subsides lack of NA viral function. The rationale behind this construct was to increase the level of cross-protection against heterologous H3N2 viruses with a single bivalent vaccine construct. After two vaccinations the novel LAIV induced antigen-specific systemic and mucosal antibody responses in the respiratory tract. In addition, vaccinated pigs had no or minimal lung lesions and undetectable levels of virus in the lungs after challenge either with H1N1 or H3N2 IAV-S strains. However, those results should be carefully interpreted as the H3N2 challenge virus was also undetected in the lungs of 4 out of 5 pigs from the challenge control group. Those vaccines proved to protect in presence of MDAs and VAERD was never reported (129).

CONCLUSIONS

IAV-S can cause important health issues in pigs and the subsequent economic damage to the swine industry. Although only three subtypes of IAV-S are circulating the origins, genetic and antigenic diversity of those viruses show great regional differences. IAV-S populations are highly dynamic. However, the impact of IAV-S may not be exclusively related to swine industry. The first pandemic virus from the twenty-first century was caused by an influenza A virus generated in swine containing genes from avian, human and swine origins, and "variant" viruses have been repeatedly isolated from humans since 2010 (14, 33). Pigs and humans share the same influenza receptors pattern in their respiratory tract (49) and inter-species transmission of influenza A viruses from pigs-to-humans and from humans-to-pigs occur in both directions (60). Therefore, efficient prevention and control of IAV-S may not be only a benefit for swine health but for human health.

Currently, the main tool to control IAV-S infection is by vaccination. The desirable vaccine should be easy and safe to administer, generate a robust immune response to confer heterologous or even heterosubtypic broad protection, function in the presence of MDAs or active immunity, and not induce VAERD. None of the vaccines described here comply with all the characteristics described. The inoculation route has an impact in the immune response generated. For instance, IM or intradermal route vaccines generate higher humoral responses based in HA neutralizing antibodies, which are very strain specific, while IN vaccines generate a robust cell-mediated response and mucosal antibodies, which are less strain specific. However, IN route may not be a desirable or practical route to vaccinate large numbers of sows or adult pigs. LAIVs are the only vaccines that generate mucosal immunity and are a promising tool in the prevention of IAV-S, but they can replicate in the host and reassortment with field strains cannot be ignored. In fact, reassortment capabilities of LAIVs were demonstrated in experimental conditions and in the field (115, 130). Although this reassortment did not result in increased virulence, further research will be needed to evaluate the impact of this process on influenza A virus's epidemiology. All vaccines mentioned were tested in controlled laboratory environments, which greatly differ from those encountered in the field. Most pigs in the field have already a pre-existing immunity at the time of vaccination, either from MDAs or due to previous infections. Some studies demonstrated that MDAs reduced the efficacy of inactivated and, to a lesser extent, LAIV vaccines. However, other experimental vaccines, such as viral vector vaccines, have never been tested in the presence of MDAs or active immunity. Differences in vaccination rates and region-specific perceptions may also impact vaccine research and commercial products available. For example, while VAERD has been broadly described using commercial and experimental inactivated vaccines in the USA, it has never been described in Europe. This together with the fact that vaccine uptake is much lower in Europe and that regulatory requirements are stricter to update current vaccines when compared with the US, may explain the presence of very old vaccines in the European market. In addition,

this context may also explain why United States researchers have actively evaluated innovative vaccine technologies such as LAIVs whereas in Europe efforts focused primarily on the optimization of inactivated vaccines. In China, the availability of only monovalent or bivalent vaccines contrasts the much more complex epidemiological situation in that region.

Although considerable work has been performed to create novel vaccines, investigate their value, and evaluate alternative platforms to control the spread of IAV-S, much more research needs to be done. It is the responsibility of researchers throughout the world to continue working together, not only on improved vaccination strategies, but also to significantly booster worldwide surveillance in an effort to maintain the clearest possible picture of IAV-S epidemiology.

AUTHOR CONTRIBUTIONS

JM drafted the first manuscript. JM, DP, AM, and MB wrote the paper. All authors contributed to the article and approved the submitted version.

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Comparison of Humoral Immune Responses to Different Forms of *Salmonella enterica* Serovar Gallinarum Biovar Gallinarum

Nam-Hyung Kim[1,2], *Eun-Jin Ha*[1,2], *Dae-Sung Ko*[1,2], *Kang-Seuk Choi*[2,3] and *Hyuk-Joon Kwon*[1,2,4]*

[1] Laboratory of Poultry Medicine, Seoul National University, Seoul, South Korea, [2] Research Institute for Veterinary Science, College of Veterinary Medicine, BK21 for Veterinary Science, Seoul, South Korea, [3] Laboratory of Avian Diseases, College of Veterinary Medicine, Seoul National University, Seoul, South Korea, [4] Farm Animal Clinical Training and Research Center (FACTRC), GBST, Seoul National University, Seoul, South Korea

Fowl typhoid is caused by *Salmonella enterica* serovar Gallinarum biovar Gallinarum (SG), and live attenuated, rough vaccine strains have been used. Both humoral and cellular immune responses are involved in protection, but the humoral responses to different forms of SG antigens are unclear. In this study, we compared humoral responses to a killed oil-emulsion (OE) smooth vaccine (SG002) and its rough mutant vaccine (SR2-N6) strains using proteomics techniques. We identified two immunogenic outer membrane proteins (OmpA and OmpX), and the selected linear epitopes were successfully applied in peptide-ELISA. Our peptide- and total OMP-ELISAs were used to compare the temporal humoral responses to various SG antigens: OE SG002 and SR2-N6; live, killed [PBS-suspension (PS) and OE)] and mixed (live and PS) formulations of another rough vaccine strain (SG 9R); and orally challenge with a field strain. Serum antibodies to the linear epitopes of OmpA and OmpX lasted only for the first 2 weeks, but serum antibodies against OMPs increased over time. The rough strain (SR2-N6) and mixed SG 9R induced higher serum antibody titers than the smooth strain (SG002) and single SG 9R (OE, live and PS SG 9R), respectively. Infection with the field strain delayed the serum antibody response by ∼2 weeks. Mucosal immunity was not induced by any formulation, except for infection with the field strain after SG 9R vaccination. Thus, our results may be useful to understand humoral immunity against various SG antigens and to improve vaccine programs and serological diagnosis in the field.

Keywords: ***Salmonella serovar*** **Gallinarum biovar Gallinarum, humoral immunity, vaccines, natural infection, Peptide-ELISA**

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***Correspondence:**
Hyuk-Joon Kwon
kwonhj01@snu.ac.kr

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INTRODUCTION

Salmonella enterica serovar Gallinarum biovar Gallinarum (SG) is a pathogen causing fatal and persistent infection, fowl typhoid (FT) (1, 2). Both humoral and cell-mediated immune responses are required to prevent mortality and achieve bacterial clearance (3). A live vaccine strain, SG 9R, mimics infection of pathogenic field strains, and has been used to prevent FT worldwide (4).

The potent immunostimulatory effect of lipopolysaccharide (LPS) is mediated by O-Ag and lipid A, which induce T cell-independent humoral and TLR4-mediated innate immune responses, respectively (5). Although LPS induces a strong humoral immune response to concomitantly inoculated antigens, LPS on the surface of bacteria may also shield or compete with outer membrane proteins (OMPs), resulting in decreased immunogenicity of OMP (6, 7). Therefore, while SG 9R is a rough strain with defective outer-core and O-antigen regions (O-Ag) of lipopolysaccharide (LPS), it may induce a different humoral immune response from field strains against OMP (8). The protective efficacy of OMP vaccines has already been established, and protective OMPs of *S.enterica* serovars have been identified for vaccine development (9, 10).

Although SG 9R has been commonly used in the field, it displays potential pathogenicity and may cause mortality and gross lesions in the liver under immunosuppressive conditions (8). Therefore, SG 9R was not recommended for use in chicks under 6 weeks old (w-o) who are most susceptible and may become carriers (4, 11). For this reason, killed vaccines, if possible, need to be considered, but basic data on the differences in humoral immune responses to different forms of SG antigens (oil-emulsion, killed, smooth vs. rough SG; live vs. killed with or without oil adjuvant vs. a mixture of live and killed SG 9R; or field strain) are insufficient. In addition, humoral immunity against natural infection with field strains is unclear. Humoral immunity to live or killed bacteria is the sum of antibodies directed to multiple antigens and their epitopes. Therefore, investigations of a single epitope-specific antibody in the antiserum against different antigens using single peptide epitopes may provide more insights into the kinetics of humoral immunity. In this study, we compared humoral immune responses to smooth and rough SG strains and identified immunogenic OMPs and their linear epitopes. We developed linear epitope-based peptide-ELISAs to compare humoral immune responses to different forms of SG antigens, and the results were compared with data from the OMP-ELISA.

MATERIALS AND METHODS

Bacteria, Serum Samples, and Experimental Birds

A commercial rough vaccine strain, SR2-N6 (DAE SUNG Microbiological Lab., Uiwang-si, Korea), and its parent strain SG002 were used to compare the effect of LPS on humoral immunity, and a commercial rough vaccine strain, SG 9R, was purchased from the manufacturer (Nobilis; Intervet International, Boxmeer, the Netherlands) (12). SG0197, a virulent strain isolated from commercial chickens in 2001, was used to observe the immune response of challenged chickens (12). The strains were cultured in Luria-Bertani broth (Duchefa Biochemie, Groot Bijgaarden, Belgium) with shaking at 37°C overnight.

One d-o male Hy-Line brown layer chicks without SG vaccination were purchased from a farm (Yangji Farm, Pyeongtaek-si, Korea) and reared for animal experiments to compare humoral immune responses to different forms of SG antigens. Feed and water were supplied *ad libitum*.

Fifty-six field serum samples obtained for serological tests from 6 layer and breeder farms were used to determine humoral immunity to SG in the field. In detail, L1D included 10 samples from 1-day-old (d-o) layer chicks, L12W included 10 samples from 12-w-o layer chickens vaccinated at 10 w-o, L19W included 10 samples from 19-w-o layer chickens vaccinated twice at 8 and 15 w-o, and L41W included 10 samples from 41-w-o layer chickens vaccinated twice at 7 and 16 w-o. PS18W and PS23W included 10 and 6 samples from 18 and 23-w-o parent stocks, respectively.

2D-Gel Electrophoresis, Immunoblotting, and LC-MS/MS

Total bacterial proteins were extracted via cell lysis with 7 M urea, 2 M thiourea, 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), and 2.5% dithiothreitol (DTT) and quantified with the Bradford protein assay. OMPs were extracted using the previously described sodium lauroyl sarcosine (SLS) method (13), with some modifications. Briefly, the cultured bacteria were centrifuged and the pellet was washed with 50 mM Tris-HCl. After centrifugation, lysis buffer (50 mM Tris-HCl and 150 mM NaCl) was added, and the cells were lysed by ultrasonication. The supernatant was ultracentrifuged at 100,000 × g for 1 h. Pellets were resuspended in 2% SLS and 50 mM Tris-HCl, and then incubated at room temperature for 40 min. After another ultracentrifugation step, pellets were stored by adding 1% Triton X-100 to the lysis buffer.

2D-gel electrophoresis of total proteins or OMPs was performed using isoelectric focusing (pH 3-10 for whole bacteria or pH 4-7 for OMPs) and 14% SDS-PAGE gels, and separated proteins were electrotransferred to nitrocellulose membranes for western blotting (ProteomeTech, Seoul, Korea) (14). Membranes were incubated with anti-SR-N6 (1:10,000 dilution) and anti-SG002 (1:5,000 dilution) serum samples. LC-MS/MS was performed as described below. The analysis was performed using a nano ACQUITY UPLC and LTQ-Orbitrap-mass spectrometer (Thermo Electron, San Jose, CA). One of the mobile phases for LC separation was 0.1% formic acid in deionized water, and the other was 0.1% formic acid in acetonitrile. The flow rate was 0.5 μl/min, and the transfer tube temperature was set to 160°C. The MS/MS data were interpreted using SEQUEST software (Thermo Quest, San Jose, CA, USA), and the generated peak lists were compared using the MASCOT program (Matrix Science Ltd., London, UK).

B Cell Epitope Prediction and Peptide Synthesis

B cell epitopes were predicted by the IEDB B cell epitope prediction program (http://tools.iedb.org/bcell/), and they were located on the 3D structure files of corresponding proteins generated with PyMOL 2.2 (Schrodinger, New York, USA). Selected peptides were synthesized with a modification of the N-terminus by adding aminocaproic acid

for better performance of the peptide-ELISA (Cosmogenetech, Seoul, Korea).

ELISA

Synthesized peptides (1 μl/ml) or SG 9R OMP extracts (105 ng/ml) in 100 mM sodium bicarbonate/carbonate coating buffer (pH 9.6) were used to coat an immunoplate (SPL Life Science, Pocheon-si, Korea) at 4°C overnight. Antigen-coated wells were washed twice with PBST (PBS containing 0.5% Tween 20) and blocked with 1% bovine serum albumin (BSA) (GenDEPOT, Katy, USA) at room temperature for 2 h. After washing the plates as described above, the primary antibody, which was serum or bile juice (1:300 in PBST containing 1% BSA), was added, incubated for 30 min, and then the plate was washed 4 times with PBST. The secondary antibody, an HRP-conjugated goat-anti chicken IgG or IgA antibody (Bethyl, Laboratories, Montgomery, USA; diluted 1:10,000 in PBST containing 1% BSA), was added for 30 min, and the plate was washed as described above. TMB substrate (SurModics, Eden Prairie, USA) was added for 10 min, and the OD was measured at 450 nm after the addition of stop solution. We used a commercial *Salmonella* D group ELISA kit to test the anti-O-Ag antibody according to the manufacturer's recommendation (BioChek BV., Reeuwijk, the Netherlands).

Inactivation and Preparation of Oil-Emulsion (OE) SG

Cultured bacteria were centrifuged and washed once with PBS. Bacteria were inactivated at 65°C for 2 h in a water bath and cooled gradually to room temperature. The inactivation was confirmed by culture on Mueller Hinton Agar (Duchefa Biochemie, Groot Bijgaarden, Belgium). The live and heat-inactivated bacteria were diluted to 1×10^7 cfu/100 μl and 1×10^9 cfu/100 μl in PBS, respectively. The live and killed mixture was prepared by mixing the same volume of both preparations of bacteria to obtain 200 μl. The OE bacteria were prepared by emulsifying heat-inactivated bacteria with oil adjuvant (Montanide ISA 70, Seppic Co., Courbevoie, France) at a ratio of 3 to 7 ($\sim 3.3 \times 10^8$ cfu/100 μl of OE) (**Table 1**).

Animal Experiments

Fifteen (5 chickens in each group) 3-w-o male brown layer chickens were divided into SR2-N6, SG002, and negative control groups to compare the humoral immune responses to smooth (SG002) and rough (SR2-N6) strains, respectively. OE SR2-N6 and OE SG002 were inoculated via the intramuscular route (100 μl/chicken), and serum samples were collected weekly for up to 3 weeks postinoculation (wpi). Bile juice samples were collected from the gall bladder at 3 wpi with a 1 ml syringe. Specific antibodies in serum (IgG) and bile juice (IgA) samples were measured using the ELISA.

Forty 3-w-o male brown layer chickens were assigned to OE SG 9R (10), live SG 9R (10), mixed SG 9R (10), PS (PBS –suspension) SG 9R (5), and negative control groups (5) to compare the humoral immune responses to different forms of SG 9R. All groups were inoculated via the intramuscular route (100 μl/chicken), and serum samples were collected weekly for up to 3 wpi. Bile juice samples were collected at 3 wpi as described above. Specific antibodies in serum and bile juice samples were measured using the ELISA.

TABLE 1 | Inoculated vaccines and field strain.

Sample	Solvent	Dose (cfu/chicken)	Inoculation route
SR2-N6	PBS with the ISA 70 adjuvant	3×10^8	IM
SG002	PBS with the ISA 70 adjuvant	3×10^8	IM
SG9R	PBS	1×10^7	IM
OE SG9R	PBS with the ISA 70 adjuvant	3×10^8	IM
PS SG9R	PBS	1×10^9	IM
MX SG9R	PBS	1×10^7(SG9R)+1×10^9(PS SG9R)	IM
SG197	PBS	1×10^6	*per os*

Fifteen 4-w-o chickens were infected with the field strain (SG0197, 1×10^6 cfu/0.1 ml/chicken) *per os,* and serum samples were collected from the surviving chickens weekly for 4 wpi. Bile juice samples were collected after 3 days of starvation at 4 wpi, as described above. Specific antibodies in serum and bile juice samples were measured using the ELISA.

Twenty 6-w-o chickens were divided into SG 9R vaccine and no vaccine groups to compare the mucosal immune responses. The SG 9R vaccine group was vaccinated with SG 9R (1×10^7 cfu/100 μl/chicken) via the intramuscular route, and both groups were challenged with SG0197 at 2 wpi (8 w-o). After 2 wpi, SG0197 bile juice samples were collected as described above, and specific antibodies were measured using the ELISA.

All animal experiments were approved by the Institutional Animal Care and Use Committee of BioPOA Co. (permission number BP-2019-C31-1).

Statistical Analysis

Analyses were performed with SPSS Statistics version 26.0 (SPSS, Chicago, IL, USA). One-way ANOVA was used to analyze significant differences between the groups, followed by the Bonferroni *post-hoc* test (**Figure 2C** 1 wpi and 3 wpi, **Figure 2D** 1 wpi, **Figures 5A,C**). When unequal variance was observed, the Welch test was used for the analysis, and the Games-Howel test was performed as the *post hoc* test (**Figure 2B** 2 wpi, **Figure 2C** 2 wpi, **Figure 2D** 2 wpi and 3wpi, **Figure 3A** 2 wpi, **Figure 3B** 2 wpi, **Figure 3C** 1 wpi, **Figure 3D** 1-3 wpi). Data with a non-normal distribution were subjected to the Kruskal-Wallis *H*-test, and the Bonferroni correction was used as the *post-hoc* test (**Figure 2A** 1-3 wpi, **Figure 2B** 1 wpi and 3 wpi, **Figure 3A** 1 wpi and 3 wpi, **Figure 3B** 1 wpi and 3 wpi, **Figure 3C** 2 wpi and 3 wpi, **Figures 4**, **5B,D**, **6**). If only two groups were analyzed, the significance was determined with the *t*-test for data with a normal distribution (**Figures 5E–G**), and the Mann-Whitney *U*-test was used for data with a non-normal distribution

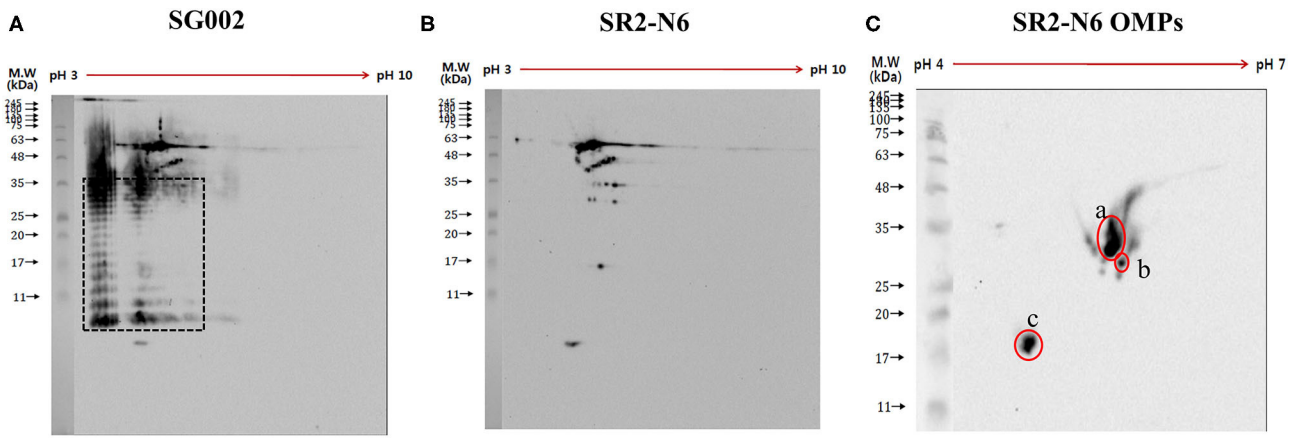

FIGURE 1 | Antigens of SG002 were tested against antiserum of SG002 **(A)** and SR2-N6 **(B)** using 2DE western blotting. Different blots obtained in **(A,B)** were analyzed using LC-MS/MS (**Supplementary Table 1**). Distinct ladders at different isoelectric points represented the O antigen (black dotted rectangle). Omp extracts of SR2-N6 were also tested against the antiserum of SR2-N6 **(C)** using 2DE western blotting. Three spots (a, b, and c) were analyzed with LC-MS/MS (**Table 2**).

(**Figures 5H,I**). Statistical significance was considered when the $p < 0.05$.

RESULTS

Comparison of Humoral Immune Responses to Smooth (SG002) and Rough (SR2-N6) Strains

In contrast to anti-SR2-N6 serum samples, anti-SG002 serum samples showed a strong antibody reaction to O-Ag (at least two distinct ladders at different isoelectric points) (**Figure 1A**, black dotted rectangle). The different spots recognized by the anti-SR2-N6 serum sample were analyzed with LC-MS/MS. Interestingly, most of the spots were not OMP and included translation elongation factor G, GroEL, phosphoglycerate kinase, elongation factor Tu, electron transfer flavoprotein subunit beta, *etc*. (**Supplementary Table 1**). To identify immunogenic OMPs, we performed 2D-gel electrophoresis and immunoblotting with OMPs of SR2-N6 and anti-SR2-N6 serum samples to identify major antigens. The three major antigen spots were identified to be OmpA (spots a and b) and OmpX (spot c) (**Figure 1C**, **Table 2**). We selected candidate peptides for the peptide-ELISA according to the amino acid sequences of OmpA (CAR36850) and OmpX (CAR36706) (**Table 3**).

A pilot study with the synthesized peptides (OmpA-N-L1, OmpA-N-L2, OmpA-N-L3, OmpA-N-L4, OmpX-L1, and OmpX-L2) and anti-SG002 and anti-SR2-N6 serum samples revealed that the reactivity of OmpA-N-L1, OmpA-N-L2, and OmpX-L1 was too low to differentiate responses from anti-SG002 and anti-SR2-N6 serum samples. We selected OmpA-N-L3, OmpA-N-L4, and OmpX-L2 for the peptide-ELISA. According to the results, the anti-SR2-N6 antibody titer was significantly higher than the anti-SG002 antibody titer in the OmpX-L2 and OMP-ELISA at 1 week postinoculation (wpi) (**Figures 2C,D**). All the anti-SR2-N6 and anti-SG002 serum samples showed significantly higher OD values than the negative control only for the first 2 weeks using peptide-ELISAs, except for OmpX_L2. However, in the OMP-ELISA, significantly higher OD values were observed than the negative control, with a gradual increase during the observation period.

TABLE 2 | Proteins predicted by the LC-MS/MS analysis.

Spot label	NCBI BLAST	Protein name	Score	Mass
a	WP_065702086.1	porin OmpA [*Salmonella enterica*]	4039	37640
b	WP_065702086.1	porin OmpA [*Salmonella enterica*]	1735	37640
c	WP_058343733.1	outer membrane protein OmpX [*Salmonella enterica*]	3409	17570

TABLE 3 | B cell epitopes of OmpA and OmpX tested in the peptide-ELISA.

Protein/location	Peptide name	Sequence (N- to C-terminus)
OmpA/N-terminus	OmpA-N-L3	TKSNV PGGPS
	OmpA-N-L4	TNNIG DANTI GTR
OmpA/C-terminus	OmpA-C-L1	QLYSQ LSNLD PKDGS
	OmpA-C-L2	GESNP VTGNT CDNVK
OmpX	OmpX-L2	GKFQT TDYPT YKHDT

Comparison of Humoral Immune Responses to Live, Killed and Mixture of Live, and Killed Rough Vaccine Strains (SG 9R)

The OD values of anti-OE SG 9R, anti-live SG 9R, and anti-mixed SG 9R serum samples were not significantly different from each other, and produced higher OD values than the anti-PS SG 9R serum samples and negative control samples at

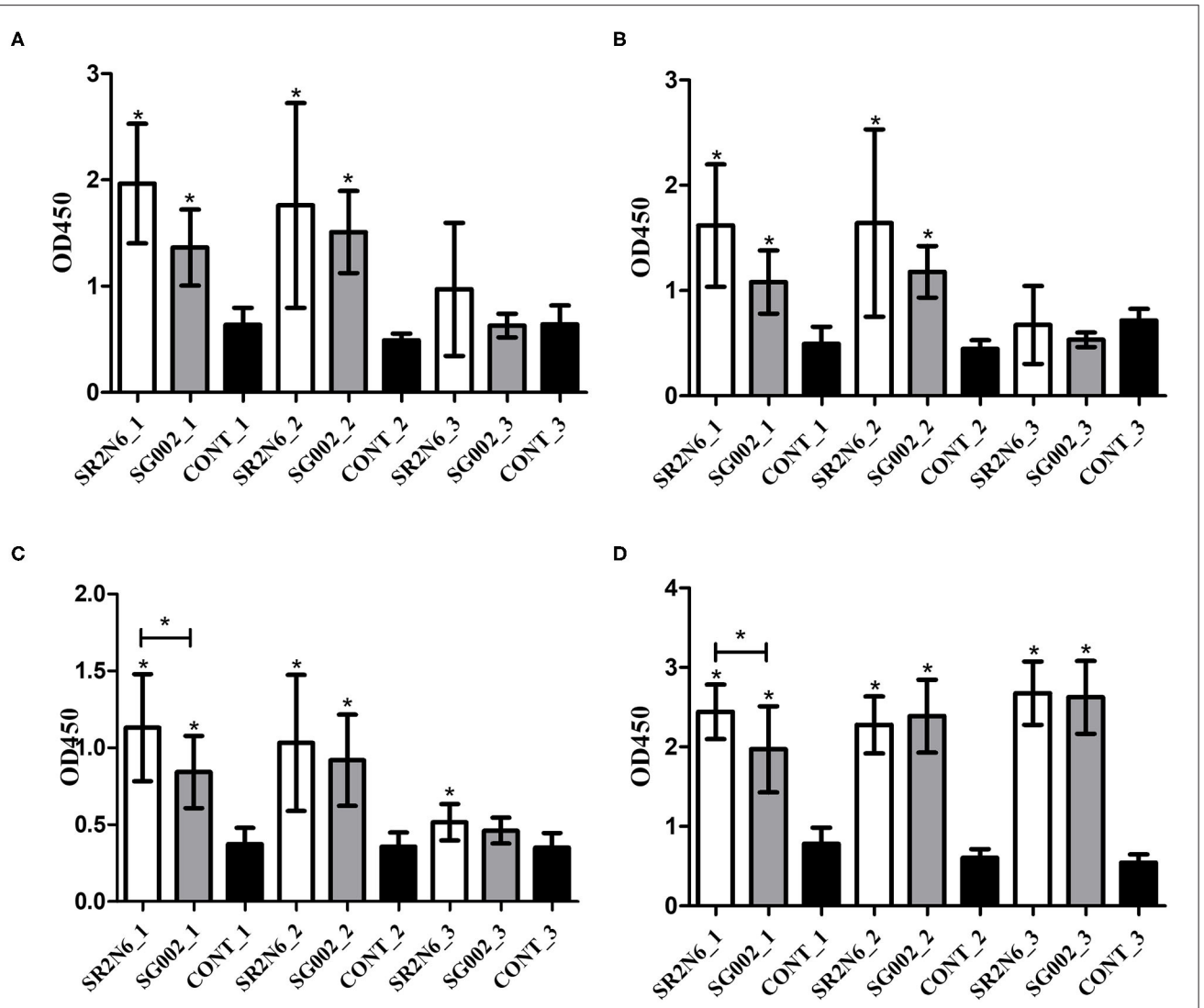

FIGURE 2 | Humoral immune responses to the smooth strain (SG002) and rough strain (SR2-N6) measured using ELISAs (mean with SD): OmpA-N-L3 **(A)**, OmpA-N-L4 **(B)**, OmpX-L2 **(C)**, and OMP ELISAs **(D)**. *Indicates a significant difference [*P*-values - **(A)** SR2-N6 vs. CONT at 1 wpi (0.000) and 2 wpi (0.001); SG002 vs. CONT at 1 wpi (0.008) and 2 wpi (0.000); **(B)** SR2-N6 vs. CONT at 1 wpi (0.000) and 2 wpi (0.005); SG002 vs. CONT at 1 wpi (0.01) and 2 wpi (0.000); **(C)** SR2-N6 vs. SG002 at 1 wpi (0.046); SR2-N6 vs. CONT at 1 wpi (0.000), 2 wpi (0.002), and 3 wpi (0.003); SG002 vs. CONT at 1 wpi (0.001) and 2 wpi (0.000); **(D)** SR2-N6 vs. SG002 at 1 wpi (0.035); SR2-N6 vs. CONT at 1 wpi (0.000), 2 wpi (0.000), and 3 wpi (0.000), SG002 vs. CONT at 1 wpi (0.000), 2 wpi (0.000), and 3 wpi (0.000)].

1 and 2 wpi in the peptide-ELISAs (**Figure 3**). Interestingly, anti-mixed SG 9R showed significantly higher OD values than the negative control in the OmpA-N-L3 and OmpA-N-L4 peptide-ELISAs at 3 wpi. According to the results of the OMP-ELISA, anti-OE SG 9R, anti-SG 9R, and anti-mixed SG 9R serum samples showed significantly higher OD values than the negative control samples with a gradual increase over time. Anti-PS SG 9R serum samples did not show significantly higher OD values than the negative control in either peptide- or OMP-ELISAs.

We tested two additional peptides (OmpA-C-L1 and OmpA-C-L2) in the C-terminal domain of OmpA using the peptide-ELISA (**Table 3**). The anti-OE SG 9R serum samples showed significantly higher OD values than the negative control at 1 and 2 wpi, but not at 3 wpi (**Supplementary Figure 1**).

Humoral Immunity Against Natural Infection With a Field Strain (SG0197)

SG0197 infection caused 86.7% (13/15) mortality within 4 weeks (7/15 at 2 wpi, 5/15 at 3 wpi and 1/15 at 4 wpi); therefore, the numbers of serum samples were 8 at 2 wpi, 3 at 3 wpi, and 2 at 4 wpi as the number of surviving chickens decreased. The serum samples from the challenged group only showed significantly higher OD values than the negative control group at 3 wpi using peptide-ELISAs (**Figure 4C**) but significantly higher OD

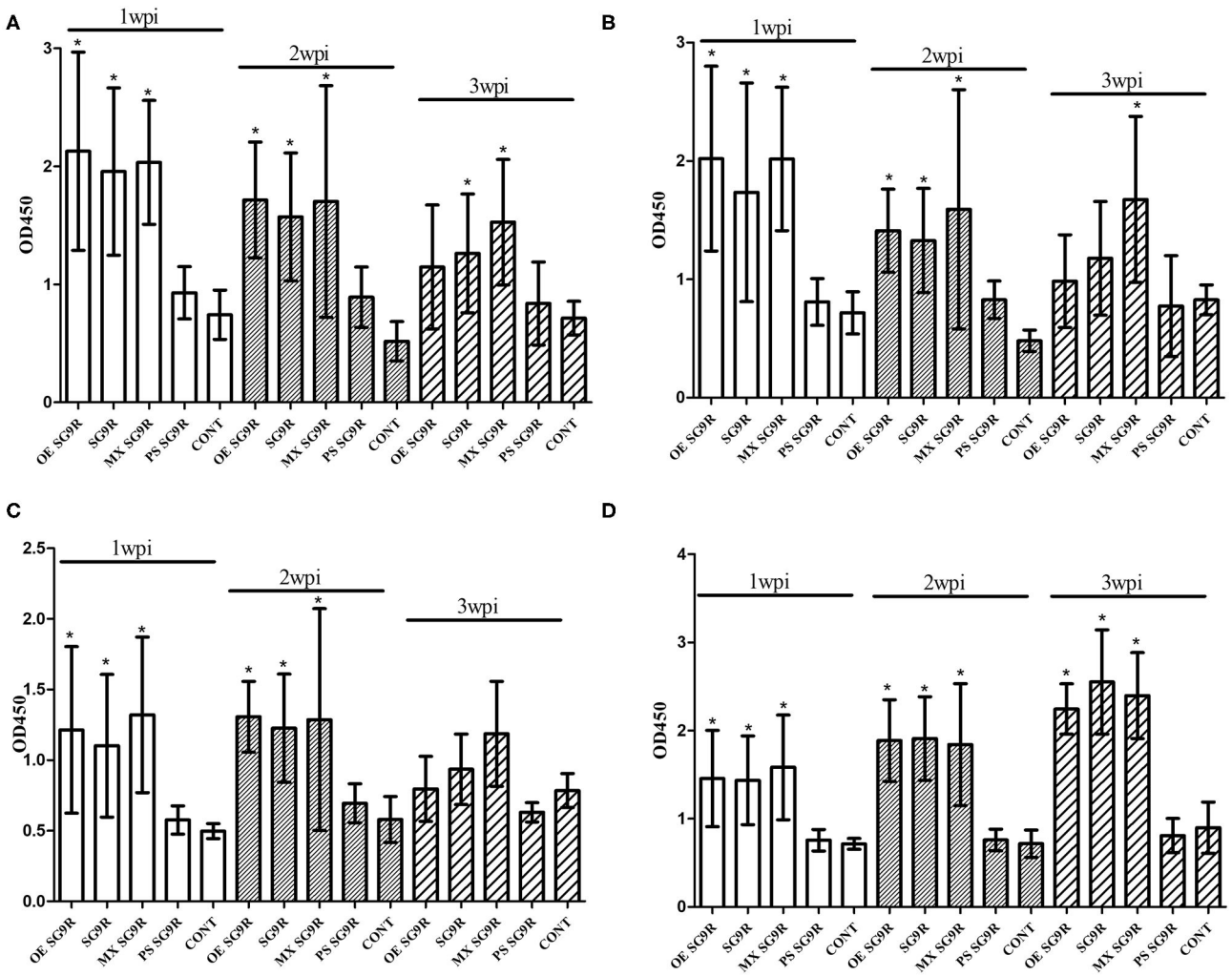

FIGURE 3 | Humoral immune responses measured using ELISAs under various conditions (mean with SD): OmpA-N-L3 **(A)**, OmpA-N-L4 **(B)**, OmpX-L2 **(C)**, and OMP-ELISA **(D)**. SG 9R was inoculated as killed adjuvanted (OE), live, mixed with live and killed (MX), or killed bacteria in a PBS suspension (PS) and compared with the negative control (CONT). *Indicates a significant difference compared with CONT [*P*-values - **(A)** OE SG 9R at 1 wpi (0.000) and 2 wpi (0.000); SG 9R at 1 wpi (0.000), 2 wpi (0.000), and 3 wpi (0.048); MX SG 9R at 1 wpi (0.000), 2 wpi (0.000), and 3 wpi (0.000); **(B)** OE SG 9R at 1 wpi (0.001) and 2 wpi (0.000); SG 9R at 1 wpi (0.019) and 2 wpi (0.000); MX SG 9R at 1 wpi (0.000), 2 wpi (0.001), and 3 wpi (0.022); **(C)** OE SG 9R at 1 wpi (0.000) and 2 wpi (0.000); SG 9R at 1 wpi (0.000) and 2 wpi (0.000); MX SG 9R at 1 wpi (0.000) and 2 wpi (0.006); **(D)** OE SG 9R at 1 wpi (0.013), 2 wpi (0.000), and 3 wpi (0.000); SG 9R at 1 wpi (0.009), 2 wpi (0.000), 3 wpi (0.000); MX SG 9R at 1 wpi (0.008), 2 wpi (0.004), and 3 wpi (0.000)].

values at 3 and 4 wpi than the negative control group using the OMP-ELISAs (**Figure 4D**).

Comparison of Mucosal Immunity Against Various SG Antigens

The anti-OE SG 9R, anti-SG 9R, anti-mixed SG 9R, anti-PS SG 9R, anti-OE SR2-N6, and anti-OE SG002 IgA levels in bile juice samples were not significantly different from the negative control using peptide- and OMP-ELISAs (**Figures 5A–D**).

The inoculation of SG0197 in 8-w-o male brown layer chickens did not cause mortality in either the SG 9R vaccine or no vaccine (CONT) group at 2 wpi. The SG 9R vaccine and no vaccine groups did not display different OD values for the peptide-ELISA (**Figures 5E–G**). However, the SG 9R vaccine group showed significantly higher OD values than the no vaccine group using the OMP-ELISA ($P < 0.05$) and O-Ag-ELISA (**Figures 5H,I**).

Humoral Immunity Against SG in the Field

When the field serum samples were tested using peptide-ELISAs, L12W showed higher OD values than the other samples, although the differences were not significant. The OMP-ELISA revealed significantly higher OD values in the L12W, L19W, and L41W groups than in the negative control (**Figure 6D**).

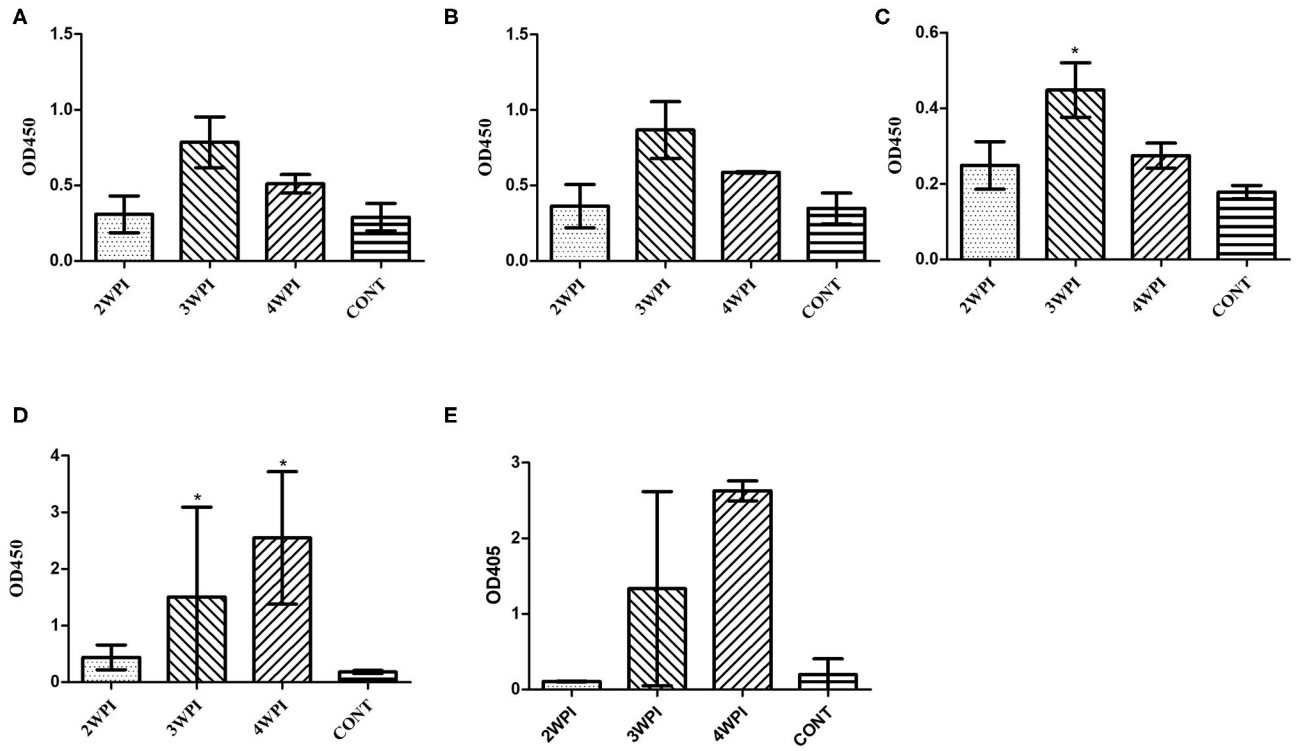

FIGURE 4 | Evaluation of humoral immunity in response to challenge with a field strain (mean with SD) using the OmpA-N-L3 **(A)**, OmpA-N-L4 **(B)**, OmpX-L2 **(C)**, OMP-ELISA **(D)**, and *Salmonella* D group O-Ag ELISA **(E)**. The field strain SG0197 was infected *per os* at 4 weeks of age, and serum samples were collected weekly for 4 weeks. *Indicates a significant difference compared with CONT [*P*-values - **(C)** 3 wpi (0.016); **(D)** 3 wpi (0.036) and 4 wpi (0.017)].

DISCUSSION

Fowl typhoid vaccines are categorized into live attenuated and inactivated vaccines, and live attenuated vaccine strains are subdivided into rough and smooth strains (15–17). As O-Ag of LPS hides OMPs and induces strong activation of specific B cells, the immunogenicity of OMPs of smooth strains may be less than rough strains (18). Our western blotting results from 2D-gel electrophoresis with whole bacterial lysates revealed predominant humoral immunity to O-Ag, and the results of the peptide- and OMP-ELISAs of SG002 and SR2-N6 supported the hypothesis that OMPs of the rough strain are more immunogenic than OMPs of the smooth strain (**Figures 1**, **2**). The increased immunogenicity of OMPs may be due to unrestricted exposure to B cells without shielding by O-Ag and the absence of a competing strong immunogen monopolizing most of the resources of humoral immunity. Considering the already improved protective efficacy of OMP vaccines and antigenic conservation among Gram-negative bacteria, the potential value of rough strains to become universal vaccines needs to be demonstrated in future studies (19).

SG 9R has been used worldwide due to its better protection efficacy, but the humoral immune responses to live, killed and a mixture of live, and killed SG 9R have never been compared. The significantly lower immunogenicity of PS SG 9R than SG 9R was unexpected because a killed rough strain of *Salmonella* serovar Typhimurium generated a higher antibody titer than the live rough strain (18). We killed the bacteria at 65°C for 2 h, while the authors of the previous used 100°C for 30 min and subsequent treatment with 1% human serum albumin and 0.16% formaldehyde. Therefore, the additional treatment with albumin and formaldehyde may have resulted in different results. Interestingly, a synergistic effect of PS SG 9R and live SG 9R was apparent and may reflect cooperative stimulation of humoral immunity by dead and live bacteria. Therefore, this new formulation without the use of the carcinogen formaldehyde may be useful to improve the protective efficacy of conventional live vaccines against infection with virulence variants recently detected in the field (20).

The oil adjuvant significantly increased the immunogenicity of OE SG 9R compared with PS SG 9R and generated similar serum antibody titers to SG 9R. Because of its potential pathogenicity, SG 9R vaccination of chickens aged <6 w-o is not recommended (8, 21, 22). Because the highest susceptibility and likelihood of infection are observed during the prevaccination ages (1 d-o to 6 w-o or age before vaccination), clinical measures, including early inoculation with adjuvanted killed vaccines, can be considered to protect chicks from vertical and/or horizontal transmission of SG.

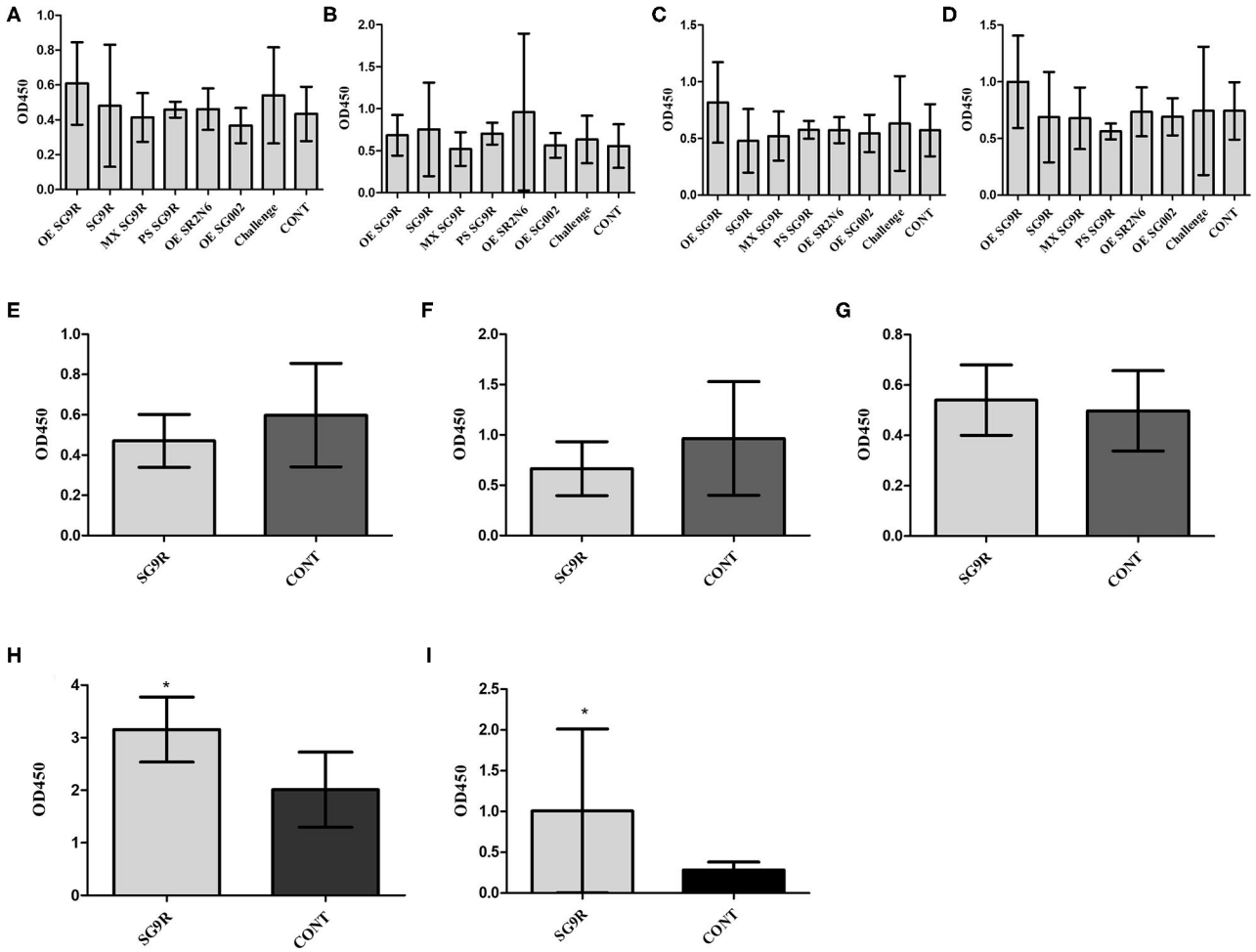

FIGURE 5 | The mucosal immunity of bile was measured with IgA secondary antibody with the same ELISA (mean with SD): OmpA-N-L3 **(A,E)**, OmpA-N-L4 **(B,F)**, OmpX-L2 **(C,G)**, OMPs **(D,H)**, and O-Ag IgA ELISA **(I)**. Birds were inoculated with the vaccine at 3 weeks of age, and bile juice was collected at 3 wpi **(A–D)**. Eight-week-old brown layer chickens with or without SG 9R vaccination were challenged with SG0197 at 6 weeks of age, and bile juice samples were collected at 10 weeks of age **(E–I)**. *Indicates a significant difference compared with CONT [*P*-values - **(H)** (0.001) and **(I)** (0.001)].

As invasive *Salmonella enterica* serovars penetrate the cell as an intracellular pathogen, researchers assumed that live bacteria might not produce sufficiently high titers of specific antibodies in the bloodstream. SG did not stimulate the initial immune response via proinflammatory cytokines or chemokines due to the absence of flagella, and the ability of SG to evade the immune system was very remarkable, even when the systemic infection had progressed (23, 24). Consistent with previous reports, infection with a field strain might delay humoral immunity by ∼2 weeks compared with SG 9R inoculation (**Figure 4**) and might result in insignificant mucosal antibody levels compared with the negative control (**Figure 5**). However, we should consider their different routes of infections. The observation that surviving chickens mounted antibodies against OmpX peptides and OMPs may support the importance of humoral immunity in survival. Additionally, a significant increase in the IgA titer in the bile juice of SG 9R-vaccinated chickens may support the importance of humoral immunity induced by SG 9R vaccination (**Figure 5**). Because most commercial layer farms inoculate animals with SG 9R vaccines, testing IgA levels in bile juice, intestinal washes and feces may be useful for the differential diagnosis of FT and an estimation of the risk of SG exposure.

Considering temporary increases in OD values obtained from peptide-ELISAs during the first 2 weeks after SG vaccine inoculations, the higher OD value of L12W may be related to the SG 9R vaccination at 10 weeks of age (**Figure 6**). Thus, the peptide-ELISAs were able to detect specific antibodies induced by recent SG 9R vaccination under both experimental and field conditions. However, the OMP-ELISA revealed significantly higher antibody levels in vaccinated flocks than in unvaccinated flocks. All field samples were tested using the O-Ag ELISA, and no positive sample indicating a field strain or SE infection was examined (data not shown). Therefore, these assays may be useful to verify the efficacy of the inoculated vaccine and

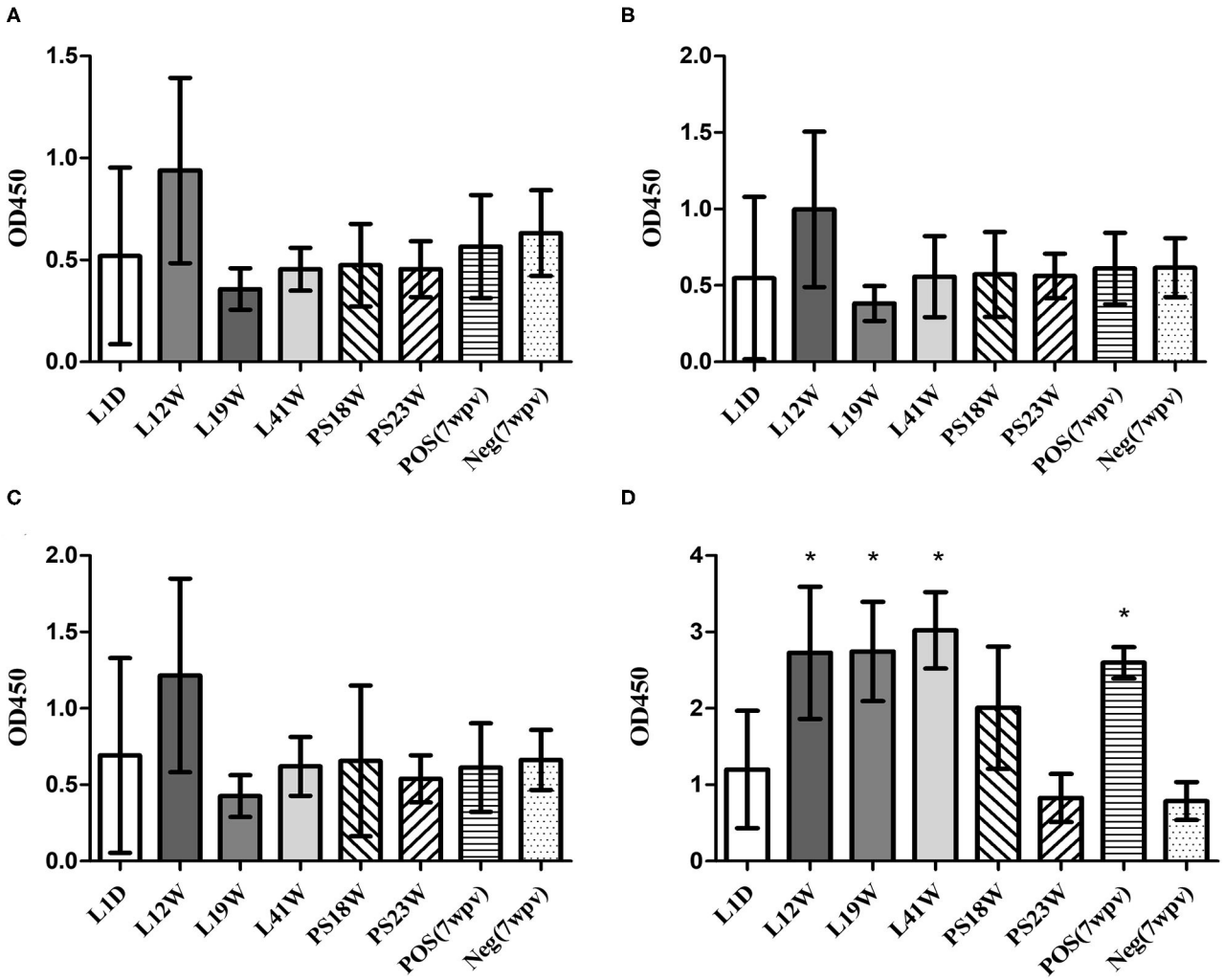

FIGURE 6 | Humoral immunity against SG in the field (mean with SD): OmpA-N-L3 **(A)**, OmpA-N-L4 **(B)**, OmpX-L2 **(C)**, and OMP-ELISA **(D)**. The field serum samples from the L1D (1 d-o), L12W (12 w-o, vaccination at 10 weeks of age), L19W (19 w-o, vaccination at 8 and 15 weeks of age), and L41W (41 w-o, vaccination at 7 and 16 weeks of age) groups were collected from laying hens, and the PS18W (18 w-o) and PS23W (23 w-o) samples were collected from parent stocks. *Indicates a significant difference compared with Neg. [*P*-values - **(D)** L12W (0.002), L19W (0.002), L41W (0.000), and POS (0.028)].

monitor unlawful vaccination with parent stocks in the field in combination with the O-Ag ELISA.

The immunogenic OMPs of *Salmonella enterica* have been reported, and OmpA and OmpX are known to be protective antigens (25–27). Although we selected OmpA and OmpX due to their immunodominance, the rapid but short-lived antibody responses induced by these antigens were unexpected and have not been reported. Additionally, our study is the first to investigate the kinetics of the production of specific antibodies against linear epitopes of OmpA and OmpX compared with OMPs. In summary, with the gradual increase in the titers of antibodies against OMPs over time, there may be other OMPs inducing long-lasting antibody responses. Although the immune dominance of the C-terminus of OmpA was reported previously, we could not find any difference between the results of C- and N-terminal peptide-ELISAs (**Supplementary Figure 1**) (28).

In conclusion, rough strains are better than smooth strains in terms of the immunogenicity of OMPs, and a mixture of a live and killed rough vaccine strains may potentiate the efficacy of the conventional live vaccine. The evasion of humoral immunity by the field strain was demonstrated again, but SG 9R may be useful to prime mucosal immunity against infection with a field strain. Additionally, combined serological tests with peptide, OMP, and O-Ag ELISAs may be useful for the differential diagnosis of FT in the field.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of BioPOA Co.

AUTHOR CONTRIBUTIONS

N-HK, H-JK, and K-SC substantially contributed to conceptualization, data curation, and analysis of the study. H-JK supervised all surveillance components. E-JH and D-SK contributed to analysis of data. N-HK prepared the initial draft, figures, and tables. N-HK and H-JK contributed to the writing and editing of the manuscript. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.598610/full#supplementary-material

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frontiers in Veterinary Science

ORIGINAL RESEARCH
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Immunization With a Combination of Four Recombinant *Brucella abortus* Proteins Omp16, Omp19, Omp28, and L7/L12 Induces T Helper 1 Immune Response Against Virulent *B. abortus* 544 Infection in BALB/c Mice

Tran Xuan Ngoc Huy[1,2†], *Trang Thi Nguyen*[2†], *Alisha Wehdnesday Bernardo Reyes*[2], *Son Hai Vu*[2], *WonGi Min*[2], *Hu Jang Lee*[2], *John Hwa Lee*[3] *and Suk Kim*[2*]

[1] *Institute of Applied Sciences, Ho Chi Minh City University of Technology - HUTECH, Ho Chi Minh City, Vietnam,* [2] *Institute of Animal Medicine, College of Veterinary Medicine, Gyeongsang National University, Jinju, South Korea,* [3] *College of Veterinary Medicine, Chonbuk National University, Iksan, South Korea*

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***Correspondence:**
Suk Kim
kimsuk@gnu.ac.kr

[†] *These authors have contributed equally to this work*

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Protective efficiency of a combination of four recombinant *Brucella abortus* (*B. abortus*) proteins, namely outer membrane protein (Omp) 16, Omp19, Omp28, and 50S ribosomal protein L7/L12 was evaluated as a combined subunit vaccine (CSV) against *B. abortus* infection in RAW 264.7 cell line and murine model. The immunoreactivity of these four recombinant proteins as well as pCold-TF vector reacted with *Brucella*-positive serum individually, but not with *Brucella*-negative serum by immunoblotting assay. CSV-treated RAW 264.7 cells significantly induced production of IFN-γ and IL-12 while decreased IL-10 production at the late stage of infection compared to PBS-treated control cells. In addition, the enhancement of nitric oxide production together with cytokines secretion profile in CSV-treated cells proved that CSV notably activated bactericidal mechanisms in macrophages. Consistently, mice immunized with CSV strongly elicited production of pro-inflammatory cytokines TNF-α, IL-6 and MCP-1 compared to PBS control group. Moreover, the concentration of IFN-γ was >IL-10 and titers of IgG2a were also heightened compared to IgG1 in CSV-immunized mice which suggest that CSV induced predominantly T helper 1 T cell. These results suggest that the CSV used in the present study is a potential candidate as a preventive therapy against brucellosis.

Keywords: *Brucella abortus*, combined subunit vaccine, T helper 1 T cell, humoral immunity, macrophages

INTRODUCTION

Brucellosis remains an extremely common zoonotic disease worldwide caused by *Brucella* species that are designated as category B of potential bioterrorism agents. This potential is due to the various biological and pathogenic characteristics of *Brucella* species including being infectious via the aerosol route, being notoriously debilitating disease, having no safe and effective available vaccine for humans as well as requiring prolonged antibiotic treatment and having relapse rates

of 5–10% after successful treatment (1, 2). In addition, *Brucella* is an intracellular pathogen which calls for the use of intracellular-acting antibiotics which are limited. Furthermore, the most effective regimens and treatment durations are still controversial (3). Therefore, significant research efforts have been carried out to seek and develop new and better therapies against *Brucella* infection.

Recently, there are numerous advances in immunology, genomics, proteomics, biochemistry as well as recombinant technology that have been utilized in the development of subunit vaccines through recombinant proteins (4). This kind of vaccine is able to reduce drawbacks of live attenuated vaccines including reversion to virulence, abortion in pregnant animals and infection to humans (5). Consequently, more studies have been reported on the protective efficiency of recombinant *Brucella* proteins as subunit vaccine against *Brucella* infection such as Outer membrane protein (Omp) 28, 50S ribosomal protein (L7/L12), Omp16, Omp19, lumazine synthase, etc… (6–9). Interestingly, combined subunit vaccine (CSV) using more than two recombinant proteins recently has been reported to confer higher potential immune response against *Brucella* infection than single subunit vaccine (10–13). Among them, recombinant proteins L7/L12 and Omps were considered as potential immunogens and demonstrated to induce strong protective effects against *Brucella* infection as well as others bacterial infections. Therefore, in this study, we evaluated the ability of a combination of four *B. abortus* recombinant proteins L7/L12, Omp16, Omp19, Omp28 as a CSV to induce immune response against *B. abortus* infection in RAW 264.7 cell line and BALB/c mouse models.

MATERIALS AND METHODS

Bacterial Strains and Cell Culture

The smooth, virulent, wild-type *B. abortus* 544 biovar 1 strain (ATCC 23448) was cultured in Brucella broth at 37°C until stationary phase. *B. abortus* RB51 vaccine strain was used as positive control. *E. coli* DH5α was purchased from Invitrogen. *E. coli* cultures grown at 37°C in LB broth or agar supplemented with 100 μg/mL of ampicillin were used for expression of recombinant proteins. RAW 264.7 cells (ATCC, Rockville, USA) were grown at 37°C in 5% CO_2 atmosphere in RPMI 1640 containing 10% (vol/vol) heat-inactivated FBS with or without antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) depending on the experiment.

Recombinant Protein Expression and Purification

Preparation and expression of plasmids and purified recombinant proteins were obtained as described previously (13). Briefly, the fully coded sequences of the four *B. abortus* genes *Omp16*, *Omp19*, *Omp28,* and *rplL* were amplified using their respective primer pairs (**Table 1**). The individual amplified DNA fragments were cloned into a pCold-trigger factor (pCold-TF) vector. Recombinant proteins, namely rOmp16, rOmp19, rOmp28, and rL7/L12 were expressed in *E. coli* DH5α, and were purified using HisTALON gravity columns purification kit. The expression and immunoreactivity of these recombinant proteins were analyzed by SDS-PAGE and western blot assay, respectively.

Mice Immunization and Bacterial Challenge

Twenty 12-week-old female BALB/c mice were distributed into four groups of five mice each. Each animal was intraperitoneally (IP) injected with a mixture of incomplete Freund's adjuvant (IFA) (Sigma-Aldrich, USA) and 100 μg of a combination of rOmp16, rOmp19, rOmp28, and rL7/L12 at a ratio of 1:1:1:1 in a total volume of 200 μL at weeks 0, 2, and 5. The other two groups were injected IP with phosphate buffered saline (PBS) or pCold-TF (100 μg) combined with IFA in a total volume of 200 μL at weeks 0, 2, and 5. Mice group used as positive control was IP immunized with 1×10^6 CFUs of vaccine strain *B. abortus* RB51 in 100 μL PBS at day 0. Serum samples were collected via tail vein from all mice at week 7 after the first immunization to evaluate cytokine levels as well as IgG1 and IgG2a production. At week 7, mice were IP challenged with

TABLE 1 | Primer sequences used for cloning *B. abortus* genes *Omp16*, *Omp19*, *Omp28,* and *rplL*.

Gene	Forward primer	Reverse primer	Restriction enzyme (forward)	Restriction enzyme (reverse)
Omp16	5′-CCC GGATCC[a] ATGCGCCGTATCCAGTCGATT-3′	5′-ACC AAGCTT TTACCGTCCGGCCCCGTTGAG-3′	*Bam*HI	*Hind*III
Omp19	5′-AGCA GGATCC ATGGGAATTTCAAAAGCAAG-3′	5′-ATA CTGCAG TCAGCGCGACAGCG-3′	*Bam*HI	*Pst*I
Omp28	5′-GATC GGATCC AACACTCGTGCTAGCAATTTT-3′	5′-GATC AAGCTT TTACTTGATTTCAAAAACGAC-3′	*Bam*HI	*Hind*III
rplL	5′-AGC TCTAGA A TGGCTGATCTCGCAAAGATC-3′	5′-ATC CTGCAG CTTACTTGAGTTCAACCTTGGC-3′	*Xba*I	*Pst*I

[a] *Enzyme recognition sequences are underlined.*

approximately 2 × 10^5 CFUs of *B. abortus* 544 virulent strain in 100 μL PBS.

Cytokine and Antibody Measurement From Serum Samples

The levels of IL-10, IFN-γ, TNF-α, IL-6, MCP-1 and IL-12p70 in serum samples were determined using a cytometric bead array kit (BD CBA Mouse Inflammation Kit, USA) and analyzed using a FACSCalibur flow cytometer.

The CSV-specific antibody titers IgG1 and IgG2a were measured using indirect ELISA. Briefly, the immunoassay 96-well plates were coated with 100 μL of a combination of four recombinant proteins (6 μg/mL) at a ratio of 1:1:1:1 in coating buffer (50 mM carbonate-bicarbonate coating buffer, pH 9.6) per well at 4°C, overnight. CSV-coated plates were washed, blocked, incubated with serial dilutions of sera, then with secondary antibody and results were analyzed following the previous method (13).

Cytokine and NO Production in RAW 264.7 Cells

Overnight culture of RAW 264.7 cells at a concentration of 2 × 10^5 cells per well in 96-well culture plates were pre-treated with lipopolysaccharide (LPS), pCold-TF or CSV for 4 h with PBS as control. The cells were washed with PBS, incubated in fresh medium (RPMI 1640 with 10% heat-inactivated FBS) and then infected with *B. abortus* at multiplicity of infection of 50. The cells were centrifuged at 150 × *g* for 10 min and incubated at 37°C in 5% CO_2 for 1 h. Further, the cells were washed and added with fresh medium containing 50 μg/mL of gentamicin and treated with LPS, pCold-TF or CSV for 4, 24 and 48 h. At different time points (4, 24, and 48 h), 50 μL of cell culture supernatant from each well was collected to evaluate cytokine production using a cytometric bead array kit

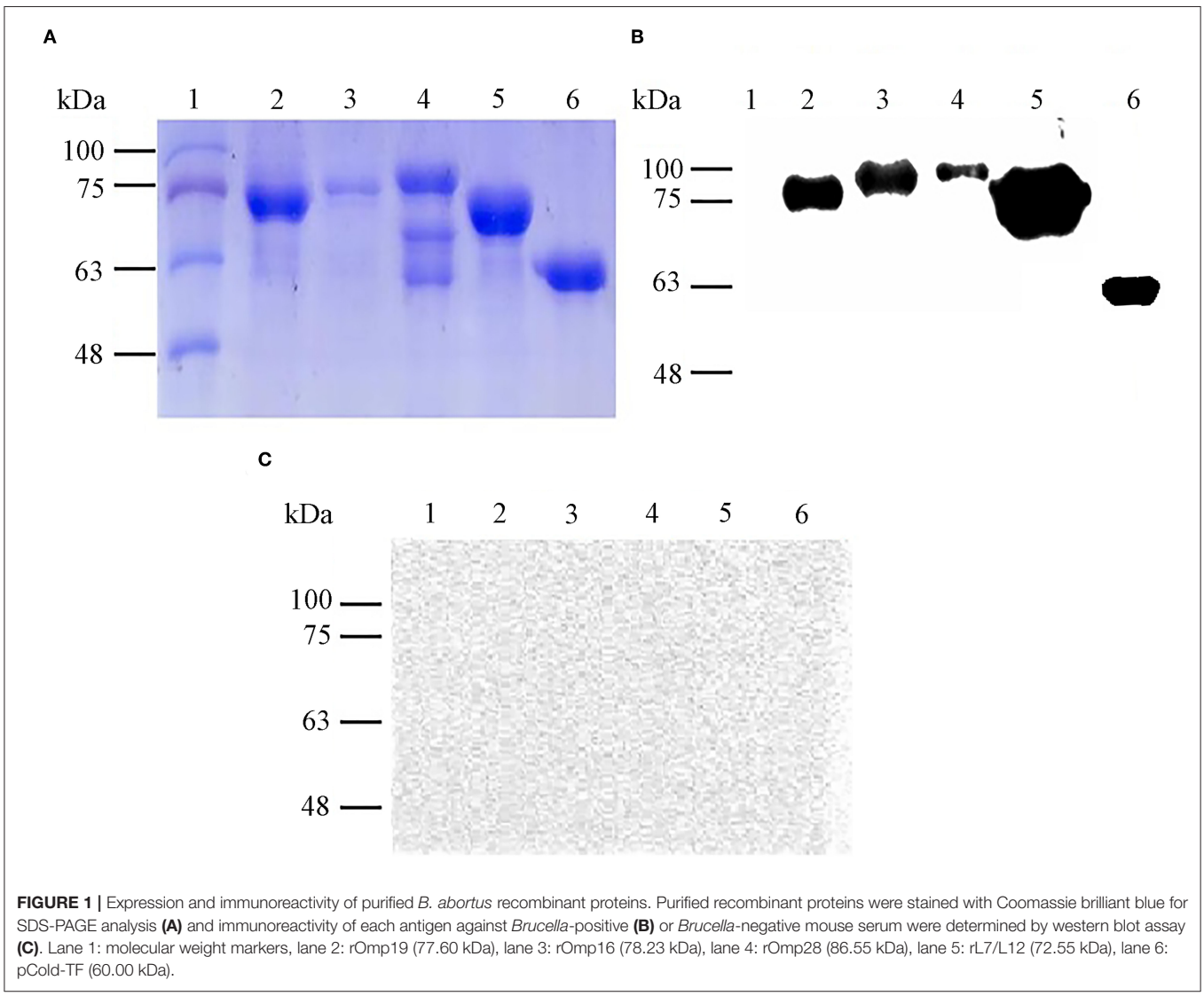

FIGURE 1 | Expression and immunoreactivity of purified *B. abortus* recombinant proteins. Purified recombinant proteins were stained with Coomassie brilliant blue for SDS-PAGE analysis **(A)** and immunoreactivity of each antigen against *Brucella*-positive **(B)** or *Brucella*-negative mouse serum were determined by western blot assay **(C)**. Lane 1: molecular weight markers, lane 2: rOmp19 (77.60 kDa), lane 3: rOmp16 (78.23 kDa), lane 4: rOmp28 (86.55 kDa), lane 5: rL7/L12 (72.55 kDa), lane 6: pCold-TF (60.00 kDa).

(BD CBA Mouse Inflammation Kit, USA) which was analyzed using a FACSCalibur flow cytometer. Another 50 μL of cell culture supernatant was collected to measure nitric oxide (NO) production using Griess reagent system (Promega, USA) according to the manufacturer's instruction.

In vivo Bacterial Clearance Efficiency Assay

Two weeks after infection, all mice were sacrificed, and the spleens were collected, weighed, and homogenized in PBS. The homogenized spleens were serially diluted, plated on Brucella agar and incubated at 37°C for 3 days. The number of CFU per spleen was counted. Unit of protection was calculated as the mean $\log_{10}$ CFU of PBS group minus $\log_{10}$ CFU of vaccinated group.

Statistical Analysis

The results for each of experiment are expressed as the mean ± standard deviation (SD). Data were analyzed by GraphPad InStat using unpaired, two-tailed Student's *t*-test. Results with $P < 0.05$ were considered statistically significant.

RESULTS

Protein Purification and Immunoreactivity of Recombinant Proteins

By Coomassie brilliant blue staining, the approximate molecular masses of purified proteins rOmp19, rOmp16, rOmp28, rL7/L12, and pCold-TF were 77.60 kDa, 78.23 kDa, 86.55 kDa, 72.55 kDa, and 60.00 kDa, respectively (**Figure 1A**). On the other hand, the immunoreactivity of all purified proteins was measured by western blot assay. The results showed that these proteins reacted with *Brucella*-positive mouse serum and even with pCold-TF (**Figure 1B**) but all of them did not react with *Brucella*-negative serum (**Figure 1C**).

Cytokine and NO Production in RAW 264.7 Cells Culture Supernatant

Macrophages are one of the first line of innate immune response against invading *Brucella* mediated by releasing an impressive panel of cytokines (14). Herein, cytokines production from cell culture supernatant was measured using flow cytometry assay. At the early stage of infection (4 h post infection), LPS-treated cells strongly produced 1.21-fold increase in IFN-γ and 4.51-fold increase in IL-12 but 2.27-fold decrease in IL-10, whereas no difference was observed in CSV-treated cells compared to PBS-treated cells. Interestingly, CSV-treated cells significantly induced 1.14-fold higher in IFN-γ and 1.98-fold higher in IL-12 production at 24 h post infection and continuously increased at 48 h, conversely, IL-10 level showed 6.14-fold and 4.26-fold decrease at 24 and 48 h post infection, compared to PBS-treated cells, respectively (**Figure 2**).

NO has been proven to control *Brucella* infection in macrophages (15). Therefore, in this study, NO production was measured to evaluate the antimicrobial mechanism employed by macrophages to combat *Brucella* infection. There was no significant difference in NO production at the early stage of infection (4 h post infection) in all treated and untreated cells. However, it was noteworthy at the late infection (24 and 48 h post infection) that all treated cells increased NO production compared to control cells whereas LPS-treated cells were the most predominant in NO production. Notably, at 48 h post infection NO level was observed to be continuously increased in CSV-treated cells and 1.47-fold higher than pCold-TF-treated cells (**Figure 3**).

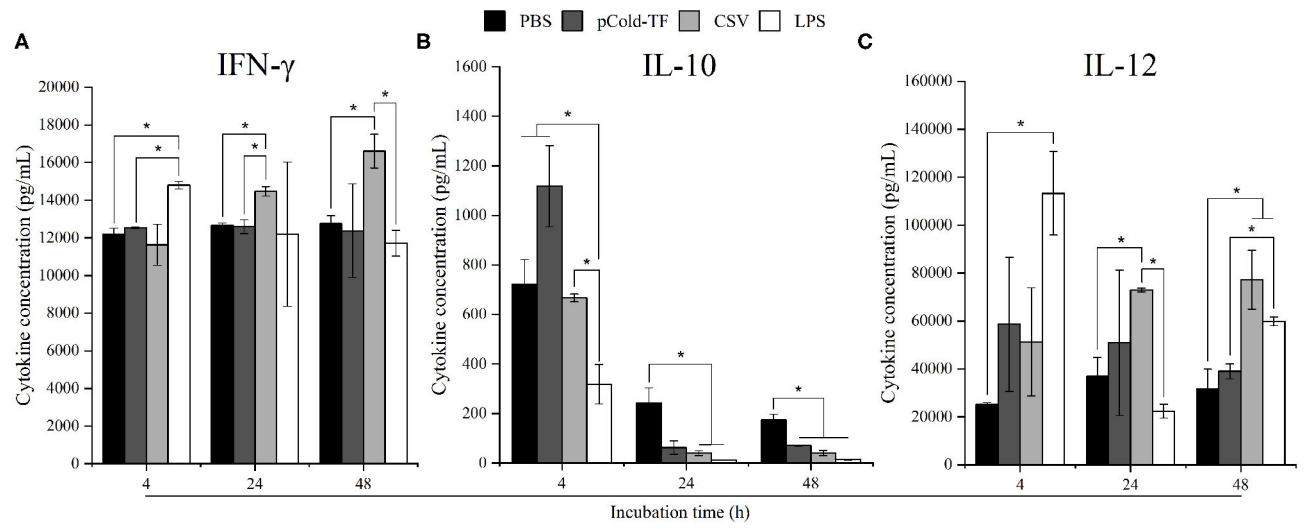

FIGURE 2 | Cytokine concentration in RAW 264.7 cell culture supernatants. RAW 264.7 cells were pre-treated with LPS, pCold-TF or CSV for 4 h prior to infection. After infection, 50 μL of cell culture supernatant was collected and analyzed for cytokine production including IFN-γ **(A)**, IL-10 **(B)** and IL-12 **(C)**. The data are represented as the mean ± SD of duplicate samples from at least two independent experiments. Asterisks indicate statistically significant differences (*$P < 0.05$).

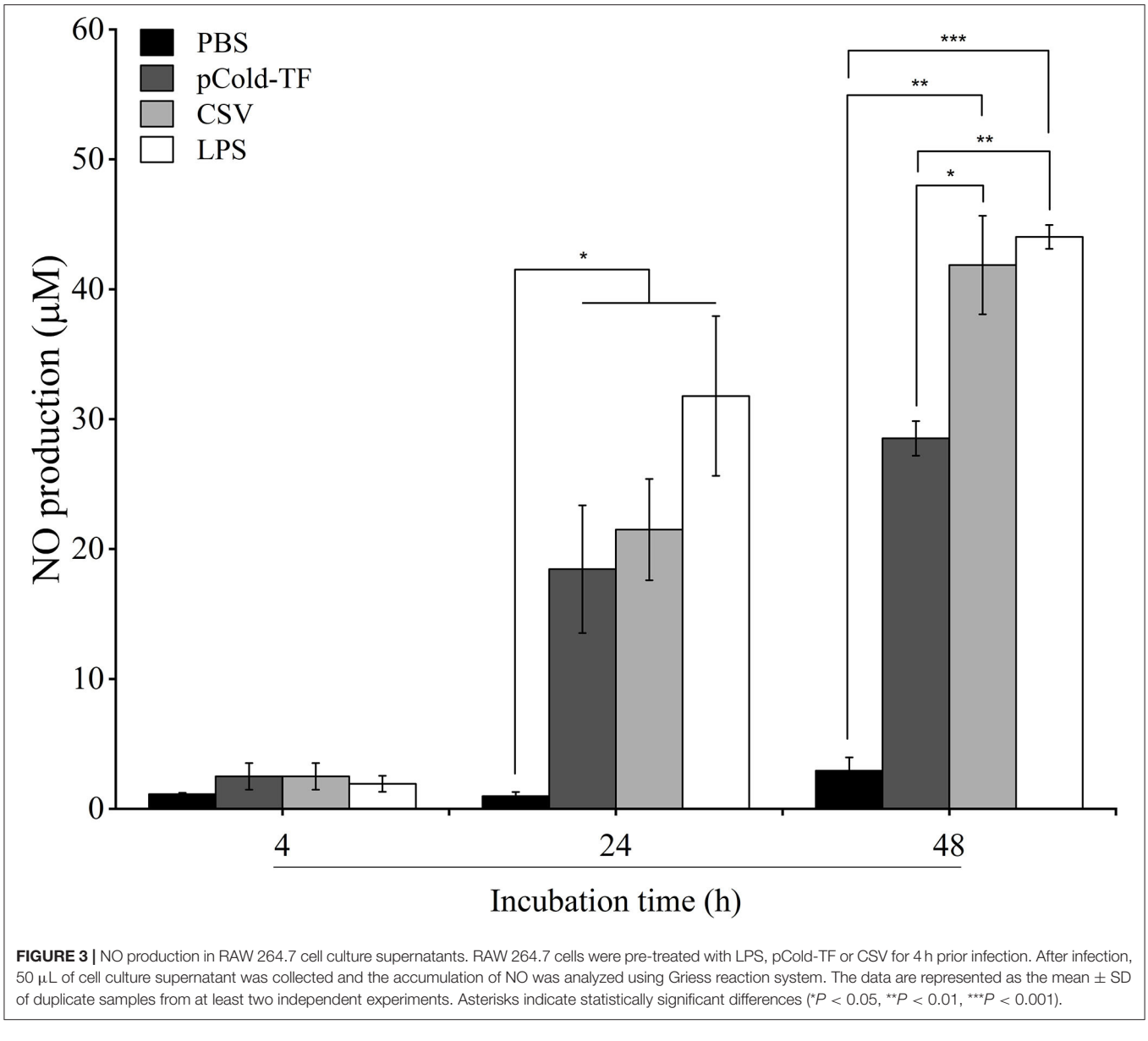

FIGURE 3 | NO production in RAW 264.7 cell culture supernatants. RAW 264.7 cells were pre-treated with LPS, pCold-TF or CSV for 4 h prior infection. After infection, 50 μL of cell culture supernatant was collected and the accumulation of NO was analyzed using Griess reaction system. The data are represented as the mean ± SD of duplicate samples from at least two independent experiments. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Cytokine Secretion Analysis in Serum Samples

At week 7 after the first immunization, sera were collected from all mice and cytokines levels were analyzed. CSV-immunized mice produced 1.65, 4.24, and 4.86-fold increases of pro-inflammatory cytokines TNF-α, IL-6 and MCP-1, respectively compared to PBS group. Moreover, CSV group displayed considerably increased IFN-γ production by 1.71-fold and decreased IL-10 production by 2.10-fold compared to PBS. Besides, CSV group elicited higher IFN-γ levels than IL-10 levels of approximately 1.56-fold. On the other hand, mice immunized with pCold-TF vector showed induced enhancement of TNF-α, IL-6 and MCP-1 levels compared to PBS group as well as induced IL-10 level that is ∼1.43-fold higher than the IFN-γ level. Whereas, attenuated vaccine group RB51 remarkably induced highest IFN-γ level which is known to plays critical role in fighting against *Brucella* infection (**Figure 4**).

Induction of Humoral Immunity by Eliciting Specific IgG1 and IgG2a Antibody in Immunized Mice

Specific IgG1 and IgG2a antibodies produced by B lymphocytes, play substantial role in neutralization and opsonization which facilitate the phagocytosis of *Brucella* by some professional phagocytes. In this study, ELISA was utilized to measure the presence of CSV-specific IgG1 and IgG2a antibodies in the serum samples. The results showed that CSV group induced the highest IgG1 and IgG2a production in which the IgG2a/IgG1 ratio was 1.02. Besides, pCold-TF group displayed increased production of CSV-specific IgG1 and IgG2a compared to PBS group with

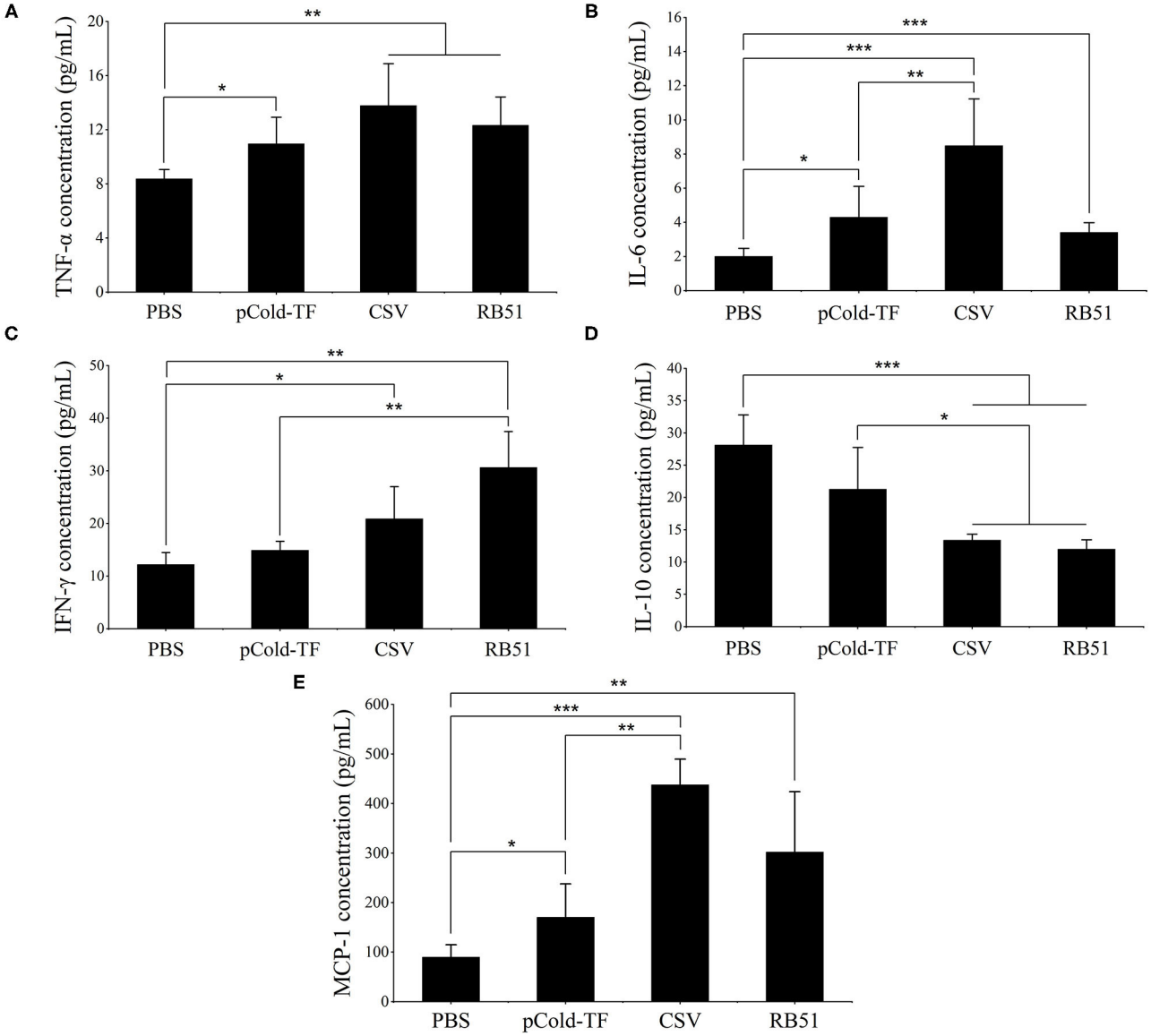

FIGURE 4 | Cytokine concentration in the sera of immunized mice. Concentration of TNF-α **(A)**, IL-6 **(B)**, IFN-γ **(C)**, IL-10 **(D)** and MCP-1 **(E)** were analyzed using cytometric bead array. The data are represented as the mean ± SD of each group of five serum samples. Asterisks indicate statistically significant differences (*$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$).

IgG1/IgG2a ratio of approximately 1.02. On the other hand, RB51 group induced higher production of IgG2a than IgG1 by 1.37-fold (**Figure 5**).

Protection Against *B. abortus* in Immunized Mice

After three rounds of immunization, vaccinated and control mice were challenged by IP infection with *B. abortus*. Furthermore, CSV group conferred significant degree of protection with 1.41- and 1.26-log unit of protection compared to control mice receiving PBS and pCold-TF, respectively. RB51-immunized mice displayed highest degree of protection, approximately 1.70-log protection than PBS group. Mice immunized with pCold-TF vector exhibited induced 0.15-log protection compared to PBS group but not significant (**Figure 6**, **Table 2**).

DISCUSSION

Brucella has the ability to survive, replicate and persist within professional and non-professional phagocytes. It is able to avoid degradation within phagolysosome fusions, reaching its safe haven-endoplasmic reticulum (ER). Once safely residing in ER, *Brucella* is able to evade bactericidal mechanisms within

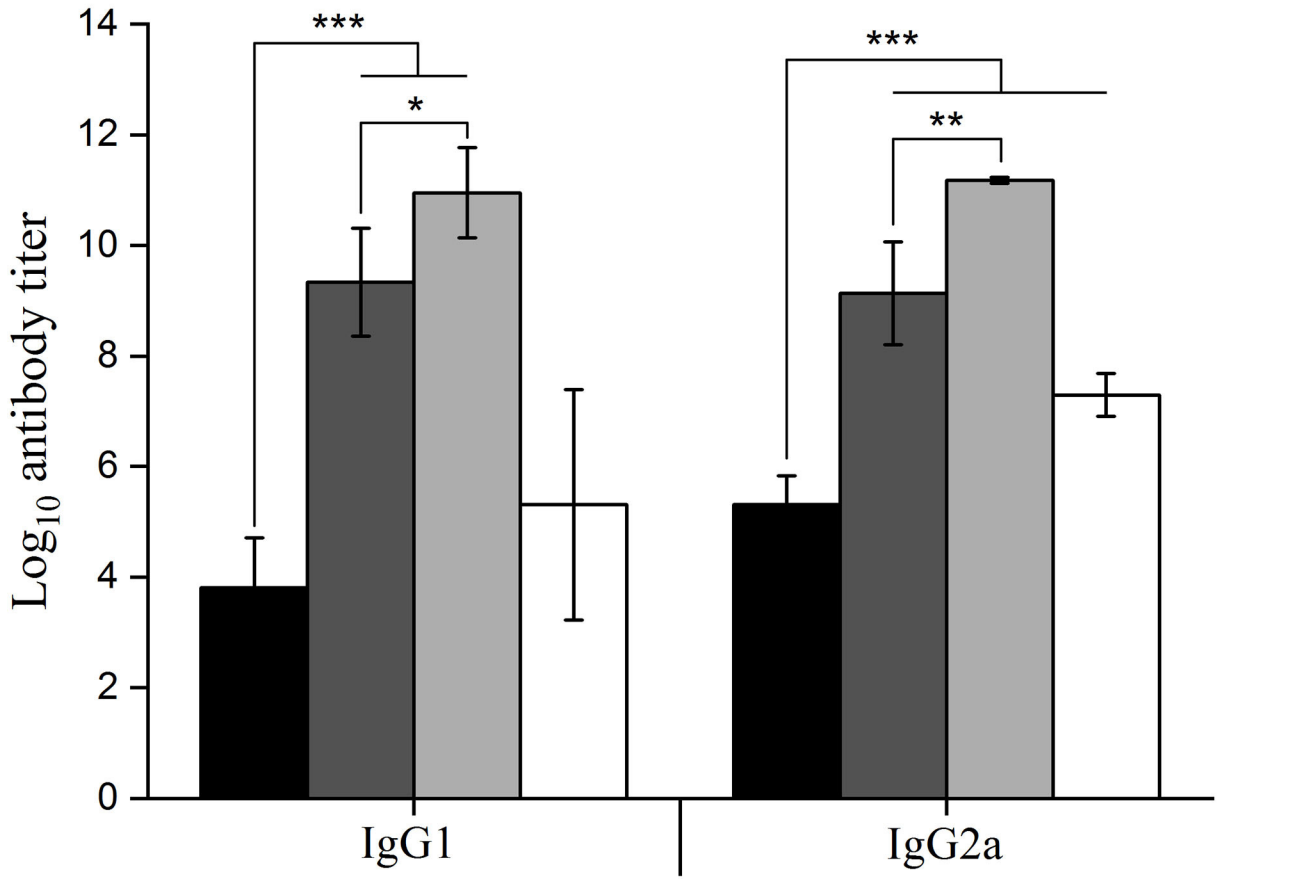

FIGURE 5 | IgG1 and IgG2a antibodies production in the sera of mice immunized with different vaccines. Sera from CSV, pCold-TF, RB51, or PBS-immunized mice were collected at week 7 after the first immunization. CSV-specific IgG1 and IgG2a antibodies titer were determined by ELISA. The data are represented as the mean ± SD of each group of five serum samples. Statistically significant differences are indicated by an asterisk (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

the professional phagocytes as well as pursuit of the humoral immunity (16). On the other side, the complex immune systems of mammals have evolved over vast periods of time when facing battles against pathogens. Among them, activated macrophages, dendritic cells, $CD4^+$ and $CD8^+$ T cells as well as various macrophages, dendritic cells, T helper (Th) 1-derived cytokines are predominant in protection against *Brucella* which possesses stealthy strategies to serve its intracellular nature (17).

Recently, growing numbers of studies have been widely carried out toward an ideal vaccine that would be able to prevent abortion in immunized host, bacterial infections in both immunized and non-immunized host and virulence reversion, and be able to promote long periods of protection with less doses and to be produced in large scale with low cost (18). Subunit vaccine using recombinant proteins was considered as an alternative preventive therapy which is able to fulfill the requirements of an ideal vaccine. Ribosomal protein L7/L12 functionally constitutes 50S ribosome encoded by *rplL* gene and plays an important role in controlling protein translational accuracy (19). Notably, it was demonstrated to be a known immunodominant antigen that stimulate strong immunity against *Brucella* infection (20, 21). The other agents of this study are Omps, essential to bacterial physiology and antibiotic resistance ability (22). *Brucella* Omps are classified based on their apparent molecular mass including group 1 (∼88-94 kDa), group 2 (∼41-43 kDa) and group 3 antigens (∼30 kDa). Among them, Omp28 belongs to group 3. Two other Omps identified as lipoproteins are Omp16 and Omp19 (23, 24). In addition, immunogenicity of rL7/L12, rOmp16 and rOmp19 were evaluated effectively against *B. suis* and *B. melitensis* (20, 25, 26). Therefore, in the present study, a combination of four recombinant proteins L7/L12, Omp16, Omp19, and Omp28 was hypothesized to have the ability to activate strong immune responses against *Brucella* infection in RAW 264.7 cell line and BALB/c mouse models.

At the onset of host immunity activation against invading pathogens, cytokine MCP-1 regulates the migration and

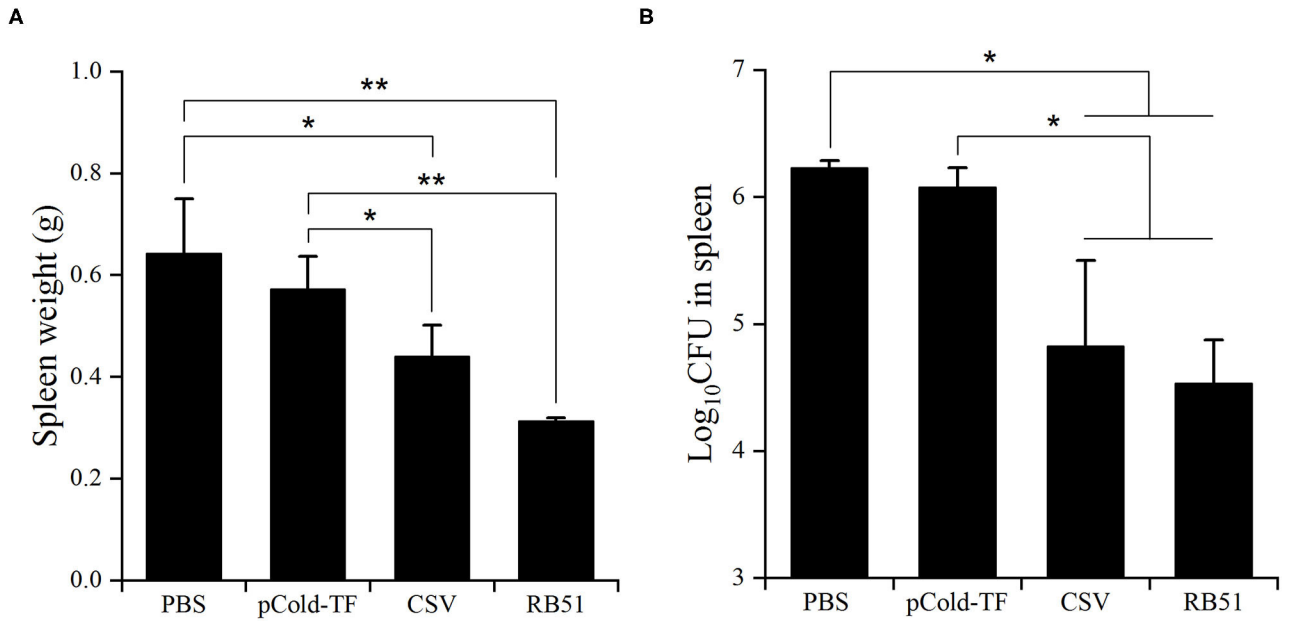

FIGURE 6 | Protection against *B. abortus* in BALB/c mice immunized with different vaccines. The average total weight of each spleen was measured **(A)** and the number of CFUs in each spleen were counted **(B)**. The data are represented as the mean ± SD of five mice. Asterisks indicate statistically significant differences (*$P < 0.05$, **$P < 0.01$).

TABLE 2 | Protection against *B. abortus* in BALB/c mice immunized with different vaccines.

Vaccine	Log_{10} CFU of bacteria in spleens (Mean ± SD)	Log protection	*P*-value[a]
PBS	6.23 ± 0.05		
pCold-TF	6.08 ± 0.15	0.15	
RB51	4.53 ± 0.34	1.70	$P < 0.05$
CSV	4.82 ± 0.68	1.41	$P < 0.05$

[a] *Significant different from PBS-immunized mice were estimated by Student's t-test.*

infiltration of macrophages, natural killer (NK) cells and T lymphocytes toward infection (27). In here, macrophages were described as sentinel of immunity, one of the first lines of innate immunity. They can display remarkable complexity of microbicidal functions including phagocytosis, phagolysosome fusion and production of reactive oxygen species (ROS), nitrite intermediates, antimicrobial peptides and degradative enzymes (28). The results in the present study showed that MCP-1 level in sera was observed to increase in CSV-immunized mice compared to PBS or pCold-TF group. In addition, production of two pro-inflammatory cytokines TNF-α and IL-6 was elevated in CSV group. These two pro-inflammatory cytokines are known as key effectors in mediating macrophages against *Brucella* infection. These cytokines promote phagolysosome fusion event as well as the production of killing effectors such as ROS, NO and lysosomal enzyme (29, 30). Furthermore, *in vitro* experiment showed significant enhancement of NO production in CSV-treated RAW 264.7 cells compared to PBS-treated cells during late stage of infection. These *in vitro* and *in vivo* experiments suggested that treatment with CSV could significantly initiate innate immunity, more particularly, activation of macrophages to elicit antimicrobial effectors that leads to restriction of an early infection.

In addition to playing a major role in innate immunity, macrophages can produce IL-12 to induce the activation of $CD4^+$ Th1 cells (31). Although IL-12 production in sera in CSV-immunized mice was not detected, CSV-treated RAW 264.7 cells induced IL-12 production compared to PBS group. This result showed that macrophages played a role as functional bridge between innate and adaptive immunity. After naive T cells were activated to differentiate into Th1 by macrophages-derived IL-12, Th1 consequently produces IFN-γ. This Th1-derived IFN-γ is a crucial cytokine in immune responses against *Brucella* infection with several important functions (32). The present results showed that not only CSV-immunized mice but also CSV-treated RAW 264.7 cells displayed increased production of IFN-γ compared to control. This is consistent with the previous studies, IFN-γ concentration was obviously up-regulated when the host was immunized with rL7/L12 or rOmp19 in context of *B. suis* or *B. melitensis* infection (20, 25). In contrast, the anti-inflammatory cytokine IL-10 is a marked cytokine for Th2 activity. It inhibits activity of Th1, NK cells and macrophages leading to increase resistance of *Brucella* infection (33, 34). Thus, ratio of IFN-γ and IL-10 can provide an immune system profile reflecting predominance of either Th1 or Th2. In this study, production of IL-10 was decreased in CSV-immunized mice and -treated RAW 264.7 cells in sera and culture supernatant, respectively.

Interestingly, at both *in vitro* and *in vivo* experiments, the concentration of IFN-γ was greater than the concentration of IL-10 in CSV-treated RAW 264.7 cells and immunized mice. These findings indicated that immunization with CSV induced Th1 T cells. Besides, humoral immune response mediated by antibodies assists in opsonisation of circulating *Brucella* in the blood of infected host. Elevated IgG1 and IgG2a antibodies production in sera in CSV-immunized mice were observed compared to PBS or pCold-TF-immunized mice. Collectively, alterations of cytokine and antibody profiles both *in vitro* and *in vivo* systems demonstrated that CSV effectively induced both Th1 and humoral immune responses.

Finally, the immunization with CSV conferred significant level of protection compared to PBS and pCold-TF groups. On the other hand, immunogenicity of pCold-TF was a notable result. It could react with *Brucella*-positive serum, induce production of TNF-α, IL-6 and MCP-1, whereas concentration of IFN-γ was <IL-10. This indicated that pCold-TF could induce Th2 immunity. Furthermore, this vector could elicit productions of IgG1 and IgG2a. The immunogenicity of this vector was primed by trigger factor component as reported by Cohen et al. (35) and Yang et al. (36). Although pCold-TF was able to elicit immune response, its protective effect against *Brucella* infection was not significant.

In conclusion, this study clearly indicated that immunization with a combination of four antigenic recombinant proteins rL7/L12, rOmp16, rOmp19, and rOmp28 could significantly induce Th1 immune response and humoral immunity, and provide superior protection effect against *Brucella* infection as compared to PBS and pCold-TF group. Although CSV could induce significantly protection effect, this study was conducted in a murine model which is not a natural host of *B. abortus*. Therefore, further investigations are needed to determine the practical efficacy of this vaccine using a bovine model which is considered as the natural host of this pathogen.

DATA AVAILABILITY STATEMENT

The original contributions generated for this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The method of animal handling and sacrifice conducted in this experiment was in accordance with established federal guidelines and institutional policies approved by the Animal Ethical Committee of Chonbuk National University (Authorization Number CBNU-2018-101).

AUTHOR CONTRIBUTIONS

TH designed and performed the experiments, analyzed data and wrote original draft. TN, AR, and SV acquired data and revised manuscript. WM, HL, and JL contributed to conception and resources. SK contributed to conception, manuscript review and revision and project administration. All authors contributed to the article and approved the submitted version.

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A Peptide-Based Enzyme-Linked Immunosorbent Assay for Detecting Antibodies Against Avian Infectious Bronchitis Virus

Liping Yin[1,2†], *Qi Wu*[1,3†], *Zhixian Lin*[1,3], *Kun Qian*[1,3,4], *Hongxia Shao*[1,3,4], *Zhimin Wan*[1,3,4], *Yuelong Liu*[1,2], *Jianqiang Ye*[1,3,4*] *and Aijian Qin*[1,3,4*]

[1] *Key Laboratory of Avian Preventive Medicine, Ministry of Education, Yangzhou University, Yangzhou, China,* [2] *JiangsuLihua Animal Husbandry Co., Ltd, Changzhou, China,* [3] *Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, China,* [4] *The International Joint Laboratory for Cooperation in Agriculture and Agricultural Product Safety, Ministry of Education, Yangzhou University, Yangzhou, China*

Infectious bronchitis virus (IBV) causes substantial loss to the poultry industry despite extensive vaccination. Assessing the antibody response is important for the development and evaluation of effective vaccines. We have developed an enzyme-linked immunosorbent assay (ELISA) for the detection of IBV-specific antibodies, using a synthetic peptide based on a conserved sequence in the IBV spike protein. This peptide-based ELISA (pELISA) specifically detects antibodies to different genotypes of IBV but not antibodies against other common chicken viruses. This assay could detect IBV-specific antibody response on as early as day 7 postinfection. In the testing with field serum samples collected from chickens administered with IBV vaccines, the sensitivity, specificity, and accuracy of pELISA were 98.30, 94.12, and 98.8%, respectively, relative to indirect immunofluorescence assay. Our data demonstrate that the pELISA is of value for the detection of IBV antibody and the evaluation of IBV vaccines.

Keywords: infectious bronchitis virus, pELISA, antibody, detection, chicken

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Vicky Lin van Santen, Auburn University, United States
Ahmed Ali, Beni-Suef University, Egypt

***Correspondence:**
Jianqiang Ye
jqye@yzu.edu.cn
Aijian Qin
ajian@yzu.edu.cn

† These authors have contributed equally to this work

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INTRODUCTION

Avian infectious bronchitis, a highly contagious disease, is caused by a coronavirus, that is, infectious bronchitis virus (IBV). The infection of IBV generally causes serious respiratory and renal diseases in broilers and lowers egg production in layers (1), resulting in significant economic loss in the poultry industry (2). Although the efficacy is far from optimal, vaccines represent one of the most effective tools for the control of IBV. As for other animal and human vaccines, assessment of antibody response is of key importance for IBV vaccine development.

The IBV genome encodes four structural proteins as well as at least 15 non-structural and accessory proteins (1). Among these proteins, the surface spike (S) glycoprotein is the major antigen that induces protective immune response against IBV (3). The S protein consists of two subunits, S1 and S2, with the S1 subunit being responsible for binding cellular receptors (4) and the major target of neutralizing antibodies. The S2 subunit is more conserved than S1 and also plays a role in inducing protective immune response (5–7), as well as facilitating membrane fusion and viral entry (5, 8, 9). It has been reported that S2 could produce cross-protection against strains that differ in their S1 subunits (7).

A feasible and practical immunoassay for antibody detection and immune response measurement is critical for vaccine development. Enzyme-linked immunosorbent assays (ELISAs)

based on whole IBV viral particles, as well as recombinant S1, nucleocapsid, and non-structural proteins, have been reported for detecting antibodies against IBV (10–13). Although these assays have achieved promising results, they have some limitations, especially in detecting antibodies induced by emergent or variant IBV strains.

Our previous studies revealed an epitope in S2 and identified the key amino acids in this epitope (14). Based on this finding, we have designed an IBV S2-based peptide and developed an ELISA for the detection of antibodies against IBV.

MATERIALS AND METHODS

Synthetic Peptide and Serum Samples

A 20-mer peptide, SCPYVSYGRFCIQPDGSIKQ, corresponding to amino acid positions 8 to 27 on the S2 protein of IBV CK/CH/2010/JT1 strain (GenBank KU361187), was synthesized (Synpeptide Co., Ltd., Shanghai, China) and used as the coating antigen for the peptide-based ELISA (pELISA). Serum samples that were used in our study included 100 serum samples collected from specific-pathogen-free (SPF) chickens (Spirax Ferrer Poultry Science and Technology Co., Ltd., Jinan, China), 250 serum samples collected from chickens that were vaccinated with IBV vaccines H120 and H52 (Lihua Animal Husbandry Co., LTD, Jiangsu, China), and sera against IBV strains Massachusetts 41 (M41), 4/91, H52, H120, and CK/CH/2010/JT1, which were prepared in our laboratory by infecting SPF chickens with 1,000 median egg infectious dose (EID_{50}) of each strain. Immune serum against QXL87 (GenBank accession no. MH743141) vaccine strain (QX-type) was obtained from Zhongchong Sino Biological Technology Co., Ltd. (Shanghai China). The other sera were kept in our laboratory, which were made from SPF chickens infected with the viruses (15).

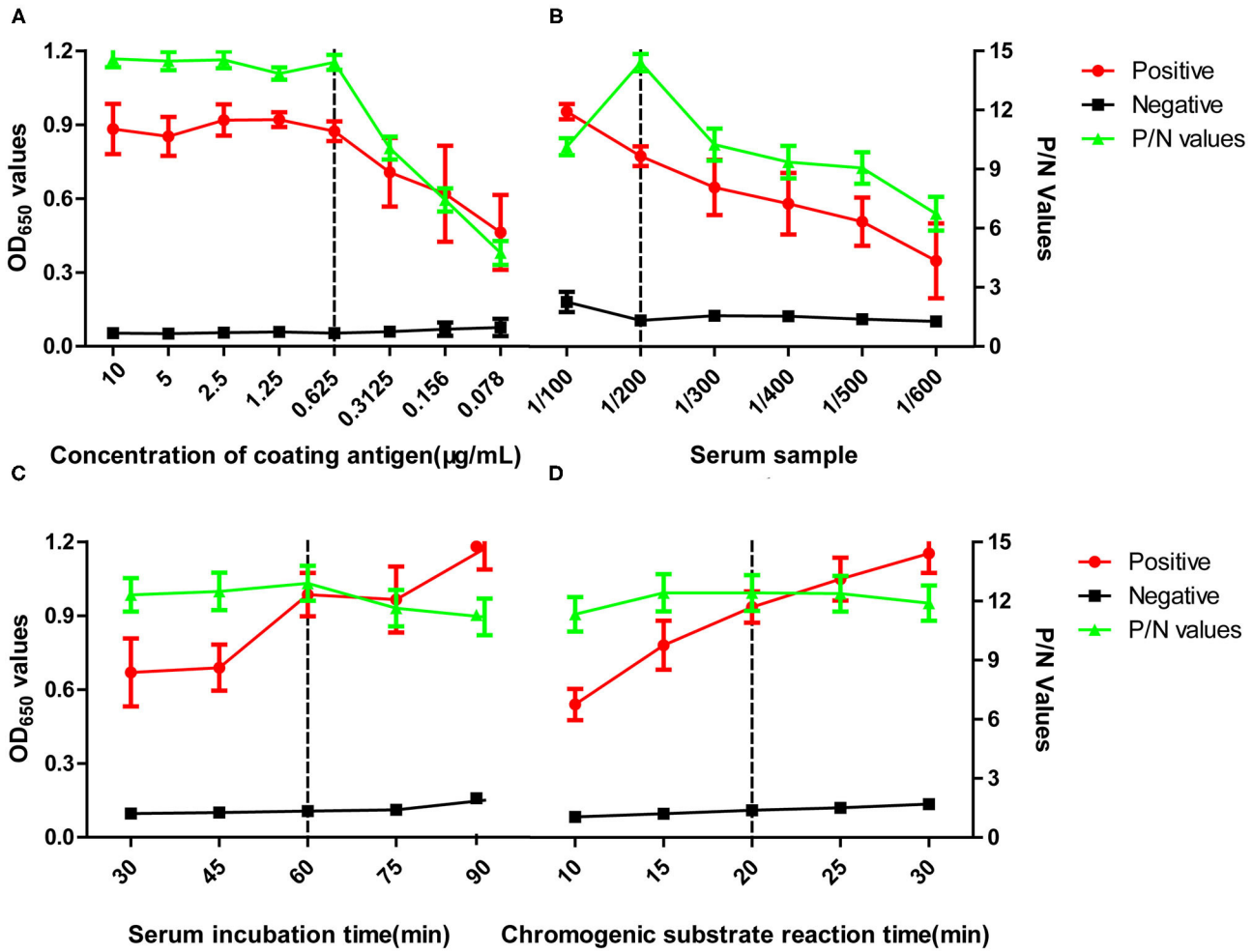

FIGURE 1 | Optimization of pELISA. Assays were performed to optimize **(A)** concentration of the coating antigen, **(B)** dilution of the serum sample, **(C)** incubation time for the serum sample, **(D)** incubation time for the TMB substrate. The vertical dotted lines indicate conditions selected for using in subsequent assays. OD values shown are the means obtained in three tests of five positive serum samples against M41, H52, CK/CH/2014/FJ14, CK/CH/2014/JT1, or 4/91 or five negative serum samples from SPF chickens. P/N values represent the ratios of mean OD values obtained for positive sera to those obtained for negative sera. Error bars represent the standard deviation (SD).

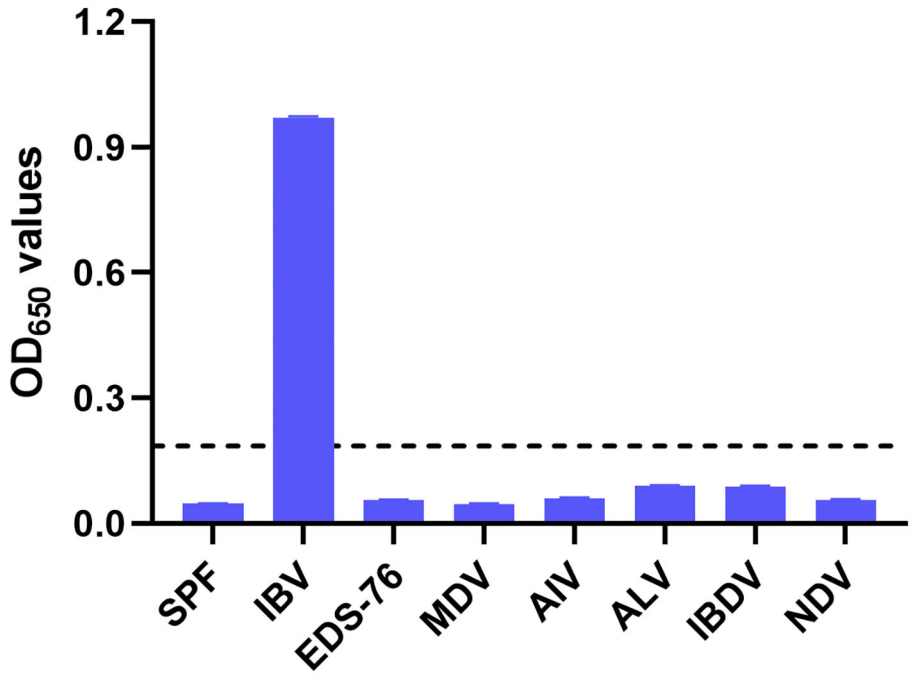

FIGURE 2 | Specificity of the pELISA. Specificity of the pELISA in detecting antibodies against common chicken viruses. Antisera raised against Newcastle disease virus (NDV), avian influenza virus (AIV, H9N2), avian leukosis virus (ALV), Marek's disease virus (MDV), egg drop syndrome virus (EDS-76V), and infectious bursal disease virus (IBDV) were tested by pELISA. Their OD_{650} values were lower than the cutoff value. The black horizontal dotted line indicates the cutoff.

pELISA Procedure

For the pELISA, 96-well polystyrene plates were coated with 0.63 μg/ml of the synthetic peptide in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. After washing with phosphate-buffered saline containing 0.05% vol/vol Tween 20 (PBST), the plates were blocked with 300 μl/well of 8% rabbit serum in PBST (Lanzhou Minhai Biological Engineering Co., Ltd., China) for 3 h at 37°C. Following three washes with PBST, 100 μl serum (1:200 dilution in PBST) was added to the wells, and the plates were incubated at 37°C for 1 h. The plates were then washed five times with PBST and further incubated with 100 μl/well horseradish peroxidase–conjugated goat anti–chicken immunoglobulin G (IgG) (1:20,000) (Jackson ImmunoResearch Laboratories, Inc., USA) for 1 h at 37°C. After washing for five times, the signals were developed by incubating the plates with 100 μL/well TMB substrate for 20 min at 37°C, followed by stopping the color development with 100 μl/well of 1% sodium dodecyl sulfate. OD_{650} values were read with an ELISA reader (BioTek, VT, USA). Each assay was repeated twice.

For the commercial IBV antibody ELISA kit (IDEXX, Westbrook, ME), the assay was performed according to the manufacturer's protocol. Briefly, 100 μl of 1:500 diluted sera was added to each well. After incubation for 30 min at room temperature and washing for three times, 100 μl conjugated antibody was added and incubated for 30 min at room temperature. The plates were washed five times, and the substrate was added in the well for color development for 15 min and terminated with stop solution. OD_{650} values were read with an ELISA reader (BioTek, VT, USA).

TABLE 1 | Reproducibility of the pELISA in detecting IBV-specific antibodies.

Samples	Intra-assay Variability		Interassay Variability	
	Mean ± SD	CV (%)	Mean ± SD	CV (%)
1	0.043 ± 0.001	2.3%	0.048 ± 0.001	2.7%
2	0.046 ± 0.001	1.6%	0.046 ± 0.001	1.9%
3	0.049 ± 0.001	2.3%	0.051 ± 0.001	1.7%
4	0.046 ± 0.001	1.6%	0.045 ± 0.001	2.5%
5	0.842 ± 0.016	1.9%	0.817 ± 0.038	4.7%
6	0.884 ± 0.009	1.8%	0.433 ± 0.025	6.5%
7	0.421 ± 0.006	2.4%	0.249 ± 0.003	1.2%
8	0.491 ± 0.008	4.4%	0.198 ± 0.002	1.3%

Every sample repeated three times, SD, standard deviation; CV, coefficient of variation.

Cutoff Value, Specificity, and Reproducibility

After the optimal dilution and peptide concentration were set, 100 negative serum samples from SPF chickens were tested by pELISA to set a cutoff value. To examine the specificity of the pELISA, positive serum samples against avian influenza virus (AIV), avian leukemia virus (ALV), Newcastle disease virus (NDV), infectious bursal disease virus (IBDV), Marek's disease virus (MDV), and egg drop syndrome virus (EDS-76V) were tested. Evaluation of the assay reproducibility within and between runs was performed with eight serum samples, four positive and another four negative, which were confirmed in indirect

immunofluorescence assay (IFA). For intra-assay (within-plate) reproducibility, four replicates of each serum sample were analyzed within the same plate, and the experiment was performed three times independently. For interassay (between-run) reproducibility, four replicates of each serum sample were run in different plates. This test was performed three times using plates coated at different times. The mean OD_{650} value, standard deviation (SD), and coefficient of variation (CV) were calculated.

Performance of the pELISA

To further evaluate the pELISA, 250 serum samples from vaccinated chickens were tested by pELISA, IFA, and the commercial ELISA kit (IDEXX, USA). The IFA was conducted according to the previously reported protocol (16). Briefly, primary chicken embryo kidney cells were grown in the 96-well plates and infected with IBV M41 strain. After 2 days, the cells were fixed with acetone and alcohol (3:2) for 5 min. The sera diluted 1:200 with PBS were added to the wells and incubated for 60 min. After washing with PBST, cells were incubated with the fluorescein isothiocyanate–conjugated rabbit–anti-chicken IgG for 60 min, followed by washing five times and observation under the fluorescence microscope. The sensitivity, specificity, and the accuracy of the pELISA and commercial ELISA kit were evaluated by comparing to the data generated by IFA.

To further evaluate the assay, five 6-week-old SPF chickens were vaccinated intranasally with 10^3 EID_{50} IBV strain H52 or 4/91; sera were collected on days 3, 7, 14, 21, and 28 and tested by the pELISA and the commercial IDEXX ELISA kit.

RESULTS

Optimization of the pELISA

To optimize the pELISA, two-fold serial dilutions of the synthetic peptide and different dilutions of the positive and negative chicken serum samples, which had been confirmed by IFA, were tested. As shown in **Figure 1**, the optimal concentration of peptide was found to be 0.63 μg/ml, and the dilution of serum samples was 1:200 (**Figure 1**), based on the criteria that the P/N value of the OD_{650} ratio between positive and negative sera was highest. At the same time, the best results could reach at 60-min incubation for serum samples and second conjugated antibody (**Figure 1**).

Cutoff Value, Specificity, and Reproducibility of the pELISA

The 100 negative serum samples collected from SPF chickens were tested to set up a cutoff value for the pELISA. When tested at a 1:200 dilution, these samples gave a mean OD_{650} value of 0.067, with an SD of 0.017; thus, the cutoff value was defined as 0.185 (2 mean ± 3 SD). In the subsequent assays, serum samples giving OD_{650} values ≥0.185 were designated positive for IBV antibodies, whereas those generating OD_{650} values <0.185

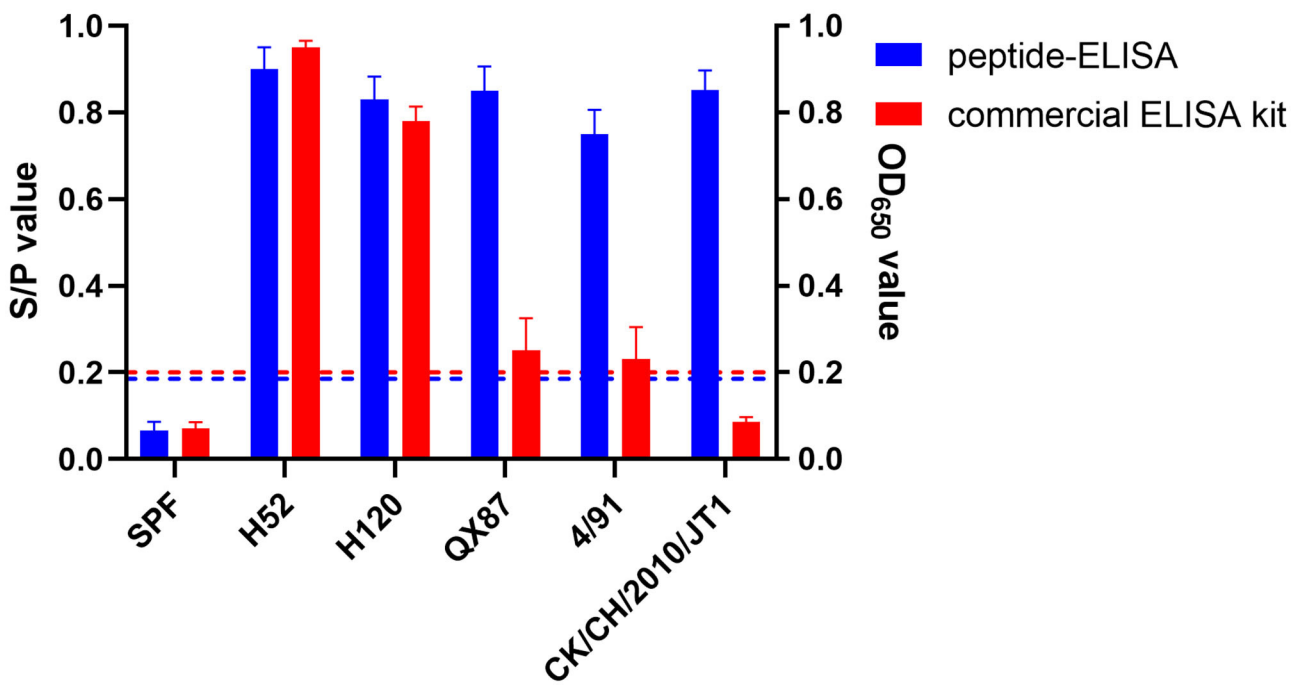

FIGURE 3 | Reactivity of pELISA and positive sera against different genotype IBVs. Two colors showed the results of pELISA and commercial ELISA kits. The blue horizontal dotted line indicates the cutoff of pELISA, and the red horizontal dotted line indicates the S/*P*-value of the commercial ELISA kit. pELISA had very good reaction with sera to different genotype IBV strains, but the commercial ELISA kit gave a strong reaction with only mass-type positive sera. H52 and H120 vaccine strains belong to mass type; 4/91 vaccine strain belongs to 4/91 type; QX87 vaccine strain belongs to QX-type; CK/CH/2010/JT1 virulent strain belongs to new cluster type.

were designated negative. The specificity of the pELISA was evaluated by testing the reactivity of sera raised against NDV, ALV, IBDV, AIV, MDV, and EDS-76V. As shown in **Figure 2**, no cross-reaction between the IBV S2 peptide antigen and these sera was detected, demonstrating the specificity of the pELISA.

Next, we examined the reproducibility of the assay. Eight selected serum samples, four positive and four negative, were tested by the pELISA in quadruplicate. The interassay CV was 1.2 to 6.5%, and the intra-assay CV ranged from 1.6 to 4.4% (**Table 1**), indicating the high reproducibility of our assay.

pELISA Detects Antibodies Against Different Genotypes of IBV

To evaluate whether our pELISA is suitable for detecting antibodies against various genotypes of IBV, chicken sera raised against IBVs 4/91, H52, H120, QX87 (QX type), and CK/CH/2010/JT1 (New cluster type) were tested in the assay. The pELISA could detect antibodies against all of these different IBVs. Interestingly, the commercial IDEXX ELISA kit could detect sera raised against the H52 and H120 strains, but showed very weak reaction with the sera to 4/91, QX87, or CK/CH/2010/JT1 (**Figure 3**), which indicated that the pELISA is a better option for the detection of antibodies to various IBV genotypes.

To further evaluate the sensitivity, specificity, and accuracy of pELISA, 250 serum samples from chickens immunized with H120 and H52 vaccines were tested with the pELISA, IFA, and the commercial ELISA kit. As shown in **Table 2**, 232 and 18 of these serum samples tested positive and negative by pELISA, respectively, whereas 233 and 17 tested positive and negative by IFA, respectively. Compared to the results of IFA, the sensitivity, specificity, and the accuracy of the pELISA were 99.14, 94.12, and 98.80%, respectively. With the commercial kit, 230 and 20 of these 250 serum samples were positive and negative, respectively. Compared to the results of IFA, the sensitivity, specificity, and the accuracy of the commercial kit were 96.57, 70.59, and 94.80%, respectively.

pELISA Effectively Detects Antibody Response in Chickens

Finally, we evaluated the suitability of the pELISA in measuring the immune response elicited by the IBV vaccine. Chickens were vaccinated with IBV H52 and 4/91 vaccines, and sera were collected at different time points postvaccination and tested by pELISA. In sera from H52-vaccinated chickens, the pELISA was able to detect antibody response as early as on day 7 postvaccination, with three of the six chickens being positive, and all chickens were positive by 14, 21, and 28 days postvaccination, whereas the commercial ELISA kit could detect positive antibodies on day 14 postvaccination. In sera from 4/91 vaccinated chickens, the pELISA was able to detect antibody response as early as on day 7 postvaccination, with two of the six chickens being positive, and all chickens were positive by 14, 21, and 28 days postvaccination. By contrast, the earliest time point when the commercial ELISA kit could detect a positive antibody response was on day 14 postvaccination, whereas no positive antibody response was detected in any of the birds on day 7 postinfection. Further, on days 14, 21, and 28, only two four and five of the chickens tested positive by the IDEXX kit, respectively (**Table 3**).

TABLE 2 | Comparison of the pELISA, IFA, and the commercial IBV ELISA kit for 250 field serum samples.

Serum samples	IFA	pELISA	IDEXX
223 serum samples[a]	+	+	+
8,283	+	+	−
8,284	+	+	−
6,817	+	+	−
6,823	+	+	−
6,878	+	+	−
6,872	+	+	−
6,819	+	+	−
8,354	+	+	−
86	−	+	−
23	+	−	+
8,358	+	−	+
8,342	−	−	+
8,348	−	−	+
8,370	−	−	+
8,366	−	−	+
8,371	−	−	+
11 serum samples[b]	−	−	−

+, Positive; −, negative.
[a]Number of serum samples tested positive by all three methods.
[b]The number of the negative serum samples by three methods.

TABLE 3 | Comparison of pELISA and a commercial ELISA kit in detecting IBV antibody response in chickens[a].

		Day 3	Day 7	Day 14	Day 21	Day 28
H52	pELISA	0/6[b]	3/6	6/6	6/6	6/6
	Commercial ELISA kit	0/6	0/6	6/6	6 /6	6/6
4/91	pELISA	0/6	2/6	6/6	6/6	6/6
	Commercial ELISA kit	0/6	0/6	2/6	4/6	5/6

[a]Chickens were vaccinated with IBV H52 and 4/91, and sera were collected on indicated days and tested by pELISA and the IDEXX ELISA kit.
[b]The number are positive number/total number.

DISCUSSION

ELISA has been used in IBV serological tests for its feasibility, sensitivity, rapidity and being suitable for large-scale use (17). In the previously reported IBV antibody detection assays, whole IBV virions and recombinant proteins were used as antigens (10–13). Although these assays have their advantages, preparation of the antigens was time-consuming and expensive. In this study, we developed a pELISA for IBV antibody detection, using a peptide based on the S2 sequence. In contrast to S1, which

is highly variable among different IBV strains, S2 is highly conserved and carries conserved epitopes (7, 14, 18). Our results demonstrated that the pELISA could detect antibodies against different genotypes of IBV. Furthermore, the assay is highly sensitive, specific, and accurate compared with the results of IFA and the commercial ELISA kit, highlighting its value as a reliable assay for the detection of IBV antibodies.

For antibody detection, different methods could give different results. The same method with different antigens also gives different results (10, 19). In our pELISA, we could detect antibody against IBV on as early as 7 day postinfection. However, the commercial ELISA kit could not detect the antibody until 14 days postvaccination, and the positive value was not very strong. Our results are similar to those of Kutle et al. (20), who found that the titer of antibody to IBV was very low on 20 days postvaccination when they used the commercial ELISA kit. The reasons could be mainly because of the surface antigen variation of the virions and different coated protein. In addition, high concentration of epitope of S2 protein could have good reaction with the antibody to the conserved epitope in different genotype. It indicates that the epitope in S2 could be an important antigen in IBV immune response.

In summary, we have developed a pELISA with a synthetic S2 peptide, which could detect antibodies against different genotypes of IBV. This assay possesses sufficient sensitivity, specificity, and accuracy and has the potential to serve as a rapid and reliable method for IBV antibody detection.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/nuccore/KU361187.1/.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and ethics Committee at Yangzhou University.

AUTHOR CONTRIBUTIONS

This manuscript was written by LY and AQ. Experiment and data analysis were performed by LY, QW, ZL, and YL. Study designed by ZW, AQ, JY, KQ, and HS. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: LY and YL were employed by the company JiangsuLihua Animal Husbandry Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Elicitation of Highly Pathogenic Avian Influenza H5N1 M2e and HA2-Specific Humoral and Cell-Mediated Immune Response in Chicken Following Immunization With Recombinant M2e–HA2 Fusion Protein

Semmannan Kalaiyarasu[1], Sandeep Bhatia[1], Niranjan Mishra[1], Dhanapal Senthil Kumar[1], Manoj Kumar[1], Richa Sood[1], Katherukamem Rajukumar[1], Boopathi Ponnusamy[2], Dhruv Desai[2] and Vijendra Pal Singh[1]*

[1] *Indian Council of Agricultural Research-National Institute of High Security Animal Diseases, Bhopal, India,* [2] *Indian Council of Agricultural Research-Indian Veterinary Research Institute, Bareilly, India*

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Edited by:
Constantinos S. Kyriakis, Auburn University, United States

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Ahmed Ali, Beni-Suef University, Egypt
Xiuqing Wang, South Dakota State University, United States

***Correspondence:**
Semmannan Kalaiyarasu
kalai_82vetmic@yahoo.co.in

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The study was aimed to evaluate the elicitation of highly pathogenic avian influenza (HPAI) virus (AIV) M2e and HA2-specific immunity in chicken to develop broad protective influenza vaccine against HPAI H5N1. Based on the analysis of Indian AIV H5N1 sequences, the conserved regions of extracellular domain of M2 protein (M2e) and HA2 were identified. Synthetic gene construct coding for M2e and two immunodominant HA2 conserved regions was designed and synthesized after codon optimization. The fusion recombinant protein (∼38 kDa) was expressed in a prokaryotic system and characterized by Western blotting with anti-His antibody and anti-AIV polyclonal chicken serum. The M2e–HA2 fusion protein was found to be highly reactive with known AIV-positive and -negative chicken sera by ELISA. Two groups of specific pathogen-free (SPF) chickens were immunized (i/m) with M2e synthetic peptide and M2e–HA2 recombinant protein along with one control group with booster on the 14th day and 28th day with the same dose and route. Pre-immunization sera and whole blood were collected on day 0 followed by 3, 7, 14, 21, and 28 days and 2 weeks after the second booster (42 day). Lymphocyte proliferation assay by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) method revealed that the stimulation index (SI) was increased gradually from days 0 to 14 in the immunized group ($p < 0.05$) than that in control chicken. Toll-like receptor (TLR) mRNA analysis by RT-qPCR showed maximum upregulation in the M2e–HA2-vaccinated group compared to M2e- and sham-vaccinated groups. M2e–HA2 recombinant protein-based indirect ELISA revealed that M2e–HA2 recombinant fusion protein has induced strong M2e and HA2-specific antibody responses from 7 days post-primary immunization, and then the titer gradually increased after booster dose. Similarly, M2e peptide ELISA revealed that M2e–HA2 recombinant fusion protein

elicited M2e-specific antibody from day 14 onward. In contrast, no antibody response was detected in the chicken immunized with synthetic peptide M2e alone or control group. Findings of this study will be very useful in future development of broad protective H5N1 influenza vaccine targeting M2e and HA2.

Keywords: avian influenza, immunity, matrix and hemagglutinin, humoral and cell mediated immunity, recombinant protein

INTRODUCTION

Influenza A viruses have been isolated from a wide range of animals including poultry, wild and cage birds, pigs, horses, dogs, sea mammals, and humans, although ducks are considered the natural reservoirs of avian influenza viruses (AIVs). Based on the antigenicity of two viral glycoproteins *viz* hemagglutinin (HA) and neuraminidase (NA), influenza A viruses are further classified into subtypes; to date, though 18 HA subtypes (H1–H18) and 11 NA subtypes (N1–N11) have been identified (1), only 16 HA subtypes (H1–H16) and nine NA subtypes are considered true influenza viruses and the remaining two, namely, H17N10 (2) and H18N11 (3), are considered influenza A-like viruses (1). In South Asia, the H5N1 virus was first reported in domestic poultry in India and Pakistan during February 2006 and followed by Bangladesh, Nepal, and Bhutan in March 2007, January 2009, and February 2010, respectively (4). All the H5N1 viruses isolated from poultry and humans in South Asia until 2010 belong to clade 2.2 (5–8). The first introduction of clade 2.3.2 H5N1 virus to South Asia was reported from Nepal in February 2010 (9, 10), followed by in India in February 2011 (11). Antigenic analysis showed 64–256-fold reduction of cross reactivity in clade 2.3.2.1 as compared to clade 2.2 viruses, which revealed that the likelihood of clade 2.2 viruses to provide cross-protection against 2.3.2.1 viruses is less (11). Between November 2014 to March 2015, clade 2.3.2.1c has been reported as the new introduction to India (12), followed by worldwide circulation of clade 2.3.4.4 including India (13–17). Due to the continuous change of clades, cross protection between the clades become uncertain.

M2 is a type III integral membrane protein forming a pH-dependent proton-selective ion channel (18, 19) produced by spliced mRNA translation of gene segment 7 of influenza virus, which also codes for M1 protein (20). The M2 protein (96 amino acids) contains three structural domains, namely, amino-terminal extracellular domain M2e (23 residues), a transmembrane domain (19 residues), and a cytoplasmic domain (54 residues) (21), which gives the native tetrameric conformation with disulfide bonds. Though M2 protein molecules are estimated to be present at low level (20–60 numbers) on each virion, they are expressed at high levels on the surface of infected cells (22). The amino acid sequence in M2e is highly conserved among influenza A viruses (23, 24). The five amino acids within the residues 10–20 of M2e were observed to be host restricted: PIRNEWGCRCN (amino acids 10–20, human isolates), PTRNGWECKCS (amino acids 10–20, avian isolates), and PIRNGWECRCN (amino acids 10–20, swine isolates) (24). Due to the low degree of variation in the M2 extracellular domain, it is considered an attractive antigenic target for developing a universal influenza vaccine.

Influenza virus HA is a homotrimeric protein molecule, and each monomer consists of two disulfide-linked subunit glycoproteins, a globular head of HA1 and a stem or stalk domain composed of the N- and C-terminal parts of HA1 and all of HA2 (25). HA is synthesized as a precursor (HA0) that is cleaved into HA1 and HA2 domains. The cleavage site of HA with the fusion peptide and N-terminal portion of HA2 is the most conserved sequence among influenza A viruses and has the potential application as a universal antigen. Although HA stem region is considered a good option for the development of the universal vaccine, the frequency of anti-stem antibodies is considerably lower than that of anti-globular head antibodies in natural infection (26) due to the physical masking of immunodominant head over the stem region and close proximity of stem epitope(s) to the viral membrane (27).

It is always advantageous to add more conserved immunogenic regions to get better cross-protection while developing a universal vaccine instead of selecting a single region. Most of the studies had been carried out to assess the immunogenicity of M2e and HA2 region of stalk domain in either mice or pigs. But it is essential to evaluate these types of conserved region-based immunogens in chicken before applying the universal vaccine strategy in poultry industry against H5N1 or other highly pathogenic AIV infections. Hence, the study was aimed to develop highly pathogenic avian influenza (HPAI) virus M2e and HA2-specific immunity in chicken to develop a broad protective influenza vaccine against HPAI H5N1.

MATERIALS AND METHODS

Identification of M2e and HA2 Conserved Region of Indian Avian Influenza H5N1 Viruses

For the identification of M2e and HA2, all the clades of Indian AIV H5N1 virus M2 and HA sequences of 2006 to 2015 outbreaks were included and analyzed by MegAlign software (DNASTAR, Inc., USA). Conserved regions of extracellular domain of M2 protein (M2e) and HA2 were identified, and the identity was compared with published M2e and HA2 sequences.

Synthesis of Avian Influenza M2e Antigen

The identified M2e (2–24 amino acid) (SLLTEVETPTRNEWECRCSDSSD) was synthesized commercially (Genscript, USA) as a synthetic peptide antigen.

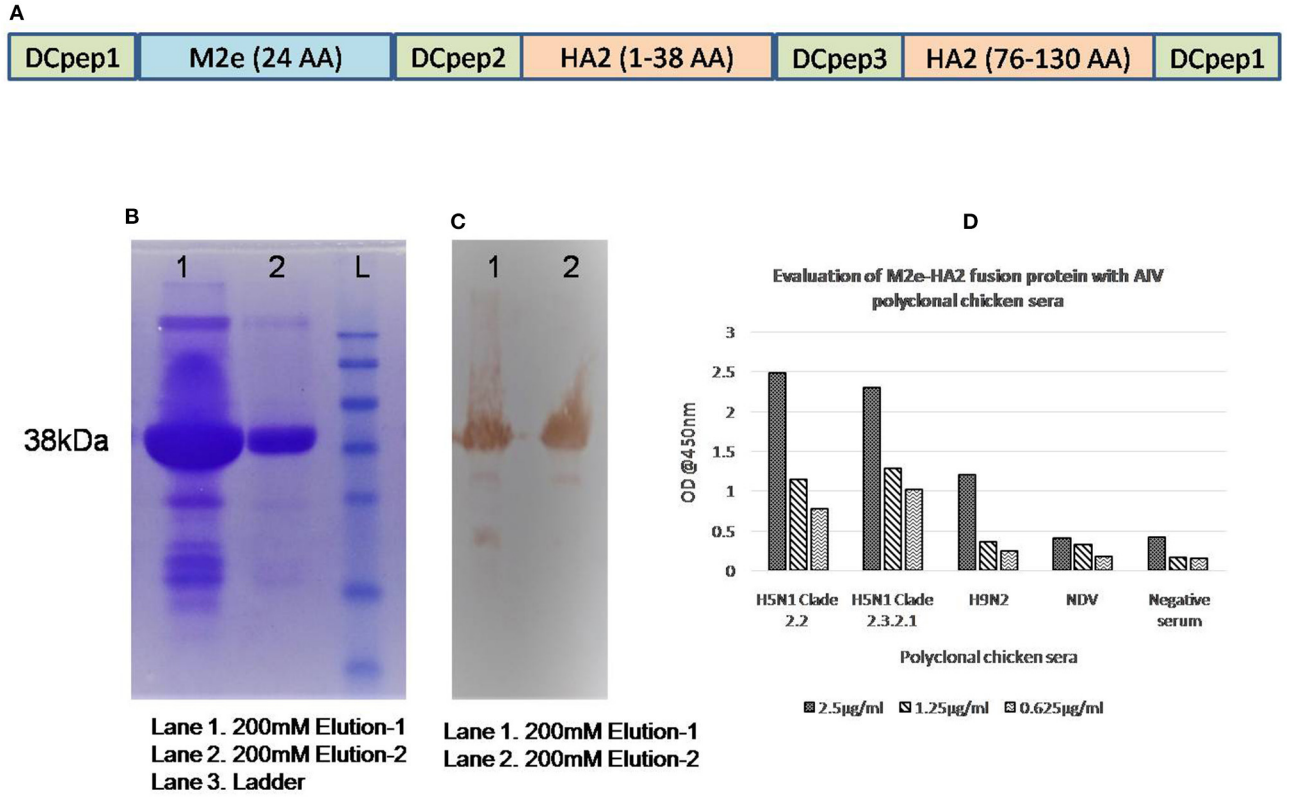

FIGURE 1 | Gene construction and expression of M2e–HA2 recombinant protein in Escherichia coli Rosetta Blue (*DE3*)*pLysS*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis and evaluation of recombinant M2e–HA2 protein by indirect ELISA with positive control sera. **(A)** Synthetic gene constructs coding for M2e (2–24 AA) and HA2 conserved regions (N-terminal 1–38 AA and Long α-helix 76–130 AA) with chicken dendritic cell binding peptides were designed by placing glycine linkers (GGG) in between them. **(B)** SDS-PAGE analysis of recombinant M2e–HA2 protein by Coomassie blue stain and **(C)** Western blot analysis of recombinant M2e–HA2 protein (38 kDa) showed high reactivity with polyclonal avian influenza virus (AIV) serum. **(D)** Indirect ELISA with recombinant M2e–HA2 fusion protein showed high reactivity with different clades (2.2 and 2.3.2.1) of H5N1 AIV, H9N2 AIV but failed to react with NDV and control chicken serum.

Expression and Characterization of M2e–HA2 Fusion Recombinant Protein

Synthetic gene constructs coding for M2e (2–24 AA) and HA2 conserved region (N-terminal 1–38 AA and Long α-helix 76–130 AA) were designed by placing glycine linkers (GGG) and chicken dendritic cell binding peptides (amino acid sequences of chicken dendritic cell binding peptides were not shown) in between them and synthesized commercially after codon optimization (Genscript, NJ, USA) in pET 32b(+) vector system along with His-Tag. Then, the gene was used for the expression of recombinant protein in BL21 (DE3) pLysS cells and purified using His-Bind purification kit (Merck Millipore, USA). Briefly, a single colony of transformed Escherichia coli Rosetta Blue (*DE3*)*pLysS* was incubated overnight on a shaker incubator in 2 ml LB medium containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) at 37°C with constant agitation (200 rpm). The next day, 500 µl of culture was inoculated in 50 ml LB broth (1/100) and grown up to an OD_{600} of 0.6 with vigorous shaking (200 rpm) at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM for expression of fusion protein in E. coli and incubated further for another 4 h at 37°C with shaking at 200 rpm. In order to produce the expression protein, bacterial suspensions were tested at 2- and 4-h intervals and analyzed on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The fusion chimeric protein (M2e–HA2) in the induced cell pellet as inclusion bodies was purified using the BugBuster® HisBind® Purification Kit (Novagen, USA) following the manufacturer's protocol. The purified M2e–HA2 recombinant protein was characterized by Western blotting with anti-His antibody and anti-AIV polyclonal chicken serum (**Figure 1**). Similarly, reactivity of M2e–HA2 fusion protein with known AIV-positive and -negative chicken sera was tested by ELISA (**Figure 1D**).

Preparation of Immunogen

M2e Synthetic Peptide and M2e–HA2 Fusion Recombinant Protein Emulsification

Six milliliters of synthetic peptides (M2e) and recombinant protein (M2e–HA2) were emulsified with Montanide™ ISA 71 VG (SEPPIC) (14 ml) in the ratio of 3:7 to prepare water-in-oil (W/O) emulsion. Sham vaccine for control birds was prepared by

emulsifying phosphate buffered saline (PBS) with Montanide™ ISA 71 VG.

Immunization of Specific Pathogen-Free Chickens With the Water-In-Oil Emulsified Synthetic Peptide (M2e) and Recombinant Protein (M2e–HA2) Antigen

Four-week-old specific pathogen-free (SPF) chickens were immunized with 0.5 ml of emulsified antigen containing 100 μg of peptide (M2e) or 100 μg of recombinant protein (M2e–HA2) per dose in breast muscle. Six birds in each group were immunized with M2e synthetic peptide (A), recombinant M2e–HA2 fusion protein (B), and six birds were sham vaccinated (C) apart from unvaccinated control birds (D). Booster was given on the 14th day and 28th day with the same dose and route. Pre-immunization sera and whole blood with ethylenediaminetetraacetic acid (EDTA) were collected on day 0, followed by 3, 7, 14, 21, and 28 days, and 2 weeks after the second booster (42nd day). All the samples were processed on the same day of collection.

Measurement of Cell-Mediated Response

Separation of Peripheral Blood Mononuclear Cells From Chicken Blood

Approximately 3 ml of peripheral blood was collected from the wing vein of each bird following sterile procedure and immediately transferred into tubes containing EDTA. Buffy coat (750 μl) was separated by centrifugation at 1,800 rpm for 40 min. A 2-ml microcentrifuge tube, 750 μl of Histopaque®-1077 was taken, and 750 μl of buffy coat was gently layered over it. Centrifugation for 30 min at 1,800 rpm was done in room temperature. The mononuclear cells were aspirated from the opaque interface of the upper layer. The cells were washed thrice with sterile PBS followed once with RPMI 1640 medium by centrifuging at 1,000 rpm for 10 min. Cells were resuspended in 1 ml of RPMI 1640 and counted using Neubauer chamber. Then, the peripheral blood mononuclear cells (PBMCs) were made into different aliquots to carryout FACS, lymphocyte proliferation assay, and cytokine and Toll-like receptor (TLR) mRNA expression studies.

Flow Cytometry Analysis of Chicken Peripheral Blood Mononuclear Cells

PBMCs of post-immunized chicken were processed for the analysis with flow cytometry using anti-chicken CD4 and CD8 fluorescein isothiocyanate (FITC) MAbs (Southern Biotech, USA). Briefly, 100 μl of PBMCs (10^5-10^6 cells) in PBS was mixed with 5 μl of MAbs (0.5 μg/μl) in individual tubes each along with an isotype control for individual birds. Cells were mixed by gentle vortexing and incubated at 37°C for 1 h. Then, the tubes were washed thrice with washing *cum* blocking buffer containing PBS, 1% bovine serum albumin (BSA), and 0.1% sodium azide (SA) by centrifugation at 4,000 rpm for 5 min. Then, the cells were resuspended and fixed with 0.5% paraformaldehyde (PFA) for 30 min at room temperature and analyzed by flow cytometry FACSCanto (BD Biosciences, San Jose, CA, USA). The results were analyzed with FACSDiVa® software (BD Biosciences, San Jose, CA, USA).

Lymphocyte Proliferation Assay

Lymphocyte proliferation assay was performed as described previously (28) using CellTiter 96® Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI). Briefly, triplicates of 1–2 × 10^5 number of PBMCs from each bird of different groups were cultured in 96-well plates in 100 μl of RPMI 1640 medium. Ten micrograms of M2e peptide or recombinant protein (M2e-HA2) were used as stimulating antigen in their corresponding groups and concanavalin A (ConA) and lipopolysaccharide (LPS) as positive controls in triplicate wells for each sample. Similarly, triplicate wells of each bird were kept as unstimulated control within the group. The cells were incubated in a final volume of 100 μl complete RPMI 1640 for 72 h, and 15 μl of dye solution [3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT)] was added to the plates and incubated further for 4 h at 37°C. Then, 100 μl of solubilization solution was added to all the wells. Then, the plates were kept on an orbital shaker for 10 min, and the absorbance was finally measured at 550 nm using TriStar2S LB 942 multimode reader (BERTHOLD Technologies, Germany). The proliferation index or stimulation index (SI) was calculated compared to negative control, and the results were expressed as the mean of triplicate wells. The proliferation index was calculated by the following formula:

Mean OD_{550} of antigen-treated well—Mean OD_{550} of blank/Mean OD_{550} of unstimulated control well.

Quantification of Cytokines and Toll-Like Receptors mRNA Expression in Chicken Peripheral Blood Mononuclear Cells

The mRNA expressions of chicken cytokines [transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, interferon (IFN)-α, IFN-β, IFN-γ, interleukin (IL)-1β, IL-6, IL-4, and IL-10] and TLRs (1, 2, 3, 4, 5, 7, 15, and 21) were quantified using primers listed in our earlier work (29, 30) by RT-qPCR using Light Cycler 480 SYBR Green I master (Roche, Germany) in Light Cycler® 480 Real Time PCR System II (Roche, Germany). Total RNA was extracted from PBMCs using RNeasy minikit (Qiagen, USA) according to the manufacturer's protocol, and cDNA synthesis was carried out using First Strand cDNA Synthesis Kit (Fermentas Life Sciences, USA) with random hexamer primer and SuperScript II Reverse Transcriptase from 1 μg of RNA. Then, SYBR green-based qPCR was performed as per the instructions of the manufacturer. Briefly, A total reaction volume of 20 μl containing 10 μl of 2× SYBR Green I master mix, 2 μl of cDNA, 1 μl of primers each (20 pmol) was used for amplification in triplicates with the following thermal profile: one cycle of 95°C for 2 min, 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The fluorescence was measured for every cycle at the end of extension, and amplification product dissociation was analyzed at the end of the PCR. Chicken β-actin gene from the same sample was used as a reference gene for normalization. Means of triplicate reactions were used to determine mean cycle threshold (Ct) value of three birds (n = 3, data points = 9), respectively. Comparative Ct value was used to determine fold changes in gene expression, calculated

as $2^{-\Delta\Delta Ct}$ (31) by using Relative Expression Software Tool (REST) 2009. Data were analyzed using REST 2009, means and SE were calculated using REST software, i.e., the results of the 2,000 random reallocations. The software uses pairwise fixed reallocation randomization test to calculate the *p*-values between groups. $p < 0.05$ was considered statistically significant.

Assessment of Humoral Immune Response by M2e Synthetic Peptide, HA Stalk Recombinant Protein, and M2e–HA2 Recombinant Protein-Based Indirect ELISA

Experimental hyper immune chicken sera of different clades were tested by ELISA using M2e synthetic peptide and M2e–HA2 recombinant fusion protein by indirect ELISA to ensure its reactivity (32). Sera from all the groups of chickens were collected at days 0, 3, 7, 14, 28, and 42 and evaluated by ELISA using M2e synthetic peptide and M2e–HA2 fusion protein separately. Briefly, ELISA plates were directly coated with 50 μl of M2e synthetic peptide or HA stalk protein or M2e–HA2 recombinant protein (1.25 μg/ml) in carbonate bicarbonate coating buffer overnight at 4°C. Next day, plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked with 5% non-fat dry milk powder (5% NFDM in PBST) for 1 h at 37°C. Then, the antigen-coated plates were washed thrice with PBST and incubated with 50 μl of 1:50 diluted (1% NFDM in PBST) serum samples for 1 h at 37°C. After washing with PBST thrice, plates were incubated with 50 μl of 1:25,000 diluted anti-chicken immunoglobulin HRPO conjugates (Sigma-Aldrich, USA) for 1 h at 37°C. The substrate reaction was developed by adding 50 μl of 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma, USA) to each well, and the reaction was stopped after 10 min with 0.4 M H_2SO_4. The optical density (OD) of each well was read at 450 nm in a TriStar2S LB 942 multimode reader (BERTHOLD Technologies, Germany).

Challenge Study

Challenge experiment was carried out in the Class III Biosafety cabinets (Isolators) inside the BSL3 animal bio-containment facility of ICAR- NIHSAD, India. The immunized chickens of groups A, B, and C (except non-vaccinated and non-infected control birds) were transferred to respective isolators A, B, and C, and all were challenged intranasally with $10^{8.0}$ EID_{50}/0.1 ml of clade 2.3.2.1 H5N1 [A/chicken/India/CA0302/2011 (H5N1)] virus and monitored continuously. Peripheral blood, oral swabs, and cloacal swabs were collected after 24 h of virus infection. PBMCs were separated from the whole blood and processed for RNA extraction. All the swabs were processed immediately and stored at −80°C until use. All the samples of challenged birds were processed following strict biosafety norms of ICAR-NIHSAD.

Post-challenge Inflammatory Cytokine mRNA Expression Analysis

Approximately 5×10^6 cells pelleted after centrifuging at 250 × g for 10 min were used for RNA extraction using an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA was quantified using a Qubit fluorometer (Invitrogen, USA), and cDNA synthesis was carried out in a 20-μl volume using a First Strand cDNA Synthesis Kit (Fermentas, USA) with random hexamer primers from 1 μg of RNA according to the manufacturer's guidelines.The post-challenge mRNA expressions of chicken inflammatory cytokines (TGF-β, TNF-α, IFN-α, IFN-β, IFN-γ, IL-1β, IL-6, IL-4, and IL-10) and TLRs (1, 2, 3, 4, 5, 7, 15, and 21) were quantified similar to pre-challenge study.

Quantification of Viral Load

Challenge virus shedding *via* oropharyngeal and cloacal route was measured by quantitative Real-Time RT-qPCR targeting Matrix (M) gene of AIV using M gene-specific primers, [(F) 5′-TGA TCT TCTTGA AAA TTT GCA G-3′; (R) 5′-CCG TAG MAG GCC CTC TTT TCA-3′] and probe (TTG TGG ATT CTT GAT GC) (33). Viral RNA was extracted from the swabs using QIAmp viral RNA mini kit (Qiagen) as per the manufacturer's protocol, and RT-qPCR was performed using SuperScript One-Step RT-qPCR kit (Invitrogen) using Roche 480 (Roche, USA) real-time cycler. The assay was performed in a total volume of 25 μl containing 12.5 μl of 2× master mix, 0.5 μl of Rox dye, 0.5 μl of Taq mix, 0.5 μl of forward and reverse primers (20 pmol), 0.5 μl of probe (10 pmol), 2.0 μl template RNA, and 8.5 μl of nuclease-free water to make a final volume of 25 μl. Positive and negative controls, no probe control were included in each assay. The cycling condition was as follows: one cycle of 50°C for 45 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s with fluorescence acquisition. The results were determined based on the Ct values, and the copy number was calculated using standard curve for influenza M gene.

Statistical Analysis

One-way ANOVA followed by Tukey's *post-hoc* analysis was used to compare multiple groups using SPSS 16.0 software. A *p*-value of <0.05 was considered to indicate a statistically significant difference between groups.

RESULTS

Identification of M2e and HA2 Conserved Region of Indian Avian Influenza H5N1 Viruses and Synthesis of Fusion Chimeric Protein

Based on the analysis of Indian H5N1 sequences and published data, the identified M2e region was conserved between all the clades of Indian AIV isolates except at amino acid positions 10, 11, 16, and 20. The identified M2e peptide was synthesized commercially (Genscript, NJ, USA) using Fmoc chemistry of solid phase method. The purity of the peptides was ensured by its high-performance liquid chromatography (HPLC) purification report and dissolved in water (14 mg/ml). Similarly, we have analyzed the HA2 region of different clades of Indian H5N1 virus and selected two conserved regions, namely, N-terminal HA2 (1–38) and LAH (long α-helix) HA2 (76–130) in which earlier was conserved 100% and later was conserved 95% except at amino

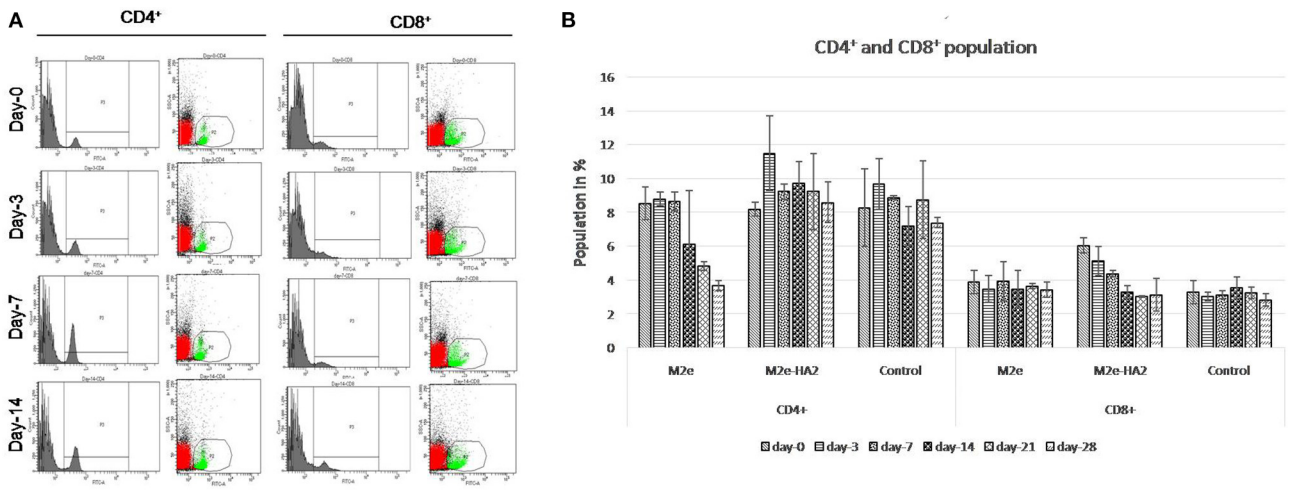

FIGURE 2 | Kinetics of immune cells ($CD4^+$ and $CD8^+$) proportion (mean ± SE) following immunization with M2e, M2e–HA2, and control groups quantified by flow cytometry at different day intervals **(A,B)**. $CD4^+$ population in the M2e–HA2 group was higher than that in the control as well as M2e group during the study period and maximum on 3DPV. Data are represented as mean ± SD ($n = 6$) and analyzed by ANOVA, and no significant difference was observed between groups at the same interval.

acid positions 116, 126, and 127, respectively. Synthetic gene construct in pET32b(+) coding for M2e-HA2 was transformed into the host, E. coli Rosetta Blue (*DE3*)*pLysS*. The addition of IPTG induced the overexpression of ∼38-kDa molecular weight recombinant protein, which was confirmed by Western blotting with anti-His and anti-AIV antibody (**Figure 1**). The expressed protein was purified by affinity chromatography using His-Bind purification kit and quantified by Qubit® fluorometer (Invitrogen, USA). The purified, pooled protein concentration was found to be 5 mg/ml and stored at −80°C. ELISA with known positive and negative AIV serum revealed that M2e–HA2 recombinant protein was highly reactive with positive serum (OD@450 nm >0.50) and failed to react with negative control (SPF chicken) serum (OD@450 nm <0.20) (**Figure 1D**).

Kinetics of $CD4^+$and $CD8^+$ Population

$CD4^+$ population in M2e–HA2 was higher than that in control as well as M2e group at all-time interval, and the maximum elevation was at 3 days post-immunization (not significant). However, M2e group showed the declining of $CD8^+$ population from day 14 and was lower than that even in the control group. The percentage of $CD8^+$ population in the M2e–HA2 group was gradually decreased from day 0, whereas there was no significant difference observed between control and M2e group (**Figure 2**).

M2e–HA2 Recombinant Fusion Protein Induced Lymphocyte Proliferation Following Immunization

Lymphocyte proliferation assay revealed that SI was increased gradually from day 0 to day 14 in the immunized group (**Figure 3**). However, the maximum fold increases in M2e and M2e–HA2 groups were 1.36 and 1.48 with their concerned antigen, respectively. At the same time, SI by LPS with all the groups was maximum and higher than that by ConA from day 14 and reached peak at day 21. Maximum SI by LPS was noticed with the M2e–HA2 group (2.61 ± 0.28-fold) followed by M2e (1.91 ± 0.38-fold), which was higher than that of the control group (1.26 ± 0.07-fold). ConA- and LPS-mediated SIs (1.04–1.26) were almost equal in the control group. Day-wise comparison revealed that SI was significantly increased from day 0 to day 14 in all immunized groups ($p < 0.05$).

Elevated Level of Pro-inflammatory Cytokine and Toll-Like Receptor mRNA Expression

Analysis of pro-inflammatory cytokines revealed that IL-1β mRNA expression was higher in the initial period ($p < 0.05$) followed by a gradual decrease in all the groups including control (**Figure 4**). Expression of IL-6 mRNA was not different in M2e and control, whereas it was gradually increased from day 0 to day 7 (eight-fold) in the M2e–HA2 group ($p < 0.01$) and then started to decline. TNF- α (LITAF) mRNA expression was maximum at day 7 of the M2e–HA2 group (18-fold) ($p < 0.01$), which was all time point higher than that of the other groups. Analysis of anti-inflammatory cytokine IL-10 and TGF-β revealed that there was no difference in the expression of TGF-β mRNA, whereas IL-10 mRNA expression was maximum (six- and eight-fold) in the M2e–HA2 group at days 3 and 7 ($p < 0.01$), followed by that in the M2e group. Analysis of IFN mRNA revealed that IFN-α was maximum (eight-fold) at day 7 of the M2e–HA2 group followed by day 14 of M2e. Regarding IFN-β and IFN-γ mRNA expression, they were maximum (18-fold and 13-fold) at the seventh day followed by the third day (4.8- and 5.3-fold), respectively, in the M2e–HA2 group ($p < 0.01$), then by that in the M2e group. Overall, all the three IFN mRNAs were expressed higher in the

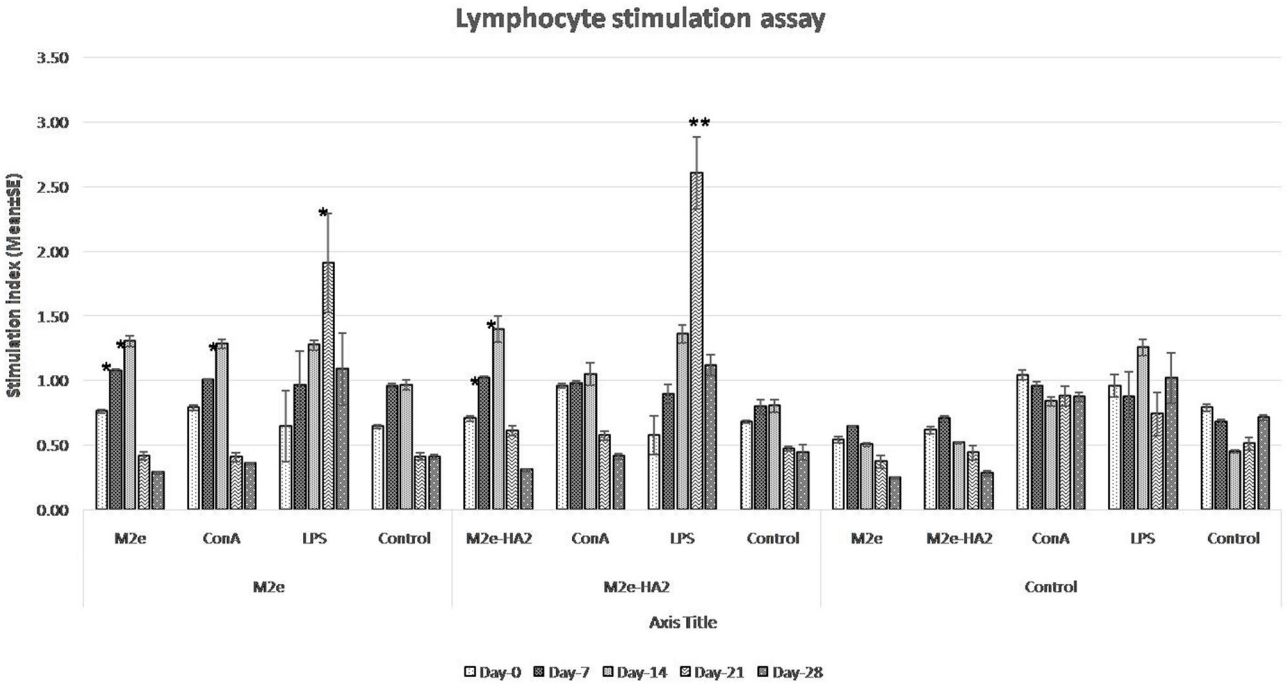

FIGURE 3 | Lymphocyte proliferation assay of M2e- and M2e–HA2-immunized and non-immunized control chickens. Stimulation index (SI) was increased gradually from day 0 to day 14 in the M2e and M2e–HA2 groups. SI by lipopolysaccharide (LPS) with all the groups was maximum and higher than concanavalin A (ConA) from day 14 and reached the peak at day 21 DPV. Data are means ± SD ($n = 6$), statistical significance (*$p \leq 0.05$, **0.01) vs. non-immunized control groups in the same time interval. The control mentioned within group is the unstimulated lymphocytes of the same group.

M2e-HA2 group followed by that of the M2e group then that of the control group at days 3 and 7 ($p < 0.05$).

All the chicken TLR mRNA expressions were analyzed by RT-qPCR. Expression of TLR1, TLR2, TLR3, TLR4, TLR5, TLR7, and TLR21 mRNA was maximum at days 3 ($p < 0.05$) and 7 ($p < 0.01$) of post-immunization in the M2e–HA2 group followed by day 14 of the M2e group (**Figure 5**). However, TLR 15 mRNA expression was maximum in day 7 of M2e–HA2 (12-fold) ($p < 0.01$) followed by day 14 of the M2e group (3.5-fold). No change in TLR mRNA expression was observed in the control group.

Detection of M2e and HA2-Specific Antibody by ELISA

Clades 2.2 and 2.3.2.1 of AIV hyper immune chicken sera were reacted well with M2e–HA2 recombinant protein and less efficiently with M2e synthetic peptide in indirect ELISA, with an average OD_{450} value up to 2.5 and 0.5, respectively, at 1:200 dilutions of serum. ELISA using M2e–HA2 recombinant protein as coating antigen revealed that the M2e–HA2 recombinant fusion protein has induced M2e–HA2-specific antibody from 7 days post-primary immunization (**Figure 6**) and then the titer gradually increased after the booster dose. At the same time, M2e peptide ELISA was also carried out with the same serum to differentiate M2e-specific antibody. M2e peptide ELISA revealed that M2e-specific antibody elicitation started from day 14 and gradually increased further after the booster. M2e-specific differential ELISA revealed that the production of HA2-specific antibody was earlier (at day 7) than M2e-specific antibody (day 14). The early elicitation of HA2-specific antibody was also confirmed by recombinant HA stalk protein-based indirect ELISA (**Figure 6C**). In contrast, only background level of antibody responses was detected in the chicken immunized with synthetic peptides M2e or control group in both the ELISA.

Drastic Reduction of Pro-inflammatory Cytokine Genes in Avian Influenza Virus-Challenged Chickens by M2e–HA2

Pro-inflammatory cytokine genes (IL-1β, IL-6, and CXCLi2) were highly upregulated in the H5N1 HPAI-infected control group, whereas the same were drastically reduced in the M2e–HA2-immunized group [$p \leq 0.01$ for IL-16 (900-fold); $p \leq 0.05$ for IL-1β (30-fold) and CXCLi2 (130-fold)]. Reduction of IL-6 and CXCLi2 was noticed in the M2e group also with lesser percentage than that in the M2e–HA2 group. At the same time, TNF-α (LITAF), IL-4, and IL-10 were slightly downregulated in the infected control group, whereas significant upregulation was noticed in the M2e–HA2 group (**Figure 7**).

Induction of $CD4^+$ and $CD8^+$ Population Depletion by Avian Influenza Virus

FACS analysis of $CD3^+CD4^+$ and $CD3^+CD8^+$ cell population after 24 h of virus infection revealed that H5N1 HPAI induced the

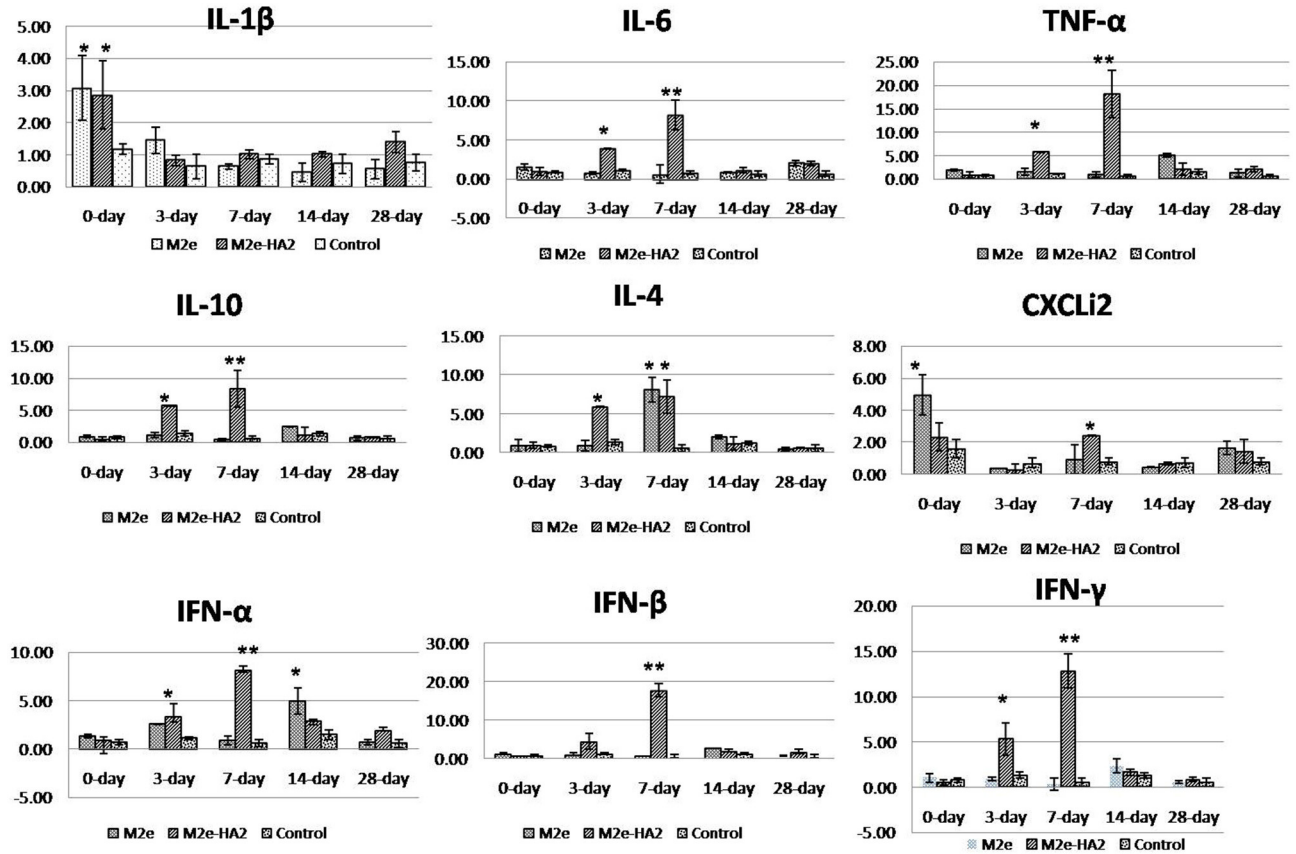

FIGURE 4 | Comparison of relative inflammatory cytokine mRNA expressions in M2e, M2e–HA2, and control chicken peripheral blood mononuclear cells (PBMCs). M2e–HA2-immunized chickens showed upregulation of interleukin (IL)-6, tumor necrosis factor (TNF)-α, chemokine CXCLi2, interferons (IFNs) (α, β, and γ) on 3 and 7 days post-primary immunization than M2e and control group. Data are means ± SD ($n = 6$), statistical significance (*$p \leq 0.05$, **0.01) vs. control non-immunized groups in the same time interval.

depletion of both populations (**Figure 8**). The M2e–HA2 group showed slight inhibition of depletion of both populations but not up to the level of the uninfected control group. However, inhibition of depletion by M2e–HA2 was comparatively higher than that in the M2e peptide group.

Upregulation of Toll-Like Receptor Gene Expression

Most of the TLR genes were downregulated in H5N1 HPAI-infected control group at 24 h of infection (**Figure 9**). At the same time, all showed significant upregulation (2–4-fold) in the M2e–HA2 group ($p < 0.01$).

Viral Shedding in Oropharyngeal and Cloacal Swabs

Viral RNA shedding analysis by RT-qPCR at 24 h of infection revealed that the control-infected birds shed more virus in their oropharyngeal swabs followed by M2e and M2e–HA2 groups in descending order (**Figure 10**). At the same time, the M2e–HA2 group showed more viral RNA in their cloacal swab than control-infected and M2e groups. However, all the birds including M2e–HA2-immunized and -challenged died at 48 h of virus infection with typical clinical signs of AIV infection.

DISCUSSION

The extracellular domain of influenza M2 protein (M2e) is highly conserved among influenza A viruses and considered an appropriate target for the development of universal influenza vaccine with broad-spectrum protection (23). Analysis of representative Indian H5N1 sequences of 2006-15 revealed the high conservation between them except at amino acid positions 10, 11, 16, and 20 of M2e. Earlier reports also reveal that M2e residues are variable between 10 and 24 but showed conservation of Arg12, Trp15, Cys17, Cys19, and Ser22, suggesting that these residues in M2e are functionally important (34), and the same conservation is noticed in Indian isolates. In earlier studies, protective M2e antibodies had been induced in a variety of ways including full-length protein with adjuvant (35), DNA administration (36), fusion to hepatitis B core protein (37, 38),

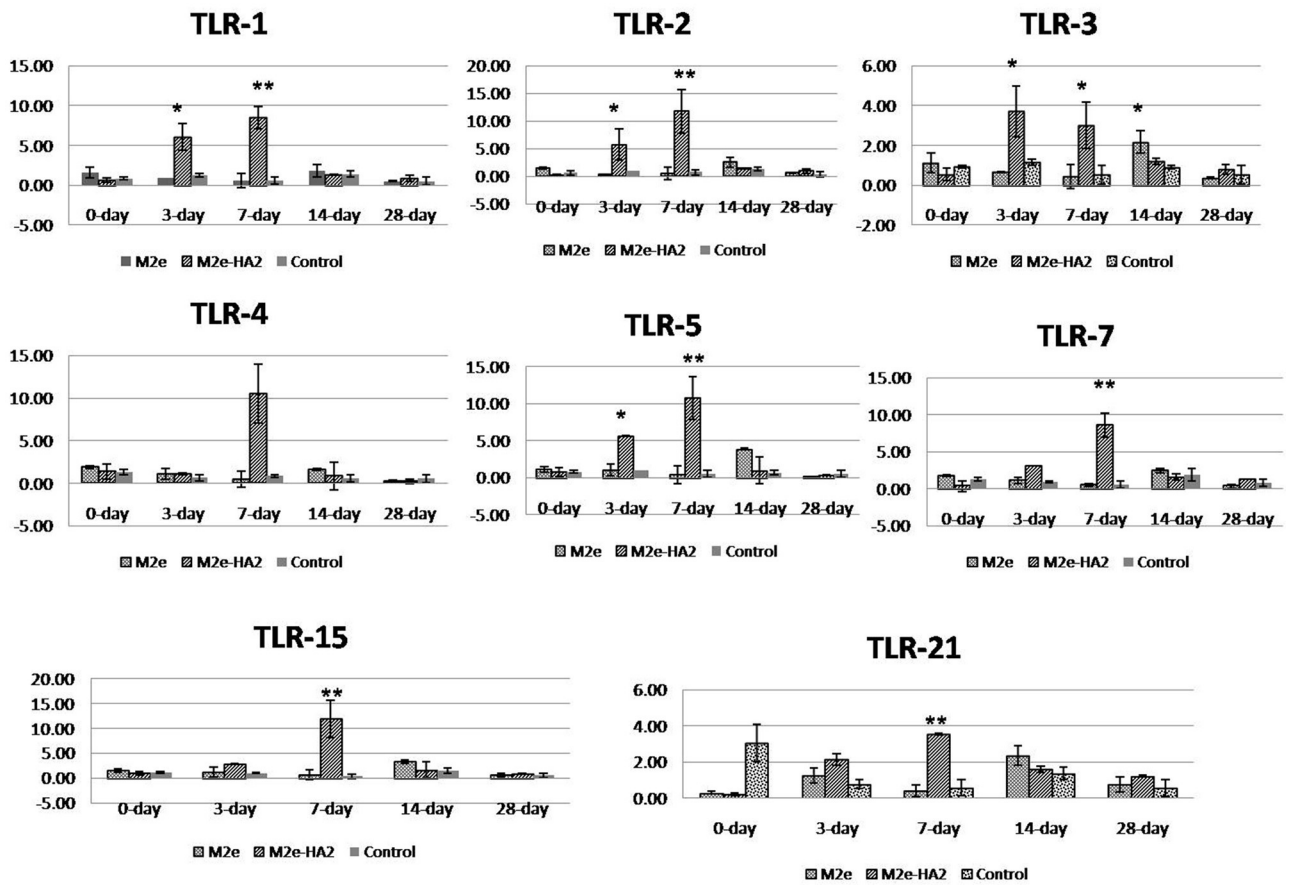

FIGURE 5 | Comparison of relative expression of Toll-like receptor (TLR) mRNAs in M2e- and M2e–HA2-immunized and non-immunized control chicken peripheral blood mononuclear cells (PBMCs). M2e–HA2-immunized chickens showed upregulation of all the TLR mRNAs than M2e and control on 3 and 7 days post-immunization. Data are means ± SD ($n = 6$), statistical significance (*$p \leq 0.05$, **0.01) vs. control non-immunized groups in the same time interval.

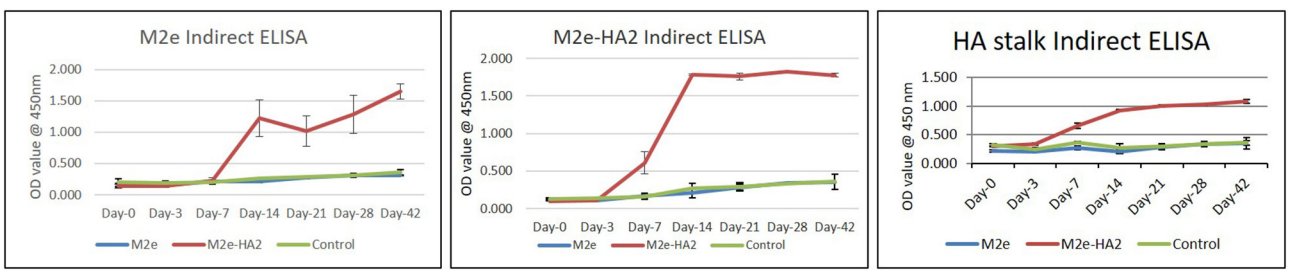

FIGURE 6 | M2e synthetic peptide, recombinant M2e–HA2 protein, and recombinant HA stalk protein-based indirect ELISA of M2e- and M2e–HA2-immunized chicken sera of different day intervals along with control group. Data are means ± SD ($n = 6$).

keyhole limpet hemocyanin (39, 40), flagellin (41), as liposomes (42), using viral vectors (43–47), tandem repeat formats (M2e–MAP) (48, 49), VLPs (23, 50), recombinant expression with CD154 epitopes (51), and chitosan nanoparticle encapsulation (52). The HA2 subunit (221 amino acids) structure is composed of two anti-parallel α-helixes and is more conserved than HA1 (53). Analysis of HA2 sequences revealed that the N-terminal region 1–38 is completely conserved among all isolates and reported to provide intra-subtype cross-protection in mice (54). Similarly, analysis of LAH (76–130 AA) revealed 95% of conservation between the Indian sequences and also reported to elicit neutralizing antibodies and efficacious protection against H3 and moderate protection against other subtypes H5, H7, H2, and H1 in mice (55).

In this study, a novel approach was attempted by making a synthetic construct to link M2e with another conserved

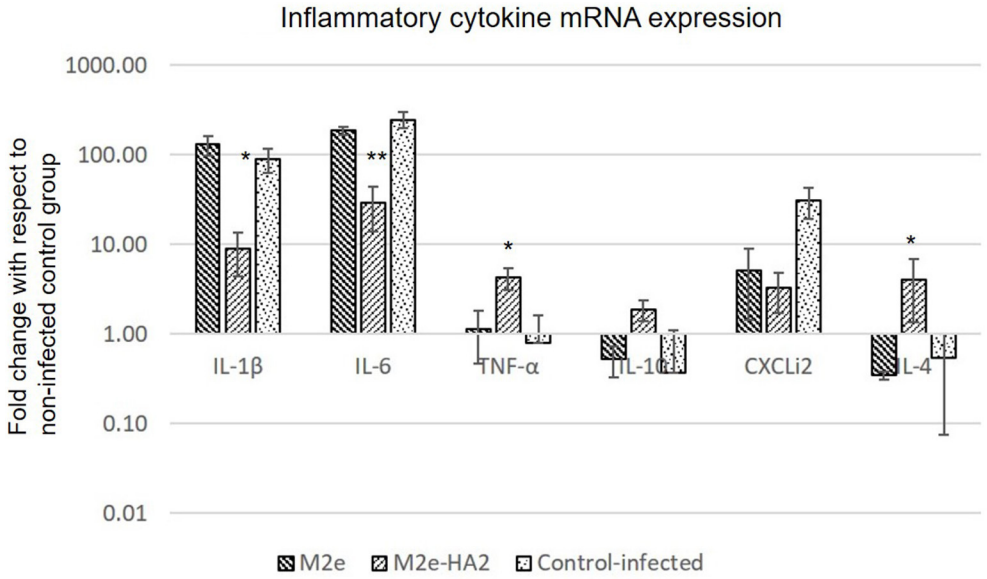

FIGURE 7 | Comparison of relative expression of inflammatory cytokines mRNA in M2e, M2e–HA2, and control chicken peripheral blood mononuclear cells (PBMCs) followed by challenge with clade 2.3.2.1 H5N1. M2e–HA2-immunized chickens showed drastic reduction of interleukin (IL)-1β and IL-6 than M2e and control. Data are means ± SD ($n = 6$), statistical significance (*$p \leq 0.05$, **0.01) vs. control-infected groups in the same time interval.

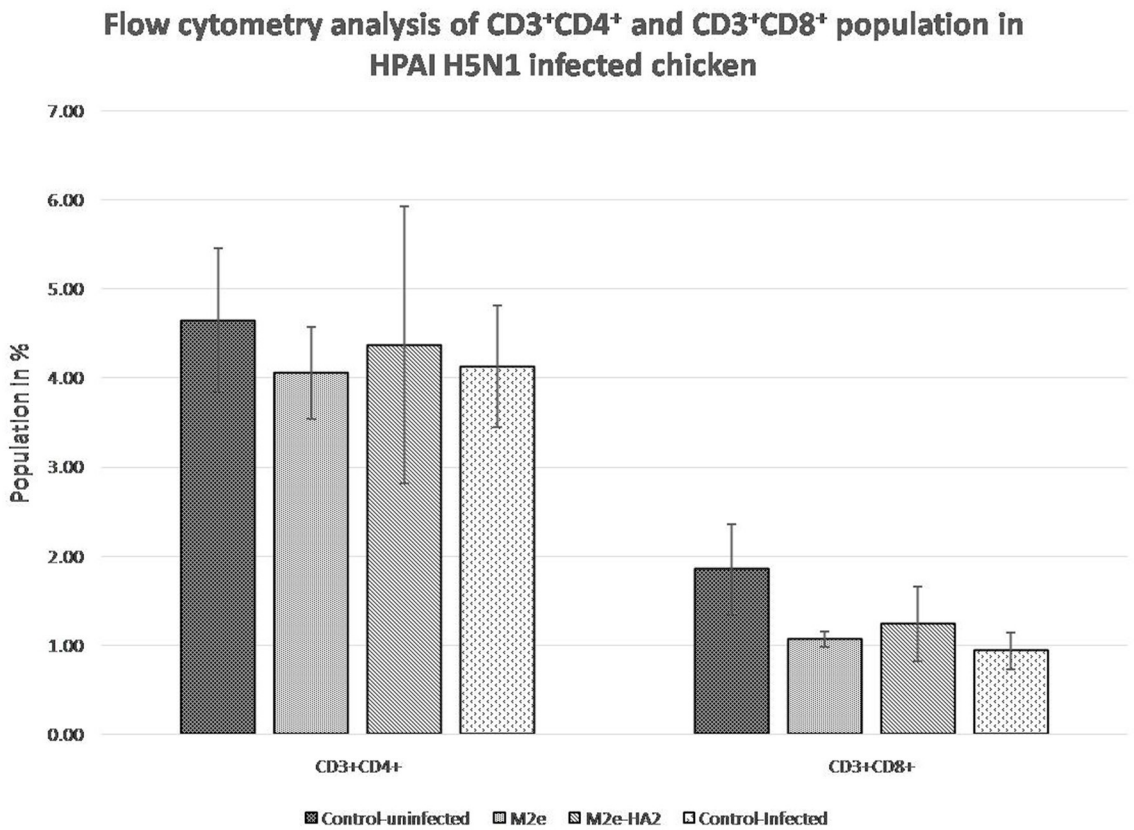

FIGURE 8 | Comparison of $CD4^+$ and $CD8^+$population in M2e- and M2e–HA2-immunized and control chickens followed by challenge with clade 2.3.2.1 H5N1. Data are means ± SD ($n = 6$), statistical significance (*$p \leq 0.05$, **0.01) vs. control non-infected group.

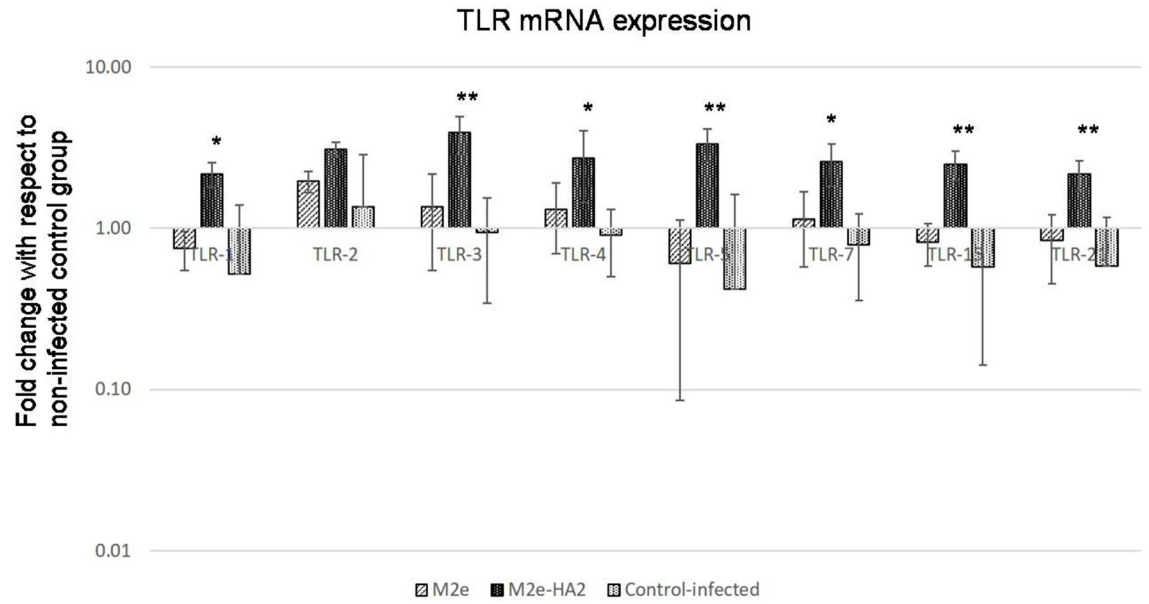

FIGURE 9 | Comparison of Toll-like receptor (TLR) mRNA expressions in M2e- and M2e–HA2-immunized and control chicken peripheral blood mononuclear cells (PBMCs) followed by challenge with clade 2.3.2.1 H5N1. M2e–HA2-immunized chickens showed upregulation of all the TLR mRNAs than M2e and control-infected. Data are means ± SD ($n = 6$), statistical significance (*$p \leq 0.05$, **0.01) vs. control-infected groups in the same time interval.

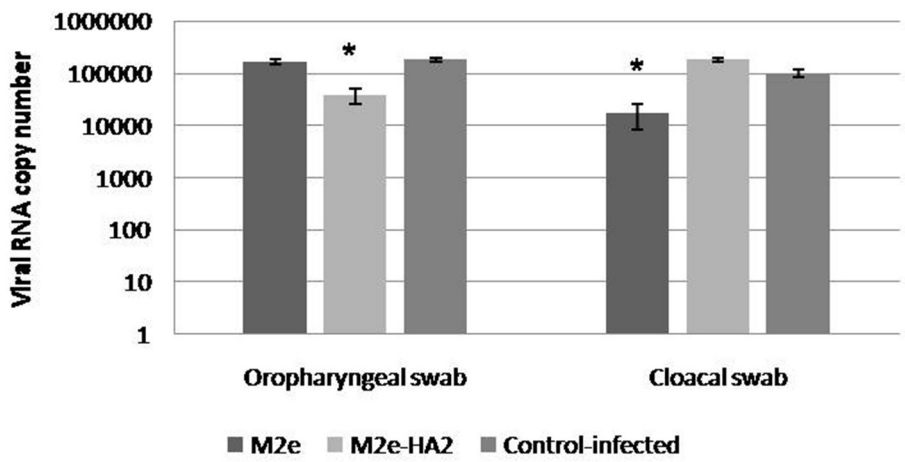

FIGURE 10 | Viral shedding detected by qRT-PCR in cloacal and oropharyngeal swabs of specific pathogen-free chickens challenged with $10^{8.0}$ EID_{50}/0.1 ml of clade 2.3.2.1 H5N1. Control-infected birds shed more virus in their oropharyngeal swabs followed by M2e and M2e–HA2 groups after 24 h. Data are means ± SD, statistical significance (*$p \leq 0.05$, **0.01) vs. control-infected group.

region of AIV, HA2 to facilitate the formation and maintenance of the larger immunogenic molecule for improving the immunogenicity of M2e (56). Combining of HA and M2e is an attractive approach for the development of broad-spectrum universal influenza vaccines, and the same had been reported by earlier workers (34, 52). Instead of selecting whole HA region, here, we have selected two immunogenic subtypic cross-protective regions, namely, HA2 (1–38) and LAH HA2 (76–130) as reported earlier (54, 55). It is expected that insertion of few conserved epitopes into recombinant proteins in any universal vaccine will lead to enhanced protective efficacy (57), hence the second subunit (HA2) of the conserved antigen was selected. In previous reports, it has been shown that HA2 (aa76–130)-based synthetic peptide vaccine using HA

from A/Hong Kong/1/1968 (H3N2) provides protection in mice against divergent subtypes H3N2, H1N1, and H5N1 (58). Therefore, we used a conserved fragment of HA2 (76–130) along with HA2 fusion peptide (1–38AA) as a second target antigen for the design of recombinant protein with broad-spectrum protection. Thus, the gene construct was designed to produce M2e–HA2 fusion recombinant protein with linkers in between them.

Cellular immune response was monitored after immunization by lymphocyte proliferation assay, flow cytometry, and cytokine mRNA analysis. Lymphocyte proliferation assay revealed that SI was increased gradually from day 0 to day 14 in the immunized group with their concerned antigen and up to 21 days with LPS than in the control group. Maximum SI with LPS at 14 and 21 days ensures the stimulation of B cells in immunized groups, which also supported by non-stimulant response of the control group. Upregulated expression of IL-10 and TNF-α mRNA expression in M2e-HA2 from 7 days onward also support the induction of humoral immunity because IL-10 and TNF-α are the important cytokines for immunoglobulin class switching, an important phenomenon in humoral immunity (59–61). Increased level of IL-4 mRNA from day 7 of the M2e–HA2 group also suggests the conversion of Th2-mediated humoral immunity (62, 63). Increased percentage of $CD4^+$ cells in the M2e-HA2 group than control and M2e indicated the enhanced cellular immune response (64).

In chickens, the currently known TLRs are TLR-1 LA, TLR-1 LB, TLR-2, TLR-3, TLR-4, TLR-5, TLR-7, TLR-15, and TLR-21. TLR-3 and TLR-7 recognize dsRNA and ssRNA molecules, respectively, in the host cells (65, 66). The chicken TLR-21 is a functional homolog of mammalian TLR-9, which induces NFκ-B production after stimulation with deoxyoligonucleotides containing CpG motifs (67). All the TLRs have been upregulated in the M2e–HA2 group at day 3 and day 7 to maximum level than those in the M2e and control groups, and the same were maximum in the M2e group on the 14th day than those in the M2e-HA2 and control groups. The results of our study indicate that conjugating M2e with HA2 effectively mediates early upregulation of TLRs than M2e alone, thereby enhancing the innate and adaptive immunity because the TLRs after stimulation by their ligands follow the cascade events of pro-inflammatory cytokine production and upregulation of co-stimulatory molecule expression, subsequently initiating adaptive immunity (68).

Although M2e-mediated humoral immunity against influenza virus has been reported in earlier studies (34, 69), most of these vaccine studies on M2 were performed in mice, while few experiments had been described for chicken with variable outcome (51, 52, 57, 70). M2e is generally a weak antigen (71)—a fact thought to be largely due to its low abundance compared with other proteins. Thus, it was hypothesized that a robust humoral immune response would be induced against M2e by linking with another conserved region of AIV, HA2, so that we could elicit immune response to two conserved regions of AIV at a time for effective response.

In this study, we also evaluated the humoral immune response of M2e–HA2 fusion recombinant protein and M2e synthetic peptide in chicken. Sera obtained from immunized chicken of all groups were found to be HI negative. Then, all the sera were tested by M2e peptide ELISA and found that the group vaccinated with M2e–HA2 fusion protein showed a positive reaction, whereas M2e alone and control group failed to produce antibody against M2e. Same types of approach were followed by earlier workers (53). The OD_{450} value of hyper immune sera against M2e–HA2 was comparatively higher than that of M2e peptide alone, indicating the abundance of HA2 antibody in natural infection than M2e. Also, we have noticed that immunization with M2e–HA2 fusion recombinant protein has induced M2e-specific antibody from day 14 of immunization whereas HA2-specific antibody was detected from day 7 of immunization and were detected by M2e peptide and M2e–HA2 recombinant protein ELISA, respectively. This observation suggests that the M2e–HA2 recombinant fusion protein elicited HA2-specific humoral immunity earlier than M2e, but at the same time, M2e-specific antibody also elicited in good amount but not earlier than HA2, and this may be due to high immunogenicity and larger molecule nature of HA2. At the same time, M2e peptide monomer was inefficient to produce an antibody response, and the same type of observation has been reported by Swinkels et al. (72), whereas immunization with M2e peptide tetrameric construct showed a significant antibody response after the booster. To the best of our knowledge, this is the first attempt to construct a fusion protein with two truncated, conserved immunogenic subunits of HA2 along with M2e to elicit a broad immune response in chicken against M2e and HA2 regions of AIV.

Recent study suggests that the host pro-inflammatory responses are one of the major contributing factors in the pathogenesis of H5N1 HPAI virus infection in chicken, and the fatal outcome could be mediated by a cytokine storm or hyper-acute dysregulation of pro-inflammatory cytokines similar to human H5N1 HPAI virus infection (73). Similar hyper-acute dysregulation of pro-inflammatory cytokines has been observed in our study also in control-infected group, whereas the cytokine response was drastically reduced in the M2e–HA2 group as a protective response. Although the cross-protective properties of M2e-based vaccines and the role of anti-M2e antibodies in cross-protection against influenza A viruses have been shown by a number of studies (41, 74–77), this M2e–HA2 fusion protein has failed to protect the chicken from a high dose ($10^{8.0}$ EID_{50}/0.1 ml) of H5N1 HPAI challenge after 48 h even after eliciting the antibodies to its conserved antigens (M2e and HA2) and inhibited the depletion of $CD4^+$ and $CD8^+$ cells to a certain extent, which is an essentiality for the novel vaccines (78–80).

In this study, we have observed that the M2e alone as a synthetic peptide was not able to induce an antibody response, whereas M2e–HA2 recombinant protein has induced antibody against both M2e as well as HA2. M2e-HA2 recombinant protein has drastically reduced the pro-inflammatory cytokines and upregulated innate immune system of chicken but failed to protect from a higher dose of HPAIV H5N1 challenge. Findings of this study indicate that despite the conservation, merely M2e and HA2-mediated immune response alone may be insufficient to protect chicken from HPAI H5N1 virus challenge, and this

will be very useful in future development of universal influenza vaccine targeting M2e and HA2 especially for chicken.

DATA AVAILABILITY STATEMENT

The original contributions generated in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institute Animal Ethics Committee (IAEC) of ICAR-National Institute of High Security Animal Diseases, Bhopal, India.

AUTHOR CONTRIBUTIONS

SK and SB designed the study. SK performed the experiments. MK, DS, BP, DD, and RS helped in the animal experiment. NM helped in the analysis. KR helped in flow cytometry analysis. SB and VS edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.571999/full#supplementary-material

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REVIEW
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Novel Vaccine Technologies in Veterinary Medicine: A Herald to Human Medicine Vaccines

Virginia Aida[1,2], *Vasilis C. Pliasas*[1,2], *Peter J. Neasham*[1,2], *J. Fletcher North*[1,2], *Kirklin L. McWhorter*[1,3], *Sheniqua R. Glover*[1,2] *and Constantinos S. Kyriakis*[1,2,4]*

[1] *Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL, United States,* [2] *Emory-University of Georgia (UGA) Center of Excellence for Influenza Research and Surveillance (CEIRS), Auburn, AL, United States,* [3] *Department of Chemistry, Emory University, Atlanta, GA, United States,* [4] *Center for Vaccines and Immunology, University of Georgia, Athens, GA, United States*

The success of inactivated and live-attenuated vaccines has enhanced livestock productivity, promoted food security, and attenuated the morbidity and mortality of several human, animal, and zoonotic diseases. However, these traditional vaccine technologies are not without fault. The efficacy of inactivated vaccines can be suboptimal with particular pathogens and safety concerns arise with live-attenuated vaccines. Additionally, the rate of emerging infectious diseases continues to increase and with that the need to quickly deploy new vaccines. Unfortunately, first generation vaccines are not conducive to such urgencies. Within the last three decades, veterinary medicine has spearheaded the advancement in novel vaccine development to circumvent several of the flaws associated with classical vaccines. These third generation vaccines, including DNA, RNA and recombinant viral-vector vaccines, induce both humoral and cellular immune response, are economically manufactured, safe to use, and can be utilized to differentiate infected from vaccinated animals. The present article offers a review of commercially available novel vaccine technologies currently utilized in companion animal, food animal, and wildlife disease control.

Keywords: veterinary vaccines, new technology vaccines, food animals, companion animals, infectious diseases, disease control and prevention

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Zhenhai Chen,
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Sherry Layton,
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***Correspondence:**
Constantinos S. Kyriakis
csk@auburn.edu

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INTRODUCTION

From Edward Jenner and Louis Pasteur in the eighteenth and nineteenth centuries, to the eradication of rinderpest in bovine and smallpox in the human populations by the twentieth century, vaccines have played a pivotal role in the survival, health, and general well-being of humans and animals (1–3).

The ultimate goal of vaccination is to generate humoral and/or cell-mediated immunity thereby inducing the production of immunological memory that confers protection against subsequent natural infection(s). The elicitation of neutralizing antibodies has long been the major goal of vaccines, however in addition to neutralizing antibodies, T-cell mediated immune responses have been shown to be crucial for effective protection against pathogens such as varicella virus, HIV, tuberculosis, and malaria (4–9).

The adaptive immune response is activated primarily through the presentation of antigens bound to a Major Histocompatibility Complex (MHC) I or II on the surface of antigen presenting cells (APCs) to T-cells and B-cells within secondary lymphoid organs. However, B-cells can take up particulate and antigen without the help of APCs provided the antigen is small enough (10). MHC-I is found in all nucleated cells while MHC-II is exclusively expressed by dendritic cells, macrophages, monocytes, B-cells, and mucosal epithelial cells (11). Nonetheless, because T cells are unable to directly interact with antigen, the mechanism of MHC presentation in conjunction with appropriate signaling plays a pivotal role in the effector cells activated and is particularly important in vaccine development in which a T-cell mediated response is desired (12). The MHC presentation is dependent on the intracellular location of the antigen processing. Cytosol derived-antigens, such as in the case of virally infected somatic cells, are processed onto MHC-I complexes and interact with CD8+ T cells, also known as cytotoxic T cells (CTLs) which directly kill infected cells (13). APCs can also present exogenously acquired antigens on MHC-I complexes, a process termed cross-presentation (14) and upon migration to lymph nodes, will activate CTLs which will migrate out of the lymph node to eliminate infected cells.

Exogenous antigens acquired by endocytosis are presented on MHC-II molecules and interact with CD4+T helper (T_H) cells. T-helper cells have various fates and effector functions which are influenced by the type of signal elicited during priming and activation. Pertinent to vaccine production, T-helper 1 (T_H1) cells produce interferon-γ and tumor necrosis factor alpha which potentiate the effector function of phagocytes and increase inflammation (15). Thus, vaccine-induced memory T_H1 cells are particularly sought for intracellular pathogens. T-helper 2 (T_H2) cells facilitate B-cell proliferation whilst antagonizing T_H1 differentiation and are therefore associated with increased humoral responses and of particular interest for vaccines targeting parasites or allergic responses (16, 17). T-follicular helper cells (T_{FH}) interact with B-cells that present antigen on MHC-II molecules (12, 18, 19). Only B cells that receive co-stimulatory signals from T_{FH} cells are able to generate high-affinity IgG antibodies or mature into memory B-cells (20). As such, vaccines aimed to generate robust B-cell memory need to also stimulate T-cell responses.

The classical inactivated and modified-live vaccines (IV and MLV, respectively), also known as first generation vaccines, have given humans and animals alike advantages over the pathogenic world that surrounds them. These vaccines have also had an economic impact due to the success that has been seen in livestock industries (21). IVs are safe and relatively inexpensive to produce, predominantly present antigens *via* the MHC-II pathway and mainly induce humoral immune responses. Due to this disadvantage, pathogens requiring a strong cell-mediated response can escape the pressure elicited by the vaccine (22). MLVs circumvent this issue, due to their ability to successfully replicate within the host and elicit protective immunity against their respective pathogens. These attenuated pathogens mimic natural infection thereby eliciting both MHC-I and MHC-II pathways. Some MLVs have been shown to elicit mucosal IgA antibodies, a unique feature to only a handful of vaccines administered *via* the oral or nasal route (23). However, MLVs pose a slight risk to animals as there were rare cases where attenuated strains regained pathogenicity, causing the spread of disease (21, 24–27). Additionally, MLVs are contraindicated in severely immunocompromised individuals due to the risk of disease (28). These classical vaccines have predominated commercial human and animal immunizations for the past 100 years. However, the aforementioned disadvantages have directed second and third-generation vaccines into the limelight of exploration.

These second and third generation vaccines have shown success in veterinary medicine thereby paving the way for advancement in human medicine (**Figure 1**). Second generation vaccines include subunit elements, conjugated/recombinant antigens, or synthetic proteins (**Table 1**). Recombinant subunit vaccines do not use virus (inactivated or live), but rather utilize antigen production through overexpression and purification of the antigen. This can be achieved through multiple routes, including the baculovirus expression vector system (BEVS). Subunit vaccines oftentimes lack the pathogen associated molecular patterns that the immune system utilizes to recognize pathogens *via* pattern recognition receptors. Because of this, subunit vaccines necessitate adjuvants with co-stimulatory activity that enhance the magnitude and quality of the immune response. Furthermore, these types of vaccines are generally recognized by antigen presenting cells *via* the intravesicular route and are consequently presented on MHC-II complexes.

Third generation vaccines include gene-based (DNA and RNA) vaccines, viral-vector platforms, and live or inactivated chimeric vaccines. DNA and RNA-based vaccines is a fundamentally new approach to vaccination, involving the use of plasmid DNA delivered through injection (**Table 2**). Advancements in molecular biology techniques have allowed us to manipulate these polynucleotides to our advantage, providing alternative routes to the classical vaccine technologies (29, 30). DNA vaccines employ the use of a plasmid containing the DNA encoding the antigen(s) of interest. Once inserted into host cells, the cellular machinery will express the antigens encoded by the DNA and an immune response will be elicited. Some advantages of DNA vaccines include the safe administration to immunocompromised individuals compared to MLVs, the potential for combining multiple plasmids for a broad-spectrum combination vaccine, and the ease of engineering compared to classical vaccines (31–33). Along with these advantages DNA vaccines induce both humoral and cell mediated responses, and function as pathogen associated molecular patterns (PAMPS) attenuating the necessity for adjuvant (32, 34, 35). More specifically, plasmid-DNA and RNA vaccines transfect cells and thus mimic intracellular pathogen protein production and typically induce strong MHC-I mediated CD8+T cell responses (36). Transfected somatic cells will present antigen on MHC-I, thereby eliciting CTLs cross-primed by dendritic cells. Additionally, APCs engulf transfected cells and present antigen on MHC-II complexes to elicit a CD4+ T-cell response (37).

Recombinant viral vector vaccines are novel technologies in veterinary medicine that utilize viruses as tools for vaccinology

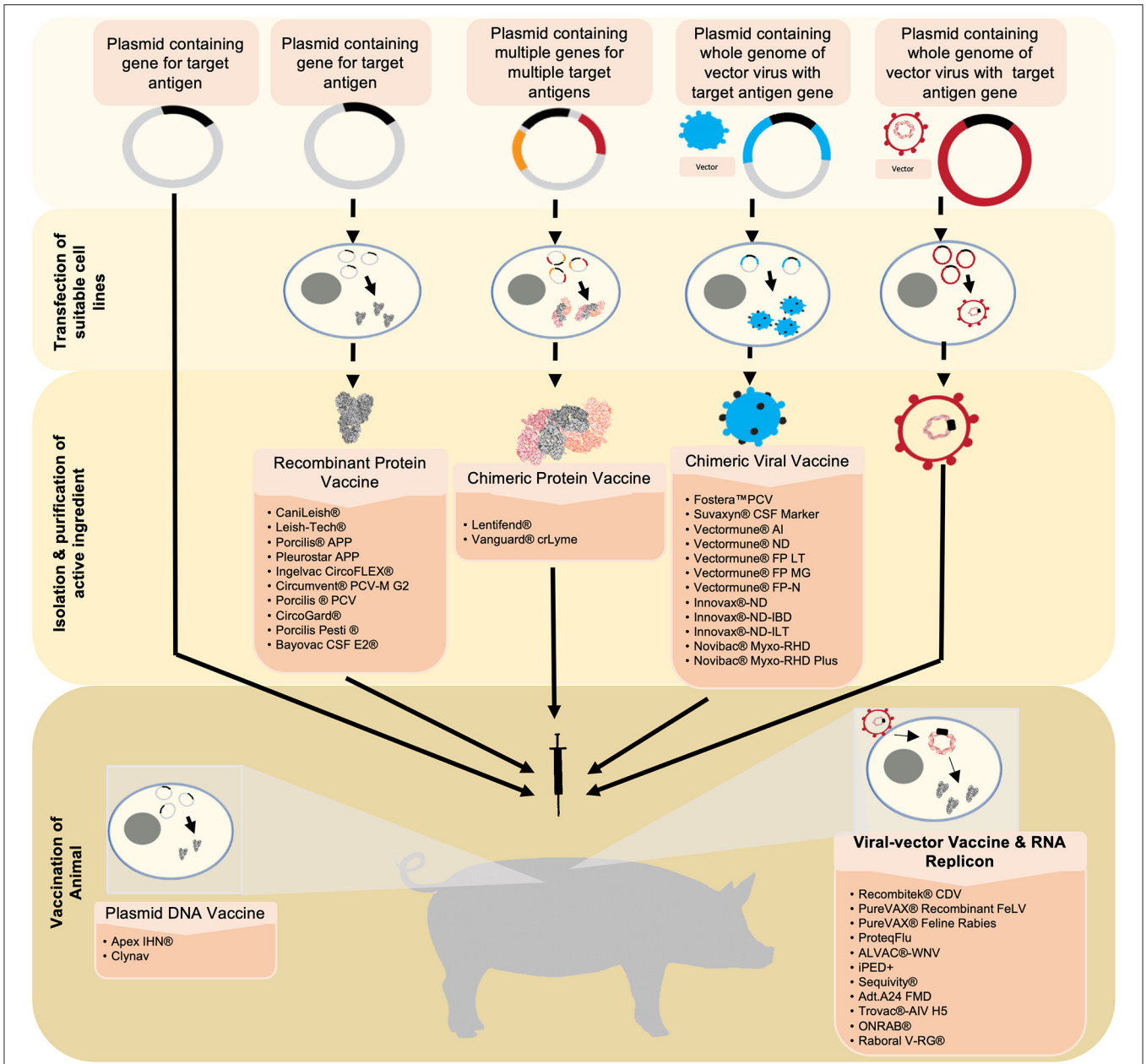

FIGURE 1 | Six novel vaccine technologies discussed in this review are simplified and summarized starting from the generation and production of antigens to the vaccination. Beginning with plasmid-DNA vaccines, the target antigen is inserted into a plasmid. This serves as the active ingredient that will be used to vaccinate the animal. Upon vaccination, the plasmid-DNA vaccine carrying the DNA encoding for the target antigen is translated into the desired protein in the vaccine recipient's cells. The antigen is then expressed from the cell, consequently eliciting an immune response. Recombinant protein vaccines and chimeric protein vaccines utilize a similar technology. However, suitable cell-lines are transfected with the plasmid in which the antigen(s) is/are expressed. The antigen(s) is/are then harvested, purified, and formulated into the vaccine. Chimeric viral vaccines utilize a plasmid containing the whole genome of a virus that will be used as a vector in addition to the target gene for the desired antigen. This plasmid is then used to transfect a suitable cell-line in which a whole virus expressing the integrated antigen is produced. This virus is harvested and purified, and formulated into a vaccine. Viral vectors utilize a virus that has been engineered to express the gene of interest. The virus is formulated into a vaccine and will release the recombinant genes into the host cells. Similar to a plasmid-DNA vaccine, the genes will be transcribed into the target antigen which will then be expressed and elicit an immune response. RNA replicon vaccines utilize a RNA segment that encodes the desired antigens encapsulated in a vesicle carrier. Once in the host's cell, the RNA is directly translated, resulting in the expression of the target antigen.

(**Table 3**). These vaccines are genetically engineered and involve the insertion of DNA encoding key antigens into a viral vector. The safety profile is similar to inactivated (killed) subunit vaccines and stimulate both cell-mediated, specifically CD8+T cell responses, and humoral immune responses (9, 38). Pox viral vectors were the first to be studied and established in

TABLE 1 | Subunit and recombinant protein vaccines.

Species	Vaccine	Manufacturer	Pathogen	Technology
Canine	CaniLeish® (LiESP/QA-21)	Virbac	Leishmania	Subunit (Cell-free, serum-free culture system)
	Leish-Tech®	CEVA Animal Health	Leishmania	Recombinant Protein
	Lentifend®	Laboratorios Leti	Leishmania	Chimeric protein
	Vanguard® crLyme	Zoetis	Borrelia burgdorferi	Chimeric Protein
Swine	Porcilis®APP	Merck Animal Health	A.pleuropneumoniae	Subunit
	Pleurostar APP	Novartis	A.pleuropneumoniae	Subunit
	Ingelvac CircoFLEX®	Boehringer Ingelheim	Porcine Circovirus Type 2	Subunit (BEVS)
	Circumvent® PCV-M G2	Merck Animal Health	Porcine Circovirus Type 2	Subunit (BEVS)
	Procilis® PCV	Merck Animal Health	Porcine Circovirus Type 2	Subunit (BEVS)
	CircoGard	Pharmgate Animal Health	Porcine Circovirus Type 2	Subunit (BEVS)
	Porcilis Pesti®	Merck	Classical Swine Fever	Subunit (BEVS)
	Bayovac CSF E2®	Bayer	Classical Swine Fever	Subunit (BEVS)

TABLE 2 | DNA vaccines.

Species	Vaccines	Manufacturer	Pathogen	Plasmid(s)
Salmonid	Apex IHN ®	Elanco (Aqua Health)	Infectious Hematopoietic Necrosis	pUK21-A2, pUK-ihnG
	Clynav	Elanco (Aqua Health)	Salmonid Alphavirus Subtype 3	PUK-SPDV-poly2#1

TABLE 3 | Recombinant viral vector vaccines.

Species	Vaccine	Manufacturer	Pathogen	Technology (viral-vector)
Canine	Recombitek® CDV	Boehringer Ingelheim	Canine Distemper Virus	Viral-Vector (canarypox)
Feline	PureVAX® Recombinant FeLV	Boehringer Ingelheim	Feline Leukemia Virus	Viral-Vector (canarypox)
	PureVAX® Feline Rabies	Boehringer Ingelheim	Rabies	Viral-Vector (canarypox)
Equine	ProteqFlu	Boehringer Ingelheim	Equine Influenza	Viral-Vector (canarypox)
	ALVAC®-WNV	Pfizer	West Nile Virus	Viral-Vector (canarypox)
Swine	Fostera™PCV	Zoetis	Porcine Circovirus Type 2	Chimeric Viral-vector (PCV-1)
	Suvaxyn® CSF Marker	Zoetis	Classical Swine Fever virus	Chimeric Viral-vector (BVDV)
	iPED+	Merck Animal Health	Porcine Endemic Diarrhea virus	RNA Replicon (VEEV)
	Sequivity®	Merck Animal Health	Swine influenza A virus	RNA Replicon (VEEV)
Bovine	Adt.A24 FMD	GenVec	Foot and Mouth Disease	Viral-vector (adenovirus)
Avian	Trovac®-AIV H5	Boehringer Ingelheim	Avian Influenza	Viral-vector (fowlpox)
	Vectormune® AI	CEVA Biomune	Avian Influenza	Chimeric Viral-vector (HVT/MD)
	Vectormune® ND	CEVA Biomune	Newcastle Disease	Chimeric Viral-vector (HVT/MD)
	Vectormune® FP LT	CEVA Biomune	Infectious Laryngotracheitis virus	Chimeric Viral-vector (fowlpox)
	Vectormune® FP MG	CEVA Biomune	Mycoplasma Gallisepticum	Chimeric Viral-vector (fowlpox)
	Vectormune® FP-N	CEVA Biomune	Newcastle Disease	Chimeric Viral-vector (fowlpox)
	Innovax®-ND	Merck Animal Health	Newcastle Disease	Chimeric Viral-vector (HVT/MD)
	Innovax®-ND-IBD	Merck Animal Health	Newcastle disease and Infectious bursal disease	Chimeric Viral-vector (HVT/MD)
	Innovax®-ND-ILT	Merck Animal Health	Newcastle disease and infectious laryngotracheitis	Chimeric Viral-vector (HVT/MD)
Wildlife	ORNAB®	Artemis Technologies, Inc.,	Rabies	Viral-vector (human adenovirus type 5)
	Raboral V-RG®	Boehringer Ingelheim	Rabies	Viral-vector (vaccinia virus)
Rabbits	Novibac® Myxo-RHD	Merck Animal Health	Rabbit Hemorrhagic Disease	Chimeric Viral-vector (myxoma virus)
	Novibac® Myxo-RHD Plus	Merck Animal Health	Rabbit Hemorrhagic Disease	Chimeric Viral-vector (myxoma virus)

the 1980's, with various backbones being utilized to induce responses to various animal pathogens, including canarypox and fowlpox backbones (39–43). Adenovirus vectors have been explored as systems of treatments for numerous infections, and even as vaccines against tumor-associated antigens (44). Positive sense RNA-containing alphaviruses have also been used

as vector backbones, and these constructs include two types: full-length infectious clones and replicon vectors. The latter type is advantageous due to their lack of structural protein genes, only containing the non-structural genomic region and the genes encoding the antigen(s) of interest (45). For alphavirus-replicons, foreign genes of interest can be inserted in the place of the structural genes generating self-replicating RNA replicons (RP) (46). Upon inoculation, the RP is engulfed by dendritic cells and consequently directs the translation of large amounts of protein in the cells resulting in the presentation of the antigen. This essentially makes them self-replicating RNA molecules. These concepts can then be extended into chimeric recombinant vector vaccines, where the principles are the same, yet the genes, and by extension antigens, of interest are taken from multiple types of the pathogen and placed within the same vector, aiming to elicit a broader immune response.

Another component that distinguishes veterinary from human vaccines is the technology that enables the differentiation of infected and vaccinated animals (DIVA), making them a critical tool in disease control and eradication (47). This technology has also made a huge impact on imports and exports as it provides a sensitive, rapid, and inexpensive method for determining pathogen free flocks and herds (48). Most DIVA vaccines, or marker vaccines, are based on recombinant deletion mutants of wild-type pathogens, where gene segments expressing viral proteins, such as the herpesvirus envelope glycoprotein (gE), have been removed. Other DIVA vaccines are based on subunit vaccines and inactivated whole virus vaccines (**Table 4**). Because DIVA vaccines elicit a different immune response from that elicited by a natural infection companion diagnostic tests, typically an enzyme linked immunosorbent assays (ELISA) can be utilized to discern those infected and those vaccinated. DIVA vaccines have been utilized in the control of Foot-and-mouth disease, Classical swine fever, Bovine rhinotracheitis, and the eradication of Pseudorabies (Aujesky's disease) in pigs (49–51).

Overall, veterinary medicine has made great strides in vaccine development for a wide array of pathogens, and has spearheaded vaccinology methodologies and designs, being years in advance compared to human vaccine technologies. In this review, current commercially available and licensed technologies being utilized in veterinary vaccinology are presented.

COMPANION ANIMALS

Canine Vaccines

Canine Distemper Virus—Recombitek® Combination Vaccines (CDV)

Canine distemper virus (CDV) belongs to the *Paramyxoviridae* family and is closely related to the human measles virus and bovine rinderpest virus. CDV is found worldwide, affects all members of the *canidae* family, and is responsible for significant disease, often resulting in high morbidity and mortality in unprotected animals. Recombitek® vaccines, produced by Merial Animal Health (now Boehringer Ingelheim Animal Health), utilize a recombinant canarypox-vector expressing both the antigenic hemagglutinin and fusion glycoproteins of CDV and are co-formulated with other MLVs (adenovirus type 2, coronavirus, parainfluenza, or parvovirus) or bacterial antigens. These vaccines are the only virus-vectored CDV vaccines licensed and commercially available for canines to-date. One of the major benefits to this vaccine is the canarypox-vector does not have the complete CDV genome nor infectious components of CDV and therefore the risk of post-vaccinal CDV encephalitis is eliminated (52). Studies have shown the Recombitek® CDV has comparable time-to-immunity to MLV-CDV vaccines, can confer moderate protection against virus challenge within hours of being vaccinated, and fully protects animals within 1 week of vaccination (53). Furthermore, unlike MLV-CDV vaccines, Recombitek® CDV can be utilized in pre-weaning disease and immunosuppressed individuals as it was shown to protect puppies in the presence of maternal antibodies whilst not suppressing lymphocyte responsiveness (54, 55). Recombitek® CDV has a significant anamnestic response and confers a 4-fold greater increase in titer upon booster vaccination (particularly when the dogs received a MLV-CDV vaccine initially) and a 36 months serologic duration of immunity (56–58). In comparison to MLV-CDV vaccines, Recombitek® CDV induces a lower serum-neutralizing titer compared to MLV-CDV vaccines in non-domestic carnivores (59).

Canine Lyme disease—VANGUARD® crLyme

Lyme disease, caused by the spirochete *Borrelia burgdorferi*, is the most common vector-borne illness in North America and Europe and infects a range of vertebrate animals including small mammals, lizards, and birds (3, 60). Previous studies have shown that 63% of dogs exposed to infected ticks, the vector transmitting *B. burgdorferi*, develop clinical signs of Lyme disease which consist of severe elbow or shoulder joint lameness of sudden onset, joint swelling of the shoulder, elbow and carpus, and acute arthritis (61). There are several commercially available canine vaccines against *B. burgdorferi* by inducing the production of outer surface protein A (OspA) borreliacidal antibodies. These antibodies form a membrane attack complex within the tick transmitting *B. burgdorferi* during the blood meal on the host (62). Because OspA is genospecific, it has been identified that targeting both OspA and outer surface protein C (OspC) is a more advantageous vaccination tactic because OspC is conserved among several of the pathogenic *Borrelia* genospecies. Nonetheless, the combination of both antigens provides complete protection from Lyme disease (63–66). VANGUARD® crLyme, created by Zoetis, is the only commercially available chimeric recombinant Lyme vaccine based on chimeric epitope-based recombinant proteins. It contains both antigens for OspA and 14 different linear epitopes derived from seven types of OspC and thus provides broad-spectrum protection (67). While investigating the efficacy and safety of VANGUARD® crLyme, researchers found the vaccine showed a 93.7% reduced incidence of *B. burgdorferi* infection and demonstrated significant humoral responses to both OspA and OspC after vaccination. Upon challenge with ticks suspected of carrying *B. burgdorferi*, vaccinated animals showed no humoral response to OspC antigen suggesting VANGUARD® crLyme prevented *B. burgdorferi* transmission

TABLE 4 | DIVA vaccines.

Species	Vaccine	Manufacturer	Pathogen
Canine	Leish-Tech®	CEVA Animal Health	Leishmania
	Lentifend®	Laboratorios Leti	Leishmania
Feline	PureVAX® Recombinant FeLV	Boehringer Ingelheim	Feline Leukemia Virus
Swine	Porcilis® Begonia	Merck Animal Health	Suid Herpesvirus 1
	Auskipra® GN	Hipra	Suid Herpesvirus 1
	Suvaxyn® CSF Marker	Zoetis	Classical Swine Fever Virus
Bovine	Adt.A24 FMD	GenVec	Foot and Mouth Disease
	Bovilis® IBR Marker Live	Intervet	Bovine Herpesvirus-1
	Hiprabovis® IBR Marker Live	Hipra	Bovine Herpesvirus-1
	Bayovac IBR Marker Vivum	Bayer	Bovine Herpesvirus-1
	Bayovac IBR Marker Inactivum	Bayer	Bovine Herpesvirus-1
	Rispoval® IBR-Marker Inactivated	Zoetis	Bovine Herpesvirus-1
	Rispoval® IBR-Marker Live	Zoetis	Bovine Herpesvirus-1

from infected ticks to vaccinated dogs (68). In contrast to these findings, the comparison of VANGUARD® crLyme to Recombitek® Lyme (the commercially available monovalent recombinant OspA vaccine) revealed VANGUARD® crLyme elicited a slower anti-OspA antibody response, had a lower serum borreliacidal activity at all post-vaccination time points, and had inferior immunogenicity (69). Grosenbaugh et al. (69), note that the variation in efficacy could be contributed to the lipidation differences of the antigens but also a mismatch between the OspC antigens used in the vaccine and the antibody assay used to evaluate the response. In a more recent study, VANGUARD® crLyme was shown to induce broadly cross-reactive antibodies to 25 recombinant OspC variants screened against sera of vaccinated animals, significantly reduce histopathological changes at the tick bite site, and prevent *B. burgdorferi*-induced synovitis and dermatitis (68).

Canine Visceral Leishmaniasis—CaniLeish®, Leish-Tec®, Letifend®

Canine leishmaniosis (CanL), caused by the protozoan *Leishmania infantum*, is a severe and chronic disease transmitted by the bite of a sandfly. Currently, leishmaniasis is endemic in the Mediterranean basin, Middle East, Central Asia, and Latin America. Importantly, domestic dogs are reservoirs for human visceral leishmaniasis in many areas (70). It is estimated that 30% of dogs in endemic areas are seropositive and some will eventually become clinically ill. Unfortunately, CanL cannot be easily cured with current therapies. Accordingly, the high prevalence implores the creation of an effective vaccine that elicits a robust and long-lasting Th1-mediated response in order to prevent the development of disease after infection. There are three vaccines available on the market to date: CaniLeish® (Virbac S.A.), Leish-Tec® (CEVA animal health), and Lentifend® (Laboratorios Leti). Leishmune® (Zoetis) was removed from the market in 2018 and will therefore not be discussed in this review.

CaniLeish® (LiESP/QA-21) was the first leishmaniasis vaccine in Europe and is indicated for the active immunization of *Leishmania* by providing a significant reduction in disease progression (71). Overall, CaniLeish® is a well-tolerated vaccine formulated with *L. infantum* Excreted-Secreted Protein (LiESP) antigens and a purified extract of *Quillaja saponaria* (QA-21) adjuvant (72). CaniLeish® has a 4 week onset of immunity characterized predominately by an IgG-2 response to ESP and a significantly strong cell-mediated Th1-dominated immune profile that remains persistent for a full year after the primary vaccination course (71, 73, 74). In a major clinical trial, CaniLeish® provided a protection of 68.4% in vaccinated animals compared to unvaccinated controls (71). Additionally, vaccinated dogs had lower mean parasite burdens due to the facilitation of a stronger macrophage-induced intracellular parasitic reduction in conjunction with autologous lymphocytes (73, 75). Unfortunately, CaniLeish® does not prevent initial entry and migration of the parasites and does not produce antibodies that can be distinguished from conventional immunofluorescence antibody tests (IFAT) diagnostic testing (71).

Leish-Tec® is licensed as another second generation vaccine in Brazil. This vaccine contains recombinant protein A2 antigens of various Leishmania species and a saponin adjuvant (76, 77). The vaccine is tolerated similarly to CaniLeish®, elicits an anti-A2 IgG1 antibody, IgG2 antibody, and Th1 immune response 1 month after vaccination (78, 79). This vaccination induces a significant reduction in the transmission of *Leishmania* spp. by sandflies that feed on anti-A2 seropositive vaccinated dogs and reduces the risk of disease progression and all-cause mortality in asymptomatic infected dogs (80, 81). In a field trial study, mean seroconversion time and cumulative incidence of infection among immunized dogs was ∼18 months and 27%, respectively while unvaccinated mean seroconversion time was ∼9 months and 42%, respectively (79). In that same study 43% of the vaccine recipients eventually developed clinical signs rending the efficacy of Leish-Tec® questionable (79). Currently, the Brazilian government advices the culling of all seropositive dogs. Fortunately, Leish-Tec® is considered a DIVA vaccine since the humoral response induced by Leish-Tec® can be detected by

A2-ELISA and does not create cross-reacting interference with conventional leishmaniosis serological diagnostic tests (79, 82).

Lentifend® contains the recombinant antigen Protein Q, a chimeric protein formed by the fusion of five antigenic determinants from four Leishmania proteins and is without adjuvant (76). Lentifend® consistently elicits a cellular and humoral immune response characterized by a significant increase in complement system proteins and an early and statistically significant increase of IgG2 antibodies against Protein Q 2 weeks after vaccination (76, 83, 84). Lentifend® has shown to be very well-tolerated, reduce circulating immune complexes, parasite burden, the incidence of clinical signs, and the number of confirmed cases, and have an overall efficacy of 72% (76, 83). Much like Leish-Tec®, Lentifend® falls into the DIVA category (85, 86).

Feline Vaccines

Feline Leukemia Virus- PureVAX® Recombinant FeLV

Feline leukemia virus is an immunosuppressive retrovirus infecting domestic and wild felids. It can be transmitted *via* direct contact or through virus shed in saliva or nasal secretions and affects multiple organ systems. It is estimated that 2.3–3.4% of all cats in North America are affected (87). PureVAX® Recombinant FeLV, produced by Boehringer Ingelheim Animal Health, is a non-adjuvanted canarypox virus-vectored vaccine that contains the mutated envelope, gag, and truncated polymerase protein of the FeLV subtype A/Glasgow-1 strain (88, 89). The immune response elicited by PureVAX® Recombinant FeLV is characterized by the activation of cell-mediated immunity by inducing FeLV-specific T cell response (89–91). Compared to other commercially available vaccines, Recombinant FeLV has similar degrees of protection from persistent viremia and integration of proviral DNA upon virus challenge and a 93% preventive fraction (92). Nonetheless, a 3-year duration of immunity after a prime and boost vaccination protocol has been shown to confer full protection against persistent viremia (93).

Feline Rabies—PureVAX® Feline Rabies

Rabies is a zoonotic, progressive neurological, and fatal infection caused by rabies virus. Rabies infection is present throughout the world, responsible for over 60,000 human deaths per year, and affects all warm-blooded animals (94). PUREVAX® Feline Rabies contains the recombinant canarypox virus (vCP65) that expresses the rabies glycoprotein gene. Inoculation of animals with vCP65 demonstrated an appropriate level of foreign gene product expression sufficient enough to induce rabies-specific serum neutralizing antibodies and T-cell responses to protect against lethal rabies virus challenge for up to 3 years (95). PUREVAX® Feline Rabies provides full protection even when co-administered with other feline vaccines illustrating the usefulness in yearly core vaccinations (96). Additionally, because this vaccine lacks an adjuvant, there is excellent local safety and minimal inflammatory reactions since chronic inflammation at the injection site is a risk factor for vaccine-induced fibrosarcomas in felines (97).

Equine Vaccines

Equine Influenza—ProteqFlu

Equine Influenza virus (EIV) is an *Orthomyxovirus* considered to be an important respiratory disease in horses. Equine Influenza has had major economic and welfare implications within the last decade and is particularly difficult to control due to the virus' inclination to readily undergo antigenic drift and shift. Unfortunately, Vaccine mismatch to the circulating strain can contribute to a significantly decreased efficacy in eliciting appropriate host immune response.

ProtequFlu (marketed by Boehringer Ingelheim, formerly Merial Animal Health) contains two modified live canarypox virus recombinants expressing the EIV hemagglutinin (HA) gene of two significantly important strains of circulating EIVs.ProtequFlu has been shown to generate significantly high IgGa and IgGb anti-influenza antibody titers pre-challenge, a long-term 6-month anamnestic IgGa and IgGb protective responses post challenge with several American lineages and induces a specific IFN-y and IL-2 mRNA expression (98, 99). In animals older than 8 months, vaccination has shown to provide protection after a single dose compared to the required two doses of inactivated vaccine and has thus been utilized as a means for emergency response to IEV outbreaks (100, 101). However, some studies found that foals <8 months did not seroconvert until the third immunization suggesting the presence of maternally derived antibodies contributes to this immunization pattern and might influence vaccination protocols (102). Regarding long-term immunity, ProteqFlu-Te® was not as robust as the whole commercial inactivated vaccines, Equilis Prequenza-Te® and Duvaxyn IE-T Plus®, or when ProteqFlu-Te® was combined in a mixed-vaccination protocol which is a common practice in the field (103).

West Nile Virus—ALVAC®-WNV & West Nile-Innovator® DNA

West Nile Virus (WNV) is a mosquito-transmitted neurotropic *Flavivirus* causing debilitating and potentially fatal disease found worldwide in birds, humans and horses (the two latter species being the dead-end hosts) (104, 105). Successful vaccination requires both the induction of neutralizing antibodies and cell-mediated immune response including the elicitation of INF-α, INF-β, and significant involvement of the complement system (104, 106, 107). IgM is critically important for the control of acute and early WNV infection followed by the presence of IgG antibodies which confer long-term protection against WNV re-infection (108, 109).

Merial Animal Health (now Boehringer Ingelheim Animal Health) developed ALVAC®-WNV, a canarypox-vectored recombinant chimeric vaccine that expresses the precursor membrane (prM) and envelope (E) genes of WNV derived from the 1999 New York Isolates (110). ALVAC®-WNV induces neutralizing antibodies and prM/E-insert-specific IFN-y+ producing cells against WNV in vaccinated horses and therefore plays a major role in anti-viral clearance (107, 111). ALVAC®-WNV vaccine was shown to be fully protective against virulent WNV challenge *via* mosquito exposure making it exceptionally applicable in the field (112). Additionally, ALVAC®-WNV

induces WNV antibodies as early as 7 days, develop protection against viremia as early as 26 days after a single dose, was fully protective against challenge, and elicited an immune response that could be recalled 9 months after appropriate primary vaccination and booster vaccination (107, 112, 113). West Nile-Innovator® DNA, a WNV DNA plasmid-based vaccine, licensed in 2005 by Fort Dodge Animal Health/Pfitzer, contained an unformulated plasmid DNA encoding the prM and E protein of WNV and a MetaStim™ adjuvant (110, 114). This vaccine resulted in a humoral and strong Th1 response however, the vaccine was discontinued by Pfizer (110, 115, 116).

FOOD ANIMALS

Porcine Vaccines

Pleuropneumonia—Porcilis® APP and PleuroStar APP

A second generation of subunit vaccines targeting the bacterium *Actinobacillus pleuropneumoniae* (APP) was previously developed by Merck Animal Health and Novartis. APP is the active agent that causes porcine contagious pleuropneumonia disease in swine through the bacterium's ApxI, ApxII, ApxII, and ApxIV toxins (117, 118). Fifteen known serotypes of APP are currently characterized, each that can cause variable pathogenicity (119). The acute form of porcine contagious pleuropneumonia is often fatal by inducing hemolytic and cytotoxic lung damage leading to pleuropneumonia (119). The disease is most severe in piglets 6–22 weeks old, usually before they go to market (119). Consequently, APP is a huge economic burden for the swine industry. Porcilis® APP and PleurostarAPP are commercially available second-generation subunit vaccines that each provides some cross protection against the 15 serotypes of *A. pleuropneumonia* (120–122). The vaccines are based on four or five purified proteins produced by *c* strains. This includes the exotoxins ApxI, ApxII, ApxIII and a 42 kilodalton outer membrane protein for the development of PorcilisAPP, and the ApxII, TbpB, CysL, OmlA, and OmlA proteins for PleurostarAPP (119, 123).

Porcilis® APP has been shown to develop a protective immunity with a peak 2–3 weeks after boost vaccination which can be maintained for up to seven weeks, confer protection in terms of clinical signs, reduced lung lesions, and reduce mortality for serovar 1 (123). In an experiment conducted by Del Pozo Sacristan et al., Porcilis® APP was evaluated in herds chronically affected by pleurisy. Vaccinated animals had significantly lower prevalence and extent of pleurisy 4.1 and 2.5%, respectively vs. the non-vaccinated animals of 18.5 and 8.0%, respectively. Vaccinated animals gained more weight than pigs in the non-vaccinated group. Additionally, antimicrobial use and mortality were reduced in vaccinated animals suggesting that although vaccination may not prevent clinical expression of APP infection, it could be useful in reducing the impact of infection (121, 123).

Porcine Circovirus Type 2—Ingelvac CircoFLEX®, Circumvent® PCV-M G2, Porcilis® PCV, CircoGard & Fostera™ PCV

Two types of circoviruses have been identified in swine, porcine circovirus 1 (PCV1) and porcine circovirus 2 (PCV2), where only the latter is considered pathogenic (124). PCV2 is the causative agent of Porcine Circovirus Associated Disease, which includes multiple clinical syndromes of swine such as Post-weaning Multisystemic Wasting Syndrome, porcine dermatitis and nephropathy syndrome, and PCV2-induced reproductive disorders (125–127).

Ingelvac CircoFLEX® (produced by Boehringer Ingelheim®), Circumvent® PCV-M G2 & and Porcilis® PCV (both produced by Merck), and CircoGard (produced by Pharmgate Biologics) are licensed subunit vaccines that were developed using a BEVS system to express the PCV-2 ORF-2 protein (128). For both Ingelvac CircoFLEX® and Circumvent® PCV-M G2 vaccines, the ORF-2 protein is used as a basis to elicit an immune response in swine against PCV-2 (129). In general, vaccination with these technologies in young piglets resulted in attenuated weight loss, shortened viremia, and lower viral load (130). Fostera™ PCV vaccine produced by Zoetis is single-dose inactivated chimeric PCV1-2 viral-vector vaccine. It utilizes the genome of the non-pathogenic PCV1 as the backbone, cloned with the ORF2 gene of PCV2 which encodes the immunogenic capsid protein of the virus (131). Vaccinated animals demonstrated increased concentration of neutralizing antibodies and anti-PCV2 IgG antibody titers which correlate with the significant reduction of viremia and replication of PCV2 compared to negative control animals (132, 133). Moreover, this chimeric vaccine induced a strong cell mediated immune response (CD3+ and CD4+ cells) that may explain the decrease of PCV2 genomic copies in the blood of immunized pigs (132).

Suid Herpesvirus-1 (Pseudorabies/Aujesky's Disease)—Porcilis® Begonia (MSD Animal Health-Intervet), Auskipra® GN (Hipra)

Suid herpesvirus 1 (SuHV-1) is an *Alphaherpesvirus* responsible for Aujesky's disease (also known as Pseudorabies). This highly contagious pathogen infects a wide range of animal species with swine being the principal reservoir and host of the virus. Disease in pigs includes a variety of clinical symptoms, neurological signs and high mortality rate up to 100% in piglets while older pigs mainly showcase respiratory signs. Infected sows demonstrate a variety of reproductive disorders such as abnormal return to estrus, abortions, stillbirth, mummified or week piglets (50). The predominant clinical symptoms in secondary hosts (cattle, dogs, and cats) are severe pruritus and neurological disorders (127). Nonetheless, this pathogen causes significant economic losses in naïve pig farm production sites and still remains a notifiable disease in the USA (134). SuHV-1 is a DNA virus comprised of several genes that contribute to the pathogen virulence but are not essential for viral replication while the tk and gE genes have been the primary target for deletion to achieve inactivation of the virus.

Porcilis® Begonia (MSD Animal Health- Intervet) is a tk and gE deletion mutant live attenuated vaccine. It is being used for the prevention of clinical symptoms and mortality by Aujesky's disease. This vaccine has been developed to protectively immunize the animals for a period of 4 months (135, 136). Auskipra® GN (Hipra) is a live attenuated gE negative Bartha K61 strain vaccine and has shown to prevent

clinical symptoms and reduce viral shedding of Chinese SuHV-1 variants (AH02 strain) (137, 138). Both of the vaccines can been used in vaccination programs to control and eradicate pseudorabies (139, 140).

Pestivirus—Suvaxyn® CSF Marker, Porcilis® Pesti, and Bayovac CSF E2®

Classical swine fever (CSF), is caused by a *pestivirus* of the family *Flaviviridae* (127). CSF virus (CSFV) is a small, enveloped virus with a single-stranded positive sense RNA genome which encodes a polyprotein, post-translationally cleaved to 12 final products, including the E2 structural glycoprotein that has a critical role in viral replication (141, 142). The eradication of CSF in several countries in Western Europe, North America and Australia is by in large credited to the Chinese lapinized vaccine (C-strain), an attenuated strain of CSF, developed by China Institute of Veterinary Drugs Control and Harbin Veterinary Research Institute in 1956 (143). However, this highly contagious viral disease remains of worldwide significance with a high mortality rate. CSFV is still endemic in many parts of the world, including most of Asia, Central and South America and multiple countries in Eastern Europe, resulting to sporadic outbreaks in highly susceptible naïve swine populations in neighboring CSF free countries (127, 144).

Pigs are typically infected with CSFV by the oronasal route, by contact of susceptible swine with infected feral or domestic pigs, or ingestion of uncooked swill, with tonsil as the initial site of viral replication. Animals in the acute form of disease, are exhibiting high fever, loss of appetite, depression, and conjunctivitis frequently succeeded by diarrhea, vomiting, cutaneous erythema and central nervous system clinical signs, days or weeks before they eventually die. Additionally, CSFV is able to cross the placenta and transmit to the fetuses resulting to mummifications, abortion, stillbirths or fetal deformities (127, 144, 145).

A promising commercially available vaccine is Suvaxyn® CSF Marker, the CP7_E2_alf chimeric vaccine which is licensed by the European Medicines Agency. The vaccine utilizes a live-attenuated bovine viral diarrhea virus (BVDV) backbone a expressing the E2 glycoprotein of CSFV (146). This is an effective strategy as the E2 glycoprotein is the major neutralizing antigen of CSFV (147, 148). In addition, the design of the CP7_E2_alf vaccine enables the serological differentiation between wild-type infected and vaccinated swine in herds (149, 150). Intramuscular (IM) and oral vaccination has been shown to confer full protection against challenge with the highly virulent CSFV strain "Eystrup" 28 days after immunization (146, 149). Challenging vaccinated animals within 2 days after immunization conferred partial protection (151). Additionally, duration of immunity has been shown to last at least 6 months after one vaccination dose (152).

Porcilis Pesti® (Merck) and Bayovac CSF E2® (Beyer AG) are licensed subunit vaccines developed using the BEVS system to express the E2 protein (153). Porcilis Pesti® has shown to be very efficacious against the low virulent strain "Glentorf" in pregnant sows, as no virus was detectable following a vaccination-challenge study and nine out of 10 litters of the vaccinated sows were protected from CSFV infection when challenged 126 days from vaccination and on day 65 of gestation (154). In a large-scale laboratory trail, both Porcilis Pesti® and Bayovac CSF E2®, were evaluated. The data revealed animals vaccinated with Bayovac CSF E2® were better protected against clinical CSF than those that received Porcilis Pesti® as the antibody response was more pronounced and the transmission probability was reduced significantly after the second dose. When sows were challenged with virulent CSF 14 days after vaccination (day 60 of gestation) with Bayovac CSF E2® and Porcilis Pesti®, 75 and 100% of the sows had viremic piglets, respectively (155). This data collectively suggests that these vaccines have reduced efficacy during an emergency field outbreak situation in which animals had not been vaccinated at least 3 weeks prior to exposure.

Porcine Endemic Diarrhea Virus –iPED+ Vaccine

Porcine Epidemic Diarrhea virus (PEDv) is a highly contagious swine coronavirus causing enteritis in all age groups with a variable virulence and mortality depending in the strain (156, 157). PEDv is an enteropathogenic coronavirus comprised of a positive sensed RNA genome that encodes a spike (S) glycoprotein located on the outer surface envelope of the virus particle. The spike (S) protein of PEDv is crucial for the virus interaction with host cell receptors and was characterized to contain many epitopes recognized by the host's immune system to incite neutralizing antibodies (158–160).

iPED+ vaccine (updated to iPED RNA) was the first *Alphavirus*-derived replicon RNA particle vaccine licensed to control PEDv. The vaccine employs a replicon vector system which utilizes a defective Venezuelan equine encephalitis virus (VEEV) like particle to deliver and propagate the PEDv S glycoprotein antigen in swine (161, 162). The iPED RNA vaccine was shown to elicit PEDv-neutralizing antibodies in dams and passively acquired PEDv-neutralizing antibodies in suckling piglets, induced clinically protective immunity and reduced viral shedding in challenged pigs, and reduced farrowing mortality in challenged sows (161, 163, 164).

Swine Influenza a Virus– SEQUIVITY®

Swine influenza A virus (swIAV) is a major respiratory pathogen in pigs resulting in delayed growth, prolonged finishing time, and consequential economic damage (165–168). Sequivity is a 3rd generation vaccine technology that employs the Sequivity™ RNA Particle Technology, an alphavirus replicon vector system derived from the attenuated TC-83 strain of VEEV (45, 169, 170). This vaccine has not been shown to be efficacious when given in the presence of maternal antibodies but does induce a strong humoral and cell-mediated immune response in animals without maternal antibodies (45, 171–173). Additionally, this vaccine platform allows the option for "Veterinary Prescription" or customized vaccines, similar to autogenous vaccines, in which individualized, single or multivalent formulations can be produced on a case-by-case basis. Accordingly, an immunogenicity and efficacy trial evaluating an H3 RP vaccine showed this vaccine platform elicited protective serologic response within 3 weeks

of receiving the boost vaccination, induced a specific IFN-γ response, prevented detectable nasal shedding and live virus within broncho-alveolar lavage fluid, and attenuated clinical disease (173).

Bovine Vaccines

Foot and Mouth Disease—Adt.A24 FMD vaccine

Foot-and-mouth disease (FMD) is caused by a highly contagious *Aphthovirus* that transmits between cloven-hoofed ungulates. The virus is a member of the *Picornaviridae* family and can be transmitted through aerosol droplets, direct contact and/or from ingestion by susceptible animals. On average 11 billion dollars (USD) is lost per annum in countries where FMD is prevalent (174). The devastating global economic impact of FMD has fast-tracked the research into FMD vaccines using novel technologies. Of interest, includes the Adt.A24 FMD vaccine, which was granted conditional licensure by the United States Department of Agriculture (USDA) to protect cattle in 2012 (175). The replication deficient Adt.A24 vaccine utilizes a human *adenovirus* construct as a vector to deliver empty capsids of the A24 FMD strain to elicit an immune response (175). Previous studies in bovine and swine has shown that the Adt.A24 vaccine prevents FMD, along with FMD viremia 7-days after initial vaccination and is most efficacious when combined with the ENABL® adjuvant (176, 177). This vaccine has no reversion to virulence, no shedding from vaccines to naïve animals, no excretion in milk from lactating dairy cattle and conferred 64% efficacy against clinical FMD (178, 179). Lastly, the Adt.A24 vaccine enables the use of a DIVA strategy for evaluating herds during an outbreak.

Bovine Herpesvirus Type 1 - Bovilis® IBR Marker Live, Hiprabovis® IBR Marker Live, Bayovac IBR Marker Vivum, Bayovac IBR Marker Inactivatum, Rispoval® IBR-Marker Inactivated, Rispoval® IBR-Marker Live

Cattle infected with Bovine Herpesvirus 1 (BoHV-1) are at risk of developing Infectious Bovine Rhinotracheitis (IBR), an acute and highly contagious disease affecting the upper respiratory tract (180). Additionally, BoHV-1 infection can also impact fertility, reproduction, and productivity. Bovilis® IBR Marker Live, Hiprabovis® IBR Marker Live, Bayovac IBR Marker Vivum, Bayovac IBR Marker Inactivatum, Rispoval® IBR-Marker inactivated, and Rispoval® IBR-Marker live are licensed vaccines for use in cattle against BoHV-1. All of these IBR vaccines have the gE- deletion; the Hipravovis® IBR Marker live also has the tk- deletion. A disadvantage to utilizing some of these modified-live gE-product is the potential for latency in immunized animals and consequent reactivation or shedding following a provoked immunosuppressive state (181, 182). It has been shown that inactivated gE-deleted vaccines reduced viral excretion more efficiently than live gE-deleted vaccines in latently infected animals induced into an immunosuppressive state (183). Nonetheless, these marker vaccines administered either IM or IN induce a robust humoral and cell-mediated immune response making them versatile and valuable (184). Bovilis IBR Marker Live has been shown to prohibit nasal secretion shedding, prevent viremia, to elicit a humoral immune response in pregnant cattle until at least 180 days post calving, and provide passive immunity to calves until at least 180 days post calving (185, 186).

Poultry Vaccines

Avian Influenzas—Trovac®-AIV H5, Vectormune®AI

Avian Influenza Viruses (AIV), are important pathogens for both poultry production and for human health. AIVs are enveloped, negative sense single stranded RNA viruses of the *Orthomyxoviridae* family and are classified as either highly pathogenic or low pathogenicity in avian species. Trovac®-AIV H5 (TROVAC-H5), produced by Boehringer-Ingelheim, contains a live recombinant fowl pox-vectored backbone that expresses an H5 HA subtype isolate synthetically generated based on a highly pathogenic AIV HA protein and altered to mimic a low pathogenicity virus. When a single dose was administered to 1-day old chicks, duration of immunity lasted at least 20 weeks providing significant and rapid protection especially within field conditions (187, 188). Importantly, this vaccine was not efficacious against animals pre-immunized against or infected with fowlpox as protection against AIV levels decreased (189).

Vectormune® AI from CEVA Animal Health, uses a similar synthetic avian IAV HA protein inserted into a turkey herpesvirus (HVT) backbone. Vaccination conferred robust and long-lasting protection in commercial flocks, prevented the development of clinical disease, and suppressed shedding of high-pathogenicity avian influenza (190, 191).

Newcastle Disease—Innovax®-ND, Vectormune® FP-ND

Newcastle Disease (ND) is a viral disease of domestic poultry, including chickens, turkeys, pigeons, pheasants, ducks and geese, of a worldwide importance (192). The infectious agent, Newcastle Disease Virus (NDV) or avian paramyxovirus serotype 1, is a highly contagious, negative sense single stranded RNA, virus of the *Paramyxoviridae* family. Transmission of NDV can occur by inhalation or by ingestion of contaminated feed or water, *via* the discharges and droppings of infected birds, and can spread rapidly through the flock. Like AIV, NDV can be further classified on the basis of its virulence, as velogenic (highly pathogenic), mesogenic (moderate pathogenicity), or lentogenic (subclinical or avirulent). Velogenic strains cause acute respiratory disease accompanied by nervous signs and high mortality that in susceptible flocks can approach to 100% (193, 194).

ND has seen advancements in commercial vaccine technologies similar to AIV. Innovax-ND, from Merck, inserts the Fusion (F) protein, a strongly immunogenic antigen, gene from NDV into an HVT vector. As with any HVT vector, vaccinated animals developed strong immunity against MD, but importantly developed protection against lethal challenge with NDV (195). A more recent and novel development in ND vaccines is Vectormune® FP-ND from Ceva which also utilizes a viral vector, however in this case it is Fowl pox.

Infectious Bursal Disease, Mareks, Disease and Infectious Laryngotracheitis—Innovax® ND-IBD, Innovax® ND-ILT

Infectious Bursal Disease (IBD) is caused by a double stranded DNA virus (IBDv) from the *Birnaviridae* family. IBD is a highly infectious disease of young domestic chickens and turkeys, characterized by immunosuppression and bursal atrophy due to depletion of B-lymphocytes. While in most cases, IBD-related morbidity is high and mortality is low, certain highly virulent strains can cause up to 60% mortality (196, 197). While IBDv targets B-lymphocytes, Marek's disease (MD) virus (MDV), also called alphaherpesvirus 2 or gallid herpesvirus 2, primarily preys on $CD4^+$ T- lymphocytes. MDV is a highly oncogenic lymphotropic virus with worldwide distribution causing lymphoproliferative disease in chickens. Marek's disease is characterized by paralysis due to widespread presence of T-cell lymphomas localized in peripheral nerves, and visceral organs (198). Another important herpesviral disease of poultry is Infectious Laryngotracheitis (ILT), which is caused by the avian Alphaherpesvirus 1 or gallid herpesvirus 1. ILT virus (ILTv) is a double stranded DNA virus transmitted to birds through aerosols and fomites. ILT is an upper respiratory track disease causing significant economic losses due to high mortality rate (up to 70%) (199).

The traditional method for immunization against MD is *via* a turkey herpesvirus-vectored live vaccine, since HVT is subclinical in poultry, and provides strongly cross-reactive antibodies against MD. This style of multi-protective recombinant vaccines has been popularized, as Merck has produced multiple variants based on this technology. Innovax® ND-IBD uses the HVT-vector, modified to include the F gene from NDV and the VP2 surface glycorptein gene from IBDv. When challenged, animals exhibited protection against NDV, IBDV, and of course MDV for up to 60 weeks (200). Another example is Innovax® ND-ILT, which provides protection against NDV and ILTV. This recombinant FPV has been edited to include the F gene and HN gene from NDV, as well as the gB gene from ILTV. The HN construct from NDV encodes the hemagglutinin/neuraminidase proteins, while the gB gene from ILTV encodes the primary surface glycoprotein antigen. Results from vaccine trials showed roughly 70% protection against ILTV, comparable to the traditional inactivated vaccine, in addition to neutralizing immunity against NDV (201).

AQUACULTURE

Salmonid Vaccines

Infectious Hematopoietic Necrosis—Apex IHN

In the aquaculture industry, DNA vaccines have seen more success than other fields and continue to be a major field of development (202). As stated previously, DNA vaccines themselves are immunogenic and function as PAMPS and thus eliminate the need for adjuvants (203). One of the major advantages to this technology in fish is the avoidance of adjuvants which have historically been shown to cause severe reactions, such as peritonitis and melanisation of the muscle tissue in fish (204, 205). Apex IHN from Novartis (now Elanco Animal Health) was developed to vaccinate against Infectious Hematopoietic Necrosis Virus (IHNV), a *Rhabdovirus* that causes extensive necrosis of hematopoietic tissue in early life stages and has a high mortality among Salmonids (206). This disease can affect both wild and farmed salmonids resulting in major economic loss. Apex IHN is a DNA vaccine encoding the glycoprotein (G), a major antigen for protective antibodies. Given IM, this vaccine induces both innate and adaptive immune responses in fish and has conferred significant protection in Atlantic salmon, Pacific salmon, and rainbow trout (207–213). Apex IHN vaccination confers a significantly attenuated mortality rate—<3% in vaccinated animals and 99% in control animals–reduces viral spread among cohabitating naïve Atlantic salmon with infected Atlantic salmon, abolishes disease transmission amongst infected Atlantic salmon cohabitating with naïve sockeye salmon, and induces a long-lasting neutralizing antibody titer (214).

Pancreas Disease - Clynav

Clynav, produced by Elanco Animal Health, is another recombinant DNA vaccine containing the puK-SPDV-poly2#1 plasmid and codes for several proteins from the salmonid alphavirus subtype 3. This vaccine has been approved in the EU and Norway and is indicated to protect against pancreas disease. This disease has a significant economic burden due to the mortality, reduced growth rates, and reduced meat quality at time of slaughter (215). Fortunately, Clynav protects against weight loss, reduces the prevalence and severity of morphological tissue lesions within the cardia, pancreas and skeletal muscle, and reduces mortality for up to 1 year after vaccination. Additionally, when compared to a traditional monovalent vaccine, Clynav provided significantly higher neutralizing antibody titers, conferred lower viremia, reduced transmission to cohabitating naïve fish, and conferred a significantly higher weight gain post challenge (216). The major criticism of these DNA vaccines is the incorporation rate in the vaccinated subjects. While the incorporation rate is negligible, however it has not been precisely estimated according to manufacturers but modeled on scenarios estimating integration (217).

EXOTIC ANIMALS

Wildlife

Rabies—ONRAB®, RaboralV-RG®

During the last 50 years there has been a significant effort to eradicate rabies virus from domesticated companion animals by establishing mandatory vaccination programs. Currently the attention has been focused on wildlife species that are critical for the prevalence of this fatal disease and transmission to humans. According to the annual Center for Disease Control and Prevention (CDC) report about Rabies surveillance in the United States during 2017, 91% of rabid cases involved feral animals mainly bats, raccoons, skunks and foxes (218). This highlights the importance of the development of different vaccine constructs to control and even eliminate the transmission of rabies by immunizing the most susceptible principal reservoir wildlife species. United States, Canada, and Europe have

established an Oral Rabies Vaccination (ORV) program, to prevent the spread of rabies to raccoons, foxes, coyotes, wolves and other species that can serve as reservoirs for rabies.

Two types of recombinant vaccines that express the rabies glycoprotein have been used in oral baits to prevent this zoonotic disease. Onrab® by Artemis Technologies Inc. (Guelph, Ontario, Canada) employs a human adenovirus type 5 (Had5) vectored vaccine. Raboral V-RG® utilizes a vaccinia virus as the backbone. ORV baits are produced by Merial Ltd (Athens, GA) and consist of an edible packet that contains the Raboral V-RG®. Administration of this vaccine has led to the eradication of the zoonotic rhabdovirus from 3 European countries (219). This is due to the higher efficacy of the vaccine in red foxes, which are the principal reservoir species in the continent (220, 221). The main objective of this vaccination program is to confer a neutralizing positive titer over 0.05 IU/ml to the targeted animals. All of the vaccinated foxes, 56% of coyotes and 62% of gray foxes have shown protective serum titers after the administration of the ORV baits (220–222). However, other mesocarnivore animals like raccoons and skunks, which are considered the primary carriers of rabies in the USA have demonstrated variable effectiveness on their immunization using ORV baits (223–225). It has been shown that Onrab® vaccine induces better protection on raccoons by inducing humoral response on 74–77% of the animals, instead of the 30% seropositivity achieved after the administration of the ORV baits (226, 227).

Lagomorphs

Rabbit Hemorrhagic Disease - Novibac Myxo-RHD & Novibac Myxo-RHD Plus

The etiological agent of Rabbit hemorrhagic disease (RHD) is a highly virulent *Calicivirus* that is enzootic in rabbit populations worldwide, causing frequent epidemics with significant mortality rate up to 90% in rabbits older than 5 weeks (127, 228). Another important pathogen of this animal species is Myxoma virus which is a member of the *Leporipoxvirus* genus. Myxomatosis is an acute, systemic and often fatal disease of European rabbits characterized by blepharoconjuctivitis, swellings in the eyes, skin and genitals, listlessness and anorexia (229).

Nobivac® Myxo-RHD is a live chimeric bivalent vaccine that uses a Myxoma viral vector expressing the VP60 capsid protein of the classical 009 RHD viral strain. Nobivac® Myxo-RHD Plus contains a second recombinant Myxoma virus with the VP60 protein of the emerged variant MK 1899 (230). Nobivac® Myxo-RHD confers significant protection against both of the pathogens for 12 months after a single dose administration. In an immunization study all of the vaccinated animals seroconverted showing a strong humoral response against RHDV which is essential for the prevention of this viral disease in the challenged animals (231).

DISCUSSION

Historically, vaccines in human medicine have been in the wake of veterinary medicine as there are very limited licensed approved second and third generation vaccines in human medicine. The hepatitis B vaccine was the first example of a synthetic vaccine developed using recombinant DNA technology and was licensed in 1986; Hemophilus influenza B (HIB), the first conjugate vaccine, was licensed for medical usage in 1987; The Dengue tetravalent vaccine, trade name Dengvaxia, utilizes a live-attenuated tetravalent vaccine consisting of chimeric Dengue proteins combined with the non-structural genes of the Yellow Fever 17D vaccine strain. The rVSV-ZEBOV vaccine against Ebola Zaire, approved in 2019, is a live recombinant viral replication-competent Ebola vaccine consisting of a vesicular stomatitis virus backbone with the envelop glycoprotein of the Zaire ebolavirus in place of the VSV envelop glycoprotein. A heterologous 2-dose vaccination scheme with the Zabdeno (Ad26.ZEBOV) and Mvabea Ebola (MVA-BN-Filo) vaccines are approved for use in the EU. Zabdeno is the prime vaccination and is an adenovirus type 26 vector expressing the Ebola virus Mayinga variant's glycoprotein. MBA-BN-Filo serves as the boost immunization and is a non-replicating, recombinant, modified vaccinia Ankara (MVA) vector-based vaccine encoding glycoproteins from Zaire Ebola virus, Sudan virus, Marburg virus, and the nucleoprotein from the Tai Forest virus respectively (232).

In late 2020, the United Kingdom became the first sovereign country to approve Tozinameran INN, a messenger RNA vaccine (co-produced by Pfizer and BioNTech) indicated for the prevention of SARS-CoV-2 infection, the agent responsible for the COVID-19 pandemic (233). This is the first instance in which a gene-based technology has been licensed and approved for an infectious agent. Since then, and in the midst of the pandemic, other novel and third generation vaccine candidates have been approved for Emergency Use Authorization (EUA) or are undergoing final stages toward EUA application. At the time of writing, these candidates include the Moderna mRNA vaccine, mRNA-1273, and the adenovirus-vectored vaccine AZD1222 by AstraZeneca and Oxford University (234, 235).

Continuing to optimize delivery systems, and to enhance mucosal immunity, molecular adjuvants are crucial for the synergism of vaccine development. However, to remain within the scope of licensed novel technologies in veterinary medicine, the aforementioned components will only be briefly discussed as many are still in experimental stages.

One technology in which human medicine has arguably preceded veterinary medicine is the employment of viral-like particles (VLPs). VLPs are non-infectious/void of genetic material, self-assembling complexes that bear antigens of interest and mimic the overall structure of a virus (236). The VLP technology has seen success as it activates the adaptive immune response *via* both MHC-I and MHC-II complexes and are consequently capable of stimulating robust CTLs and CD4+ T helper cells (237). Vaccines against human papillomavirus (Cervarix®, Gardasil®, and Gardasil9®) and Hepatitis B virus (Sci-B-Vac™) also utilized the recombinant technology assembled onto a virus-like particle (VLPs) (238).

Nanoparticles (NPs), similar to VLPs, are a revolutionary delivery technology widely investigated for therapeutic drugs and vaccines. Characterized for their size (<100 nm), several types of NPs composed of gold, dendrimers, carbon polymers, an liposomes have been shown to improve vaccine efficacy,

facilitate antigen uptake, and induce desired immunological responses (239). NPs offer several advantages: they can directly access lymphatic drainage systems for immune processing, can be modified to target specific subsets of immune cells, and can be delivered to specific intracellular compartments to hone in on specific immune pathways (240). As such, much of the success of the mRNA SARS-CoV-2 vaccine platform was the use of lipid NPs (241). Nonetheless, a comprehensive understanding of how NPs can be utilized to optimize vaccine delivery remains and many experimental NP candidates are currently being explored in clinical trials for influenza (NTC032293498, NCT3658629), and respiratory syncytial virus (NCT01960686, NCT02247726, NCT02624947) vaccines (240).

Some of the major gaps in vaccine development are the elicitation of mucosal immunity *via* induction of secretory IgA and the appropriate immune stimulation to the antigen *via* adjuvants. The vast majority of pathogens gain entry into hosts *via* mucosal sites, yet the majority of current vaccines provide partial or no protection at mucosal sites. In veterinary medicine, mucosal vaccines have been more successful as sprays and drinking water vaccines are routinely utilized, however, there are no licensed human vaccines for mucosal-transmitted pathogens (242). Vaccine-induced mucosal immunity is particularly challenging due to the difficulty in protecting and preserving antigen structural integrity and increasing the bioavailability of mucosal vaccines. Some experiments have seen success with the use of nanoparticle formulations by incorporating polyethylene glycol (PEG) (243). Chitosan, a non-toxic polymer has also been utilized in intranasally delivered Escherichia coli O157:H7 vaccine formations with similar success (244). Immunostimulating complexes (ISCOMs) are spherical cage-like experimental adjuvants composed of phospholipids, cholesterol, saponin, and protein antigens and have been particularly successful in mucosal immunizations resulting in secretory IgA and systemic immune responses (245, 246). This technology has been utilized in the equine influenza vaccine Equip ™ F (produced by Zoetis/Pfizer Animal Health), a subunit vaccine shown to stimulate both humoral and cell-mediated immunity (247, 248).

A promising solution to combat poor immunogenicity, for DNA vaccines specifically, are molecular adjuvants. These generally comprise plasmid-encoded signaling molecules such as cytokines, chemokines, and immune costimulatory molecules, but newer approaches include gene knockdown and systems biology (249–251). For example, Interleukin-2 (IL-2) promote differentiation of naïve T cells into effector cells and facilitates the generation of memory T cells (20). Thus, IL-2 has been one of the most extensively studied molecular adjuvants and has shown increased immunogenicity for previously low-immunogenic vaccines such as HIV, influenza, and SARS-CoV (252–255). Other immunomodulatory cytokines being evaluated as molecu lar adjuvants are IL-15, IL-12, and MG-CSF (250, 252).

The evolution of vaccine technologies mirrors the continued and rigorous advancement toward safe, efficacious, stable, and cost-effective vaccines for existing and emerging infectious pathogens. Veterinary medicine continues to trail blaze the path as evident by the numerous novel technologies already employed.

AUTHOR CONTRIBUTIONS

VA, VP, PN, JN, and KM researched data for the article and substantially contributed to the discussion of content. VA, VP, and SG drafted and generated figures for the article. VA and CK wrote, reviewed, and edited the manuscript before submission. All authors contributed to the article and approved the submitted version.

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Inherent Serum Inhibition of Influenza Virus Neuraminidases

Amanda L. Skarlupka[1] *and Ted M. Ross*[1,2]*

[1] *Center for Vaccines and Immunology, University of Georgia, Athens, GA, United States,* [2] *Department of Infectious Diseases, University of Georgia, Athens, GA, United States*

Influenza virus vaccines have been designed for human and veterinary medicine. The development for broadly protective influenza virus vaccines has propelled the vaccine field to investigate and include neuraminidase (NA) components into new vaccine formulations. The antibody-mediated protection induced by NA vaccines is quantified by inhibition of sialic acid cleavage. Non-immune inhibitors against influenza viruses naturally occur in varying proportions in sera from different species. In this brief report, the inherent ability of raw animal sera to inhibit a panel of influenza virus NA was determined. Raw sera from the same species inhibited more than 50% of influenza viruses tested from four different subtypes, but the breadth of inhibiting NA activity depended on the source of sera. Furthermore, different influenza viruses were inhibited by different sources of sera. Overall, additional studies are needed to ensure that scientific methods are consistent across studies in order to compare NA inhibition results. Through future investigation into the differences between sera from different animal species and how they influence NA inhibition assays, there can be effective development of a broadly protective influenza virus vaccines for veterinary and human use.

Keywords: neuraminidase, vaccine, ELLA, influenza, animal

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***Correspondence:**
Ted M. Ross
tedross@uga.edu

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INTRODUCTION

Influenza viruses are global zoonotic and human pathogens, and vaccination remains the main preventative measure against infection. The influenza virus is a member of the Orthomyxoviridae family. The genome is composed of eight negative single-sense RNA segments that determines the viral genus, alpha-, beta-, delta-, and gammainfluenzavirus that correspond to the species influenza A, B, D, and C viruses, respectively. Of the four influenza types, Types A and D are commonly isolated from animals, whereas influenza Types B and C are most commonly associated with human infection especially in children (1). The Type A influenza viruses are further classified into subtypes determined by the two major surface proteins, hemagglutinin (HA) and neuraminidase (NA). Currently, there are 18 HA subtypes and 11 NA subtypes that can be paired to create different influenza subtypes.

Influenza viruses are of international importance due to the widespread infection in different livestock, leading to vaccination being utilized across the veterinary field (2). Equine influenza viruses are important horse pathogens with policies in place that require horses be vaccinated for equine influenza viruses before participation in events or importation (3, 4). Furthermore, due to the transmission of influenza viruses from horses to dogs, as well as the endemic infection of influenza viruses in the canine population, canine vaccination is also recommended for dogs with high risk of exposure (5, 6). The swine

industry uses primarily whole inactivated vaccines [WIVs—reviewed in reference (7)] that are developed using split-inactivated technologies (7, 8). The poultry industry utilizes the greatest variety of vaccine platforms, including split-inactivated virus, HA protein antigens, HA DNA vaccines, and recombinant technologies with other backbone viruses (9, 10).

However, during infection both HA and NA proteins are targets for neutralizing antibodies (11). The NA glycoprotein mediates viral egress and virion de-aggregation by cleaving sialic acids as well as contributing to motility through cleaving mucins in the upper respiratory tract (12, 13). Polyclonal NA-specific sera and NA inhibition (NAI) titers reduce, modulate, and protect against disease (14, 15). Further research has identified monoclonal NA-specific antibodies that neutralize viral growth (16). Although NA antibodies hinder viral replication, the induction of NAI antibody titers following vaccination is not as great as the induction of HAI titers, potentially due to either the split-inactivated vaccines lacking a standardized concentration of NA protein or the NA protein being destroyed during the split-inactivation process (15). Recently, research and vaccine development have focused on live-attenuated viruses that elicit NA antibodies, or protein vaccines that include the NA (17).

Currently, the enzyme-linked lectin assay (ELLA), MUNANA substrate, thiobarbituric acid (TBA) fluorescent-based assay, and NA-Star chemiluminescent assay are methods for measuring antibodies against the NA molecule (18–23). As the NA glycoprotein undergoes antigenic drift, the protein's ability to cleave sialic acid can be measured and quantified using these assays. All techniques assess the elicited antibody-specific inhibition of the NA after vaccination or infection. The ELLA measures the ability of the viral NA to cleave sialic acids from a large substrate (fetuin) similar to infection when sialic acids are expressed on the surface of the host cell, whereas the MUNANA and NA-Star techniques measure cleavage of small soluble chemical substrates (24). However, only the ELLA was proposed as the assay for measuring serum NA-inhibiting antibodies as a correlate for protection for humans (25).

Components in raw sera have non-specific inhibitory activity against NA activity (20). These initial findings were conducted with ferret sera that varied using different viruses from different influenza subtypes. However, treating sera with receptor-destroying enzyme (RDE) overnight and then heat-inactivating the sera for 8 h at 55°C mitigated the non-specific inhibition without loss of NA- or HA-specific inhibitory activity (20). The animal models used for influenza virus research are growing and now include more species. Not only is there a need to compare serological results between animal models that are used for human influenza viruses, but also endemic influenza virus infection in agricultural animal species requires a consistent method to quantify the NA-inhibiting antibodies as well. Therefore, it may be necessary to handle sera from different species differently when quantifying the NA inhibition responses, which may be key to determining overall vaccine effectiveness.

Therefore, animal sera from different species were characterized for their inherent inhibition of the ELLA with a panel of influenza viruses. Sera were compared for their ability to non-specifically inhibit the NA proteins of many influenza viruses representing different viral subtypes. Sera were collected and tested from varying animal serum sources against H1 and H3 human- and swine-isolated influenza strains as well as avian-isolated viruses with N2 and N3 proteins. Overall, there are many different variables that contribute to the interpretation of the ELLA assay, and understanding the innate characteristics of the host origin of the sera is critical to conducting the assay and interpreting the results. Therefore, it is important to standardize methodologies that will allow for consistent and reproducible results to assess anti-NA antibodies.

MATERIALS AND METHODS

Viruses

All swine viruses were passaged once in Madin–Darby canine kidney (MDCK) cell culture at 37°C, which was the same growth conditions as they were received in (26). The harvested virus was centrifuged at 2,500 rpm for 10 min to remove cell debris. Human and avian influenza viruses were propagated in 11-day-old embryonated chicken eggs. Virus lots were aliquoted for single-use applications and stored at −80°C. Viral titer of the frozen aliquots was determined with a plaque assay using MDCK cell culture in plaque-forming units per ml (PFU) (**Table 1**). The panel of viruses covered a range of N1 to N3 influenza NA subtypes, including A/Brisbane/59/2007 (H1N1) (Bris/07), A/California/07/2009xPR8 (6:2 viral reassortant with six internal genes from A/Puerto Rico/8/1934, and NA and HA external genes from virus indicated) (H1N1) (CA/09), A/swine/Nebraska/A10444614/2013 (H1N1) (Sw/NE/13), A/Vietnam/1203/2004xPR8 (H5N1) (Viet/04; HA gene contains mutation in multibasic cleavage site for BSL-2-level research), A/swine/Missouri/A10444664/2013 (H1N2) (Sw/MO/13), A/swine/North Carolina/152702/2015 (H1N2) (Sw/NC/15), A/white-fronted goose/Netherlands/22/1999 (H2N2) (Wfg/Neth/99), A/quail/Rhode Island/16-018622-1/2016 (H2N2) (Qu/RI/16), A/Port Chalmers/1/1973 (H3N2) (PC/73), A/Hong Kong/4801/2014 (H3N2) (HK/14), A/swine/Missouri/2124514/2006 (H2N3) (Sw/MO/06), and A/mallard/Minnesota/A108-3437/2008 (H2N3) (Mal/MN/08).

Animal Serum

Animal serum was either commercially sourced or generated in house. Sera were confirmed to be negative for preexisting antibodies to currently circulating human influenza viruses by HAI. Ferret sera originated from 6 to 8-months female finch ferrets (*Mustela putorius furo*, spayed, female, 6–8 months, descented) purchased from Triple F Farms (Sayre, PA); porcine sera originated from piglets at Auburn University; and rhesus macaque (*Macaca mulatta*) sera originated from previous dengue virus studies performed in the lab (27). The rat (cat #: 10710C), goat (cat #: 01-6201), horse (cat #: 31874), and mouse (cat #: 01-6501; NIH Swiss mouse) normal sera were harvested from non-immune animals (Invitrogen, Carlsbad, CA, USA) and rehydrated according to the manufacturer's specification; only one lot was tested for each commercial serum. Raw serum was not diluted any further before experimentation.

TABLE 1 | Linear regression fit of the NA activity of the viruses tested in the panel.

Strain	PFU/ml	Fitted equation	R^2	NA activity reciprocal titer			
				100%	95%	90%	ELLA
Bris/07	4.2×10^8	$OD = -0.5795\log_2(Titer) + 7.72$	0.9867	160	197	243	200
CA/09	1.9×10^8	$OD = -0.5903\log_2(Titer) + 8.804$	0.9835	320	402	505	450
Sw/NE/13	1.15×10^8	$OD = -0.5694\log_2(Titer) + 7.556$	0.9867	160	196	241	200
Viet/04	1.75×10^8	$OD = -0.4799\log_2(Titer) + 5.66$	0.9738	100	119	143	130
Sw/MO/13	1.31×10^7	$OD = -0.5177\log_2(Titer) + 5.932$	0.9786	40	49	61	50
Sw/NC/15	8.45×10^5	$OD = -0.4144\log_2(Titer) + 3.913$	0.9430	10	12	15	15
Wfg/Neth/99	1.0×10^8	$OD = -0.6189\log_2(Titer) + 10.86$	0.9900	6,400	7,576	8,981	8,000
Qu/RI/16	8.0×10^9	$OD = -0.6213\log_2(Titer) + 10.82$	0.9896	6,400	7,551	8,911	8,000
PC/73	9.0×10^8	$OD = -0.6022\log_2(Titer) + 8.33$	0.9789	640	749	875	800
HK/14	3.0×10^7	$OD = -0.4438\log_2(Titer) + 3.773$	0.9903	10	12	14	13
Sw/MO/06	2.0×10^8	$OD = -0.676\log_2(Titer) + 9.549$	0.9899	320	390	477	400
Mal/MN/08	4.0×10^6	$OD = -0.6394\log_2(Titer) + 11.19$	0.9862	3,200	3,911	4,792	4,000

The plaque-forming units (PFU/ml) and the fitted linear regression equation using a minimum of five two-fold serial dilution data points with the final R-squared value are provided. From the 100% NA activity titer, the 95% and 90% NA activity titers were calculated from the fitted equation. The viral dilution used for the ELLA assay was chosen between that range.

NA Activity and Inhibition Assay

High-affinity Immunoblot 4HBX 96-well flat-bottom plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated overnight with 100 µl of 25 µg/ml fetuin (Sigma-Aldrich, St. Louis, MO, USA) in coating buffer (KPL coating solution concentrate; SeraCare Life Sciences Inc., Milford, MA, USA) and stored away from light for a maximum of 2 months at 4°C until use. Viruses were diluted to an initial dilution of 1:10 with Dulbecco's phosphate-buffered saline (DPBS) with Tween-20 and 1% BSA (DPBS-T-B), a PBS which contains 0.133 g/l $CaCl_2$ and 0.1 g/l $MgCl_2$ further supplemented with 1% BSA, and 0.5% Tween-20. Before virus addition, fetuin plates were washed three times in PBS-T (PBS + 0.05% Tween-20). Virus was diluted in two-fold serial dilutions within a range that allowed for linear regression analysis. After which, 50 µl of the viral dilutions was added to the fetuin-coated plate containing 50 µl of DPBS-T-B in duplicate. A negative control column was included containing 100 µl DPBS-T-B only. Plates were sealed and incubated for 16–18 h at 37°C and 5% CO_2. After incubation, plates were washed six times in PBS-T. After washing, a diluted lectin was added to the plates to bind exposed galactose. Specifically, 100 µl of peanut agglutinin-HRPO (Sigma-Aldrich, St. Louis, MO, USA) diluted 1,000-fold in DPBS-B (DPBS, 1% BSA). Plates were incubated at RT for 2 h. Plates were washed three times in PBS-T, and 100 µl (500 µg/ml) of o-phenylenediamine dihydrochloride (OPD; Sigma-Aldrich, St. Louis, MO, USA) in 0.05 M phosphate-citrate buffer with 0.03% sodium perborate pH 5.0 (Sigma-Aldrich, St. Louis, MO, USA) was added to the plates. Plates were immediately incubated in the dark for 10 min at room temperature (20–22°C). The reaction was stopped with 100 µl of 1 N sulfuric acid. The absorbance was read at 490 nm. NA activity was determined after subtracting the mean background absorbance of the negative control wells. Linear regression analysis was used to determine the dilution of NA antigen necessary to achieve 90–95% NA activity and was used for subsequent NA inhibition ELLAs.

From each virus titration, at least five serial dilutions within the linear range were used to calculate the linear regression after transforming the dilutions by $\log_2$. The R-squared value above 0.9 was considered acceptable. The best-fit values for the slope (m) and y-intercept (b) were used to determine the 90–95% range. The lowest titer dilution used for regression was defined as the 100% NA activity dilution. Using the fitted linear regression equation, the optical density ($OD_{100\%}$) value for 100% NA activity was calculated. Then, the $OD_{95\%}$ and $OD_{90\%}$ were calculated by multiplying $OD_{100\%}$ by 0.95 and 0.9, respectively. The range of viral dilution for 90–95% NA activity was then determined by using the $OD_{95\%}$ and $OD_{90\%}$ values in the linear regression equation to obtain lower and upper bounds for the virus dilution (Equation 1). Virus dilutions were then chosen between that range as indicated (**Table 1**).

$$
\begin{aligned}
OD &= m * \log_2(Titer) + b \qquad (1)\\
OD_{100\%} &= m * \log_2(Lowest\ Titer) + b\\
OD_{90\%} &= 0.9 * OD_{100\%} \qquad OD_{95\%} = 0.95 * OD_{100\%}\\
Titer_{90\%} &= 2^{\frac{OD_{90\%} - b}{m}} \qquad Titer_{95\%} = 2^{\frac{OD_{95\%} - b}{m}}
\end{aligned}
$$

The NI ELLA titers were determined from two-fold serially diluting sera in DPBS-T-B from 1:10 to 1:1,280. Duplicate dilutions were added to fetuin plates in 50 µl. The NA antigen was diluted to 90–95% NA activity in DPBS-T-B, and 50 µl was added to the plate. Controls were each a minimum of eight wells and included a positive NA antigen control (50 µl NA antigen + 50 µl DPBS-T-B) and a negative control (100 µl of DPBS-T-B) on each plate. Plates were incubated for 16–18 h at 37°C and 5% CO_2 after which they were washed and processed, and absorbance was read as described above. Initially, the mean background absorbance from the negative control wells was subtracted from all wells. Then, NA percent activity was determined by dividing the serum absorbance by the mean virus-positive control wells

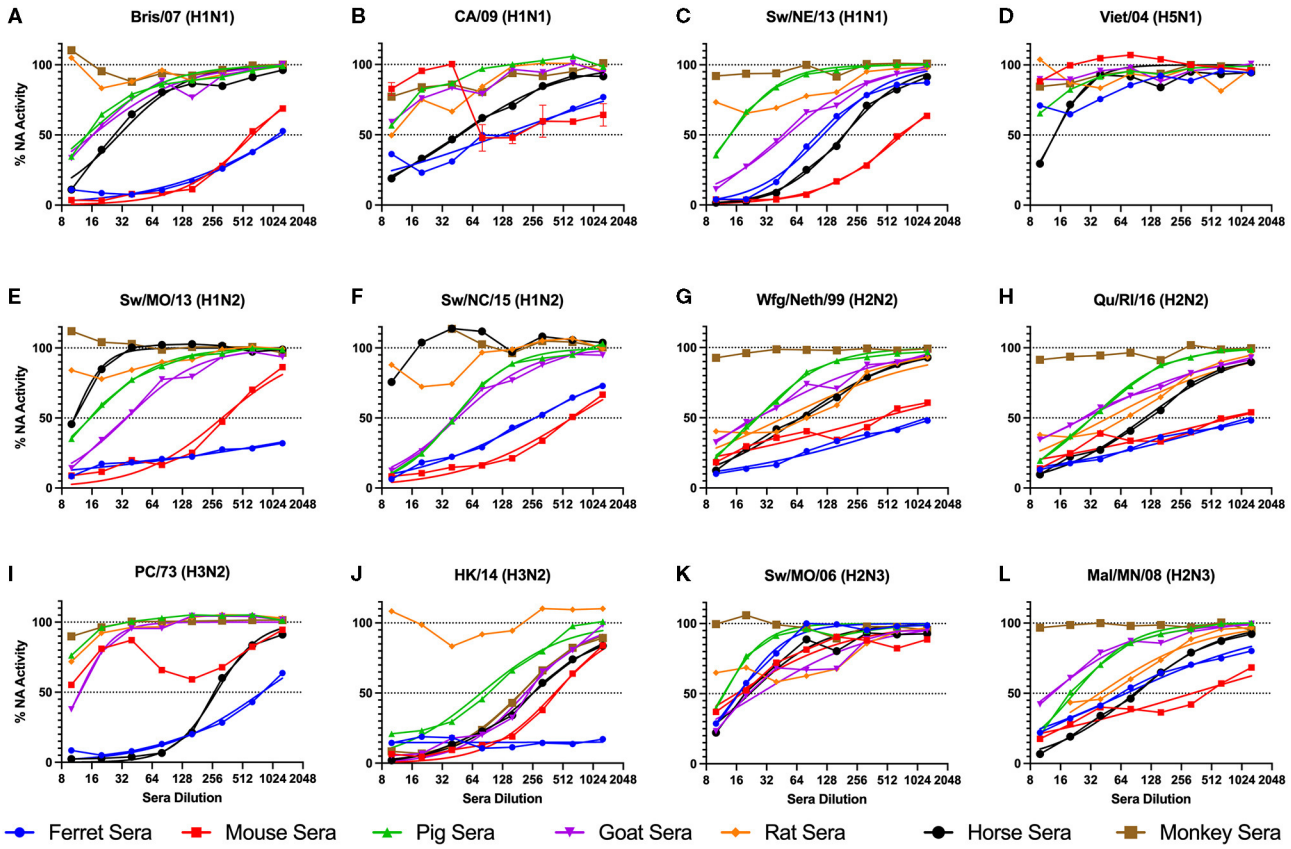

FIGURE 1 | NA inhibition of influenza virus with addition of raw animal sera. A panel of influenza viruses were tested including N1 **(A-D)**, N2 **(E-J)**, and N3 **(K,L)** NA subtypes. The sera were two-fold serially diluted from the reciprocal dilutions of 10-1,280. Non-linear regression was conducted, and the regression that resulted in estimable parameters (as indicated in **Table 2**) are shown. The NA activity was normalized to 100% of a "virus only with no sera" control.

multiplied by 100 (Equation 2).

$$NA\ Activity\ \% = \frac{Individual\ Well\ Absorbance}{Mean\ Absorbance\ of\ Virus\ only\ control\ wells} * 100 \quad (2)$$

Non-linear regression fits were performed using GraphPad Prism version 9.1.1 (223) for MacOS (GraphPad Software, San Diego, CA, USA; www.graphpad.com), and the 50% NAI titer was estimated. Briefly, the "[Agonist] vs. normalized response—Variable slope" model was chosen which fits the model presented in Equation 3, which estimates the Hill slope and the half effective concentration (EC_{50}). Outliers were not detected for or removed, and least-square regression with no weighting was used for the fitting. The model was constrained in that EC_{50} was >0. Asymmetrical profile-likelihood 95% confidence intervals of the EC_{50} were determined as well.

$$y = 100\frac{x^{Hill\ Slope}}{EC_{50}^{Hill\ Slope} + x^{Hill\ Slope}} \quad (3)$$

The lower limit of detection was 1:10, and the upper limit of detection was 1:1,280 due to the range of sera dilution tested.

RESULTS

NA Titers of Influenza Viruses

The lowest dilution of virus needed to induce 100% NA activity varied between 1:10 and 1:6,400 for different influenza viruses (**Table 1**). Three HXN2 viruses had 100% NA titers below 100, 1:40 for Sw/MO/13 (H1N2), and 1:10 for both Sw/NC/15 (H1N2) and HK/14 (H3N2). Of these, the virus titer for only Sw/NC/15 was comparatively low at 8.45×10^5 PFU/ml, while the virus titers for Sw/MO/13 and HK/14 were 2.0×10^8 PFU/ml and 3.0×10^7 PFU/ml, respectively. The avian lineage H2N2 and H2N3 viruses had the highest 100% NA titers of 1:3,200 for Mal/MN/08 (H2N3) and 16,400 for both Wfg/Neth/99 (H2N2) and Qu/RI/16 (H2N2). The virus titer was not greater for these viruses than the others, therefore indicating that the increase in activity is not due to solely an increase in replicating virus.

Animal-Specific Raw Serum Inhibition of the Influenza NA

Sera collected from seven different sources were tested for the ability to inhibit the influenza virus NA activity as tested in the ELLA assay with fetuin substrate (**Figure 1**). Each serum sample was tested against 12 influenza viruses containing either NA type N1, N2, or N3. There were four swine origin viruses and three

TABLE 2 | Non-linear regression fits of raw serum inhibition of Type A influenza viruses.

	Result	Ferret	Mouse	Pig	Goat	Rat	Horse	Monkey
Bris/07	EC_{50}	1,251	647.7	14.32	16.33		29.90	
	95% EC_{50}	997.3, 1,685	588.3, 717.2	11.79, 16.77	10.84, 21.99		24.86, 36.02	
	Adj. R^2	0.9493	0.9837	0.9541	0.8703		0.9497	
CA/09	EC_{50}	125.4					48.24	
	95% EC_{50}	78.76, 200.4					41.95, 55.31	
	Adj. R^2	0.8110					0.9799	
Sw/NE/13	EC_{50}	117.4	725.9	13.89	51.26		189.7	
	95% EC_{50}	102.9, 134.3	674.2, 785.2	13.25, 14.53	43.64, 60.15		172.5, 208.8	
	Adj. R^2	0.9821	0.9917	0.9946	0.9737		0.9903	
Viet/04	EC_{50}						14.04	
	95% EC_{50}						11.71, 16.51	
	Adj. R^2						0.8780	
Sw/MO/13	EC_{50}	23,011	318.1	15.51	35.70		10.66	
	95% EC_{50}	10,183, 75,817	258.1, 392.4	14.72, 16.30	30.74, 41.39		10.04, 11.27	
	Adj. R^2	0.9040	0.9466	0.9954	0.9721		0.9782	
Sw/NC/15	EC_{50}	270.4	636.1	41.73	45.57			
	95% EC_{50}	244.0, 300.5	538.2, 770.2	37.61, 46.28	38.96, 53.28			
	Adj. R^2	0.9891	0.9653	0.9866	0.9723			
Wfg/Neth/99	EC_{50}	1,279	425.0	26.01	25.89	47.34	69.02	
	95% EC_{50}	997.4, 1,727	265.3, 816.6	24.16, 27.96	18.98, 33.75	30.45, 68.96	62.62, 76.01	
	Adj. R^2	0.9651	0.8161	0.9920	0.9261	0.8721	0.9907	
Qu/RI/16	EC_{50}	1,214	817.3	32.91	27.97	48.04	111.1	
	95% EC_{50}	957.5, 1,611	491.6, 1749	29.91, 36.16	23.18, 33.17	34.61, 64.45	96.41, 127.9	
	Adj. R^2	0.9695	0.8347	0.9889	0.9710	0.9182	0.9804	
PC/73	EC_{50}	798.4			12.10		280.0	
	95% EC_{50}	666.1, 992.5			10.83, 13.39		262.2, 299.1	
	Adj. R^2	0.9555			0.9519		0.9936	
HK/14	EC_{50}		424.5	78.03	216.6		258.7	194.9
	95% EC_{50}		379.5, 475.3	57.20, 104.9	178.9, 261.1		240.4, 278.4	179.7, 211.4
	Adj. R^2		0.9810	0.9193	0.9618		0.9941	0.9927
Sw/MO/06	EC_{50}	16.96	16.00	11.98	27.26		21.67	
	95% EC_{50}	15.25, 18.78	10.44, 21.55	10.65, 13.23	16.58, 40.81		15.70, 28.64	
	Adj. R^2	0.9745	0.8812	0.9534	0.8365		0.8724	
Mal/MN/08	EC_{50}	72.27	329.5	21.98	12.77	39.23	87.82	
	95% EC_{50}	61.88, 84.12	222.4, 537.1	20.23, 23.84	10.09, 15.37	30.55, 48.54	81.79, 94.29	
	Adj. R^2	0.9753	0.8585	0.9883	0.9505	0.9505	0.9950	

The 50% NA inhibitory concentration estimate (EC_{50}, half maximal effective concentration), the 95% profile-likelihood confidence intervals, and the adjusted R-squared (Adj. R^2) were determined for each fit. Sera and virus pairs that resulted in an unstable estimate or did not have an estimate >10 are not shown.

avian origin viruses. The 50% NAI titers were estimable for only some virus and serum pairs (**Table 2**).

Ferret sera inhibited ELLA activity by 11 of the 12 viruses with a dilution titer >1:10 and 9 viruses with a titer >1:100 (**Table 3**). The rat sera inhibited the least number of viral NAs, inhibiting ELLA activity by three of the H2 viruses. Interestingly, not all animal sera inhibited all the same viruses (**Table 3**). For example, the Bris/07 (H1N1) virus was inhibited by ferret and mouse sera at a dilution >1:100, by pig, goat, and horse sera at a dilution >1:10, and was not inhibited by either rat or monkey sera. This variation was observed for other subtypes and host origin isolates. The Wfg/Neth/99 (H2N2) had a similar inhibition profile. The HK/14 (H3N2) virus was inhibited by the greatest number of sera. There was no distinguishable viral characteristic, such as host origin or HA or NA subtype, that was correlated with pattern of sera inhibition.

DISCUSSION

Influenza vaccine formulations, including live-attenuated virus, whole-inactivated virus, and protein subunit minutes, use NA as a vaccine component to elicit NA-specific antibodies (28). However, components in raw sera have anti-NA properties that result in inhibition of NA activity. The ELLA is used to measure antibody-mediated NA inhibition for cleaving a large substrate,

TABLE 3 | NA inhibition of raw sera stratified by host origin.

NA	HA	Host	Strain	Ferret	Mouse	Pig	Goat	Rat	Horse	Monkey	>10	>100
N1	H1	Human	Bris/07	1,251	648	14	16	<10	30	<10	5	2
	H1	Human	CA/09	125	<10	<10	<10	<10	48	<10	2	1
	H1	Swine	Sw/NE/13	117	726	14	51	<10	190	<10	5	3
	H5	Human	Viet/04	<10	<10	<10	<10	<10	14	<10	1	0
N2	H1	Swine	Sw/MO/13	>1,280	318	16	36	<10	11	<10	5	2
	H1	Swine	Sw/NC/15	270	636	42	46	<10	<10	<10	4	2
	H2	Avian	Wfg/Neth/99	>1,280	425	26	26	47	69	<10	6	2
	H2	Avian	Qu/RI/16	1,214	817	33	28	48	111	<10	6	3
	H3	Human	PC/73	798	<10	<10	12	<10	280	<10	3	2
	H3	Human	HK/14	>1,280	425	78	217	<10	259	195	6	5
N3	H2	Swine	Sw/MO/06	17	16	12	27	<10	22	<10	5	0
	H2	Avian	Mal/MN/08	72	330	22	13	39	88	<10	6	1
Number of viruses with NAI > 10				11	9	9	10	3	11	1	54	–
Number of viruses with NAI > 100				9	8	0	1	0	4	1	–	23

Viruses tested are separated by NA subtype, HA subtype, and host origin. The reciprocal NAI 50% titer for each virus and serum pair is shown from the non-linear regression estimates. The number of viruses or sera with NAI 50% titers >1:10 and 1:100 is tabulated by serum origin and by virus, respectively.

and has been used to assess the effectiveness of NA-containing vaccines and anti-NA antibodies (29–32).

In this study, seven raw animal sera were tested for inhibition of virus in the ELLA assay (**Table 2**). All sera, regardless of species, inhibited at least one influenza virus (50% inhibition) with a dilution of >1:10. Five of the seven samples inhibited 50% NAI activity at a titer of >1:100. Sera contain innate host influenza inhibitors, such as complement protein of the α-, β-, and γ-class serum inhibitors. In horse and pig sera, the α-2-macroglobulin (γ-class) is one of the major innate influenza virus-neutralizing factors (33, 34). The γ-class inhibitors express sialic acids that bind specifically to the HA protein on influenza viruses and may inhibit the NA through steric interactions. These γ-class inhibitors are inactivated through RDE treatment using *Vibrio cholerae* NA and are resistant to viral sialidase activity (34, 35). There appear to be minor innate factors that result in the ability of horse and pig sera to inhibit different viruses in the panel.

Not all sera inhibited NA activity of all viruses. There were distinct inhibition profiles against specific influenza viruses in the panel. Innate inhibitors interact with influenza viruses through competitive binding of sialic acids to the HA protein receptor-binding site (RBS) (α- and γ-class) and with mannose-binding lectins (β-class) (36, 37). Depending on the host origin of the virus, the HA RBS may have stronger affinity for α-2,3 or α-2,6 sialic acids. The glycosylation of HA proteins has been associated with mannose-binding lectins (37). Further research into the contributions of HA sialic acid binding specificity and the glycosylation of HA and NA surface proteins is needed to determine if it is significantly impacting the variation of NA inhibition observed here across the different viruses.

The innate NA inhibition of different species sera is useful for determining the appropriate treatment before conducting for ELLA assays. To account for the innate inhibitors observed here, sera may either be heat treated or RDE treated overnight at 37°C to cleave competing sialic acids from α- and γ-class inhibitors and heat inactivated at 56°C for a minimum of 30 min to inactivate the heat-labile β-class inhibitors and up to 8 h to fully inactivate the *V. cholerae* NA, when used with ferret sera (20). Immunoglobulins vary in their heat stability with IgG being more stable than IgA which is more stable than IgM (38). With researchers using different inactivation methods, it may be inappropriate to compare titers between sera heat inactivated for 30 min to RDE-treated sera that is heat inactivated for 8 h.

However, one of the major limitations of the study design was the inability to quantify within-species variability due to the limited sources of the sera. This variability can be further investigated to determine if age, sex, or husbandry practices, such as farm or laboratory origin animals, have any effect on the results. Furthermore, the serum inactivation procedure for conducting the ELLA may be different between species. To determine the appropriate method, positive control antiserum is necessary to confirm that no loss in NA-specific antibodies is observed during treatment. Given the wide panel of viruses and different animal models tested here, those samples were not available. Lastly, the wide variability in the NA activity titers observed between viruses (**Table 1**) may either be from increased enzymatic capacity, i.e., a virus' NA protein cleaves more sialic acid at a higher rate than another viral NA, or from having a higher NA content per PFU. Therefore, why different viruses had such variability in NA activity was undetermined.

In conclusion, with the increase in NA research, the RDE treatment, the inactivation time, and the temperature used to inactivate sialidases should be clearly described with the negative control data provided for each viral strain with serum species used for the assay in order to accurately interpret the results. This information will allow for comparison across species or if comparison of anti-NA serological results need to assessed within the same species.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC of University of Georgia.

AUTHOR CONTRIBUTIONS

AS: concept, writing, analysis, and statistics. TR: editing and writing. All authors contributed to the article and approved the submitted version.

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CAvant® WO-60 as an Effective Immunological Adjuvant for Avian Influenza and Newcastle Disease Vaccine

Eun-Seo Lee[1†], Young-Jung Shim[2†], W. A. Gayan Chathuranga[1], Young-Hoon Ahn[2], In-Joong Yoon[2], Sung-Sik Yoo[2] and Jong-Soo Lee[1*]*

[1] College of Veterinary Medicine, Chungnam National University, Daejeon, South Korea, [2] Choong Ang Vaccine Laboratory Co., Ltd., Daejeon, South Korea

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Constantinos S. Kyriakis,
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Madhuri Subbiah,
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University of Copenhagen, Denmark

***Correspondence:**
Sung-Sik Yoo
saintyoo@cavac.co.kr
Jong-Soo Lee
jongsool@cnu.ac.kr

† These authors have contributed equally to this work and share first authorship

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Despite the immunogenicity of vaccines currently used in poultry, several pathogens, including avian influenza virus (AIV) and Newcastle disease virus (NDV), cause enormous economic losses to the global poultry industry. The efficacy of vaccines can be improved by the introduction of effective adjuvants. This study evaluated a novel water-in-oil emulsion adjuvant, CAvant® WO-60, which effectively enhanced both the immunogenicity of conserved influenza antigen sM2HA2 and inactivated whole H9N2 antigen (iH9N2). CAvant® WO-60 induced both humoral and cell-mediated immunity in mice and provided 100% protection from challenge with 10 LD50 of A/Aquatic bird/Korea/W81/2005 (H5N2) and A/Chicken/Korea/116/2004 (H9N2) AIV. Importantly, immunization of chickens with iH9N2 plus inactivated NDV LaSota (iNDV) bivalent inactivated vaccine emulsified in CAvant® WO-60 induced seroprotective levels of antigen-specific antibody responses. Taken together, these results suggested that CAvant® WO-60 is a promising adjuvant for poultry vaccines.

Keywords: avian influenza virus, Newcastle disease virus, vaccine, adjuvant, water-in-oil emulsion, CAvant® WO-60

INTRODUCTION

Avian influenza (AI) and Newcastle disease (ND) are two of the diseases that affect poultry, causing enormous economic losses to the poultry industry worldwide (1). AI results from infection with avian influenza viruses (AIVs), which belong to the genus Influenza virus A and the family Orthomyxoviridae (2). Although infection of poultry with AIVs can be asymptomatic, it can also induce various symptoms of disease, including respiratory illnesses, reduced egg production, and severe systemic diseases with near 100% mortality rates (3, 4). AIVs can be further divided into two categories, low pathogenic avian influenza (LPAI) and high pathogenic avian influenza (HPAI), based on their genetic features and pathogenicity (4, 5). All naturally occurring HPAI strains isolated to date have been either of the H5 or H7 subtype (2).

ND results from infection with avian paramyxovirus serotype 1 (APMV-1), also called Newcastle disease virus (NDV). This virus belongs to the genus Avulavirus and the family Paramyxoviridae. NDV infection can be asymptomatic in poultry, but it can also induce disease symptoms, including depression, prostration, diarrhea, and nervous signs, with nearly 100% mortality rates (6). Based on the clinical signs in infected chickens,

NDV has been classified into four categories, the velogenic, mesogenic, lentogenic, and asymptomatic pathotypes (7). Co-infection of poultry with NDV and LPAIV–H9N2 may lead to severe clinical complications, with a higher mortality rate when compared with infection with a single virus (1, 8, 9).

Inactivated AIV and NDV antigens have been prophylactically included in water-in-oil (W/O) emulsion vaccines to control widespread outbreaks of AI and ND in enzootic countries (10). To be effective, vaccines require an appropriate antigen that matches the challenging virus. This remains a challenge in formulating AIV vaccines because AIVs often undergo mutations that alter their antigenicity (11). Furthermore, HPAIV cannot be used as a seed virus for the production of vaccines. The inability to use HPAIV as a seed virus and the difficulty predicting the antigenic shift of AIV indicate the need for new AIV vaccines based on epitopes common to various AIV subtypes and that can provide universal protection. Most universal vaccines currently under development are based on conserved epitopes in in matrix protein 2 (sM2), the stalk domain of HA (HA2), and other AIV structural proteins (12–14). A recombinant protein composed of both sM2 and HA2 (sM2HA2) has been shown to produce cross-reactive responses (15). However, the major limiting factor in the further development of vaccine remains the poor immunogenicity of antigens when administered alone (14, 16).

Attempts have been made to develop methodologies that will improve the production of cross-reactive antibodies and T-cell immune responses upon vaccination. These include the incorporation of vaccine adjuvants, consisting of chemical substances, microbial components, and/or proteins that enhance immune responses to vaccines. Adjuvants not only improve vaccine immunogenicity, but also can reduce the amount of antigen that must be administered, reduce the number of immunizations, and broaden immune responses to antigenically shifted variants. Although numerous commercial and experimental adjuvants have been tested in the last few decades, these adjuvants have limitations, including lack of efficacy, a tendency to induce systemic toxicity, manufacturing difficulties, poor stability, and high cost (17–19). For example, although aluminum-based mineral salts, the most widely used adjuvants in human and avian influenza vaccines, significantly enhance humoral responses to these viruses, their ability to enhance cellular immune responses is poor (20). Adjuvants are therefore needed that elicit both appropriate humoral immune responses and effective cellular immune responses to vaccines.

The present study evaluated a new W/O emulsion adjuvant, called CAvant® WO-60 (CAVAC, Korea), to determine whether it improves the immunogenicity of influenza antigens. The conserved recombinant sM2HA2 protein and the inactivated H9N2 (iH9N2) virus were emulsified in CAvant® WO-60 or the reference adjuvant ISA 70 VG (SEPPIC, France), followed by the immunization of mice with these vaccine formulations. CAvant® WO-60 was found to effectively enhance both the humoral and cellular immune responses of mice to these AIV vaccines. Moreover, immunization of mice with these vaccines in CAvant® WO-60 protected the mice from lethal AIV challenge. Furthermore, CAvant® WO-60 adjuvant contributed to the induction of seroprotective levels of antigen-specific hemagglutination inhibiting (HI) antibody responses to bivalent inactivated AIV-NDV vaccine in chicken. These findings suggest that CAvant® WO-60 may be a successful adjuvant for vaccines in poultry.

MATERIALS AND METHODS

Preparation of Vaccines

The sM2HA2 protein comprising conserved matrix protein 2 (sM2) and stalk domain of hemagglutinin (HA2) was constructed and purified as previously described (15). Briefly, Sequence verified synthesized sM2HA2 (662 bp) gene fragment was inserted into the multiple cloning site of the pRSETA vector (Invitrogen, USA) using *Bam*HI and *Eco*RI restriction enzyme sites. The recombinant 6xHis-M2e fusion protein was expressed in *E. coli* BL2-CodonPlus (DE3)-RIPL chemically competent cells purified by Fast protein liquid chromatography (FPLC) using immobilized metal affinity chromatography (IMAC) column (Bio-Rad, USA). The purified proteins were dialyzed using a permeable cellulose membrane (molecular mass cutoff, 12–14 kDa; Spectrum Laboratories, Auckland, New Zealand) in PBS at 4°C. The protein concentration was measured using Bradford assays (Bio-Rad, Hercules, CA). iH9N2 and inactivated NDV (iNDV) antigen formulation were performed according to the Office International des Épizooties (OIE) manual of diagnostic tests and vaccines for terrestrial animals (21). Briefly, the virus A/Chicken/Korea/116/2004(H9N2) (A/Chicken/Korea/01310/2001) and NDV (LaSota) were propagated in 10-day-old embryonated SPF chicken eggs. To determine the 50% egg infectious dose (EID50) of the propagated virus, eggs were inoculated with serially diluted virus and EID50 was calculated using the Reed and Muench method (22). The virus was inactivated using 0.2% formalin as described previously (23). The inactivated viruses were then inoculated into the 10 day old embryonated SPF chicken eggs to confirm the virus inactivation. Mineral oil-based adjuvant Montanide ISA 70 VG was purchased from SEPPIC (Paris, France); Mineral oil-based CAvant® WO-60 adjuvant was newly developed by Choong Ang Vaccine Laboratories Company (Daejeon, Korea) that forms low viscous (4.5 cP at 25°C) W/O microemulsions with small droplet size (<2 microns). For mice immunization, antigen loads of sM2HA2 15 μg/head and iH9N2 10^7 EID_{50}/dose were emulsified either with ISA 70 VG or newly formulated adjuvant CAvant® WO-60 at a 3:7 ratio (v/v) using a high shear mixer (Primix, Japan). For chicken immunization, inactivated NDV (LaSota) and LPAI (A/chicken/Korea/01310/2001) containing bivalent antigen and also 10^8 EID_{50}/dose from each antigen were emulsified either with ISA 70 VG or with CAvant® WO-60 at a 3:7 ratio (v/v).

Mice, Immunization, Virus Challenge, and Sample Collection

The specific pathogen-free (SPF) female BALB/c mice (6 weeks) were purchased from Samtako (Seoul, Korea) and housed in

TABLE 1 | Groups for the mouse experiments.

Regime	Vaccine group		Route	No. of mice tested in each group		
	Antigen	Adjuvant		ELISPOT and ELISA	Lung virus titer (3 and 5 DPI)	Survival and body weight
01	–	PBS	i.m.	5	3 for each time	5
	sM2HA2	–		5	3 for each time	5
	sM2HA2	ISA 70 VG		5	3 for each time	5
	sM2HA2	CAvant® WO-60		5	3 for each time	5
02	–	PBS	i.m.	5	3 for each time	5
	iH9N2	–		5	3 for each time	5
	iH9N2	ISA 70 VG		5	3 for each time	5
	iH9N2	CAvant® WO-60		5	3 for each time	5

PBS, phosphate buffered saline. i.m., intramuscular. DPI, day post-infection.

a temperature- and light-controlled milieu and had free access to food and water. In terms of mouse experiments, they were divided into two experiment regimes (one for the conserved influenza sM2HA2 antigen immunization and the other for iH9N2 antigen immunization) with four groups each. Each regime had 16 mice per group [5 for humoral and cellular immune responses evaluation, 6 for lung virus titration at 3 and 5 days post-infection (DPI), and 5 for body weight changes and survival rate screening after lethal challenge]. In specificity, groups of mice were intramuscularly (i.m.) immunized with PBS, sM2HA2, and sM2HA2 plus ISA 70 VG, and sM2HA2 plus CAvant® WO-60 emulsion. In each group, the dose of sM2HA2 antigen vaccinated into mice was kept constant at 15 μg/head in the total volume of 100 μl at the caudal thigh muscle of both hind limbs with 50 μl per hind limb. Mice were immunized twice every other week. Similarly, groups of other sets of mice were i.m. immunized with PBS, iH9N2, iH9N2 plus ISA 70 VG, and iH9N2 plus CAvant® WO-60 following the same immunizing schedule. The antigen dose of inactivated iH9N2 was kept constant at 10^7 EID_{50}/dose in the total volume of 100 μl at the caudal thigh muscle of both hind limbs with 50 μl per hind limb (**Table 1**).

The mouse-adapted low-pathogenic AI, namely, A/Aquatic bird/Korea/W81/2005 (H5N2) and A/Chicken/Korea/116/2004(H9N2), were used in the challenge experiments, and these were generously supplied by Dr. Young-Ki Choi (College of Medicine and Medical Research Institute, Chungbuk National University, Cheongju, Republic of Korea). The mice were anesthetized by ether inhalation following intranasal infection with $10LD_{50}$ of H5N2 or H9N2 influenza A subtypes on day 28. The survival rate was determined by death or a cutoff of 25% lost body weight, at which point the animals were humanely euthanized. All efforts were made to minimize suffering, and all of the surviving mice were humanely euthanized using CO_2 inhalation for 5 min after final monitoring. In all immunization groups, mouse sera were procured for antibody determination at the respective time points depicted in **Figure 1A** and were stored at−20°C until proceeding. Five mice from each group were sacrificed on day 24 after prime vaccination to obtain splenocytes for the analysis of antigen-specific T-cell responses.

Chicken, Immunization, and Sample Collection

The total number of 95, 6 week old (Strain: Leghorn) SPF chickens, purchased from Namdeok SPF (Korea), were divided into three experiment regimes: one regime for *in vivo* safety profile of the CAvant® WO-60 that contained two groups and other two regime for single immunization and priming-boosting immunization, which contained three groups per regime. In an *in vivo* safety profile regime both test group and control group consisted of 5 birds, chickens in the test group were vaccinated intramuscularly with 500 μl of inoculum contained 10^8 EID_{50}/dose from Newcastle disease virus (LaSota) and 10^8 EID_{50}/dose from LPAIV (A/chicken/Korea/01310/2001) antigen emulsified with CAvant® WO-60, chickens in the control group were maintain without vaccination (**Table 2**). After vaccination, the chickens were observed for general clinical signs, loss of appetite, and respiratory and gastrointestinal abnormalities for 28 days. The bodyweight of the chickens was measured at 0, 1, 2, 3, and 4 weeks after vaccination. In a single immunization regime, each group consisted of 20 birds; the first and second groups were immunized with 500 μl of inoculum containing 10^8 EID_{50}/dose from NDV (LaSota) and 10^8 EID_{50}/dose from LPAIV (A/chicken/Korea/01310/2001) antigen emulsified either with ISA 70 VG or with CAvant® WO-60 *via* intramuscular injection into the breast muscle. The birds of the third group were inoculated with 500 μl of sterile PBS. Blood samples were collected 21 days after immunization from the brachial vein as depicted in **Figure 4A**. In a priming-boosting immunization regime, the first and second groups consisting of 10 birds and the control group consisting of 5 birds were immunized twice at 2 week intervals with the same inoculum composition that was used in a single immunization experiment. Peripheral blood was collected at different time points up to 34 weeks as depicted in **Figure 4B**. Blood was incubated at room temperature for 30 min, and serum was separated from the whole blood by centrifugation at 12,000 rpm for 5 min.

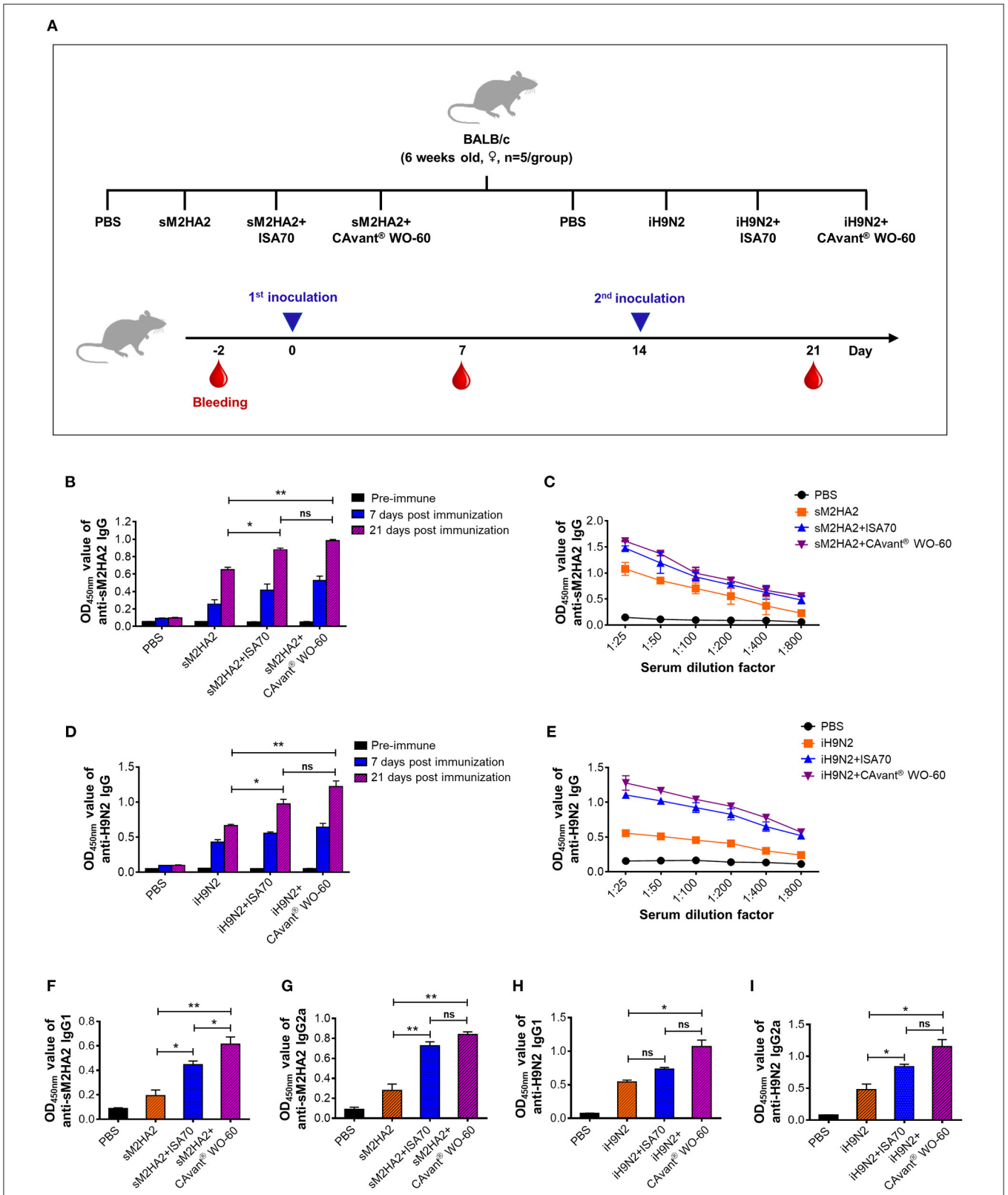

FIGURE 1 | Evaluation of the antigen-specific humoral immune response in mice. **(A)** Schematic depiction of mice experiment strategy. Mice were intramuscularly administered twice every other week. Serum samples were collected on day−2 (pre-serum), day 7, and day 21 after first immunization. The antibody response levels

(Continued)

FIGURE 1 | were detected by indirect ELISA. **(B)** Kinetics of appearance IgG at 1:100 serum dilution ratio and **(C)** comparative serum IgG antibody titers at 21 days post-immunization upon immunization with PBS, sM2HA2, sM2HA2+ISA 70 VG, and sM2HA2+CAvant® WO-60. **(D)** Kinetics of appearance IgG at 1:100 serum dilution ratio and **(E)** comparative serum IgG antibody titers at 21 days post-immunization upon immunization with PBS, iH9N2, iH9N2+ISA 70 VG, and iH9N2+CAvant® WO-60. **(F)** Systemic IgG1, **(G)** Systemic IgG2a antibody responses specific to sM2HA2 in the sera at day 21 after immunization with PBS, sM2HA2, sM2HA2+ISA 70 VG, and sM2HA2+ CAvant® WO-60. **(H)** Systemic IgG1, **(I)** Systemic IgG2a antibody responses specific to iH9N2 in the sera at day 21 after immunization with PBS, iH9N2, iH9N2+ISA 70 VG, and iH9N2+CAvant® WO-60. The bar shows the mean ± SD of $n = 5$ samples. Data from one representative experiment of two independent experiments are shown. Comparison of groups was analyzed by ANOVA and Tukey's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$ between CAvant® WO-60 adjuvanted, ISA 70 VG adjuvanted, and antigen-only group.

TABLE 2 | Groups for the chicken experiments.

Regime	Vaccine group		Route	No. of immunization	No. of chicken tested in each group
	Antigen	Adjuvant			
01	–	–	i.m.	–	5
	iH9N2+iNDV	CAvant® WO-60		2	5
02	–	PBS	i.m.	1	20
	iH9N2+iNDV	ISA 70 VG		1	20
	iH9N2+iNDV	CAvant® WO-60		1	20
03	–	PBS	i.m.	2	5
	iH9N2+iNDV	ISA 70 VG		2	10
	iH9N2+iNDV	CAvant® WO-60		2	10

iH9N2, Inactivated A/chicken/Korea/01310/2001 strain; iNDV, Inactivated Newcastle disease virus LaSota strain; PBS, phosphate buffered saline; i.m., intramuscular.

Enzyme-Linked Immunosorbent Assay (ELISA)

Ninety-six-well immunosorbent plates (Nunc, USA) were coated overnight at 4°C with 500 ng/well of sM2HA2 recombinant protein or with 1 μg/well of iH9N2 for serum IgG, IgG1, and IgG2a ELISA. After blocking for 2 h with 5% skim milk at room temperature (RT), serial twofold dilution of serum samples (1:25 to 1:800) was added into the wells, and the plates were incubated for 2 h at 37°C, treated with HRP-conjugated goat anti-mouse immunoglobulins (IgG, IgG1, and IgG2a, 1:3,000, Sigma, Korea) as the secondary antibodies, and incubated at 37°C for 2 h. The plates were then reacted with tetramethylbenzidine and H_2O_2-mixed substrate (BD Bioscience, USA) solutions for 10 min in the dark. Finally, the reactions were stopped by the addition of 2N-H_2SO_4, and the optical density (OD) values were measured at 450 nm using a scanning multi-well spectrophotometer (ELISA reader, Molecular Devices).

Splenocyte Isolation, Stimulation, and Elispot

For the analysis of antigen-specific T-cell responses, briefly, BD ELISPOT 96-well plates were coated with anti-mouse IFN-γ or IL-4 capture antibodies in 100 μl of PBS/well and incubated at 4°C overnight. The plates were blocked with complete RPMI 1640 medium containing 10% fetal bovine serum (Gibco, USA) and incubated in RT for 2 h. Freshly isolated splenocytes were added at 1×10^6 cells/well in media containing the 1 μg/well of sM2HA2 protein or M2 or HA2 peptide (**Table 3**) or 1 μg/well of inactivated H9N2 or only medium (negative control), or 0.5 μg/well phytohemagglutinin (positive control, Invitrogen, Carlsbad, CA, USA). Plates were incubated for 24 h for IFN-γ and 48 h for IL-4 at 37°C in 5% CO_2. After discarding the cells, the plates were treated sequentially with biotinylated anti-mouse IFN-γ and IL-4 antibodies, streptavidin-HRP, and substrate solution. Finally, the plates were washed with deionized water and dried for at least 2 h in the dark. Spots were counted automatically using an Immuno Scan Entry analyzer (Cellular Technology Ltd., Shaker Heights, OH, USA).

TABLE 3 | Peptides used for ELISPOT.

Protein[a]	aa position	aa sequence
HA2	19–48	GYAADLKSTQNAIDEITNKVNSVIEKMNTQ
M2	2–16	SLLTEVETPTRNEWE

[a] *Proteins of the A/EM/Korea/W149/06 (H5N1) virus. aa, Amino acid.*

Lung Virus Titration

Fifty percent tissue culture infectious dose ($TCID_{50}$) assays were performed to determine the virus titers in the lungs as previously described (21). Briefly, the lung tissues were homogenized in PBS containing an antibiotic and antimycotic solution (Gibco, USA) and centrifuged at 12,000 × *g* to remove cellular debris. Ten-fold serial dilutions of samples were added to the confluent MDCK cells at 37°C in a humid atmosphere for 1 h. An overlay medium containing L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) trypsin (Thermo Fisher Scientific, USA) was replaced without supplemented serum, and the infected cells were incubated for 48 h. After a cytopathic effect (CPE) was observed, a hemagglutination assay (HA) was performed, and the

virus titers were calculated by the Reed and Muench method (23) and expressed as $\log_{10}$ $TCID_{50}$/lung tissue.

Hemagglutination Inhibition (HI) Test

Sera from vaccinated chicken were heat-inactivated at 56°C for 30 min. Fifty microliters of sera were two-fold serially diluted in duplicate in round-bottom 96-well plates and mixed with an equal volume of 4 HA units of either NDV (LaSota) at RT for 30 min. AIV(A/chicken/Korea/01310/2001) virus was performed in V-bottom 96-well plates. Fifty microliters of 1% chicken red blood cells was added to each well and incubated for 20 min at room temperature before plate reading.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 6 software (GraphPad Software). All quantitative data were expressed as standard errors of the mean (SEM) or geometric mean (GM). Statistical significance was assessed using ANOVA followed by Tukey's multiple comparisons test. Comparison of survival was performed by a log-rank test. p-values of < 0.05 were considered statistically significant.

RESULTS

CAvant® WO-60 Adjuvant Improved Antigen-Specific Humoral Immune Responses to AIV Antigens in Mice

Groups of mice were immunized intramuscularly (i.m.) on days 0 and 14 with sM2HA2 protein (15 μg/dose) or iH9N2 (107 EID50/dose) emulsified 3:7 (v/v) in CAvant® WO-60 or ISA 70 VG adjuvant (v/v). Other groups of mice were injected i.m. with the protein or inactivated virus alone or with PBS. Serum samples collected immediately before immunization and 7 and 21 days after the first immunization were subjected to indirect ELISA using purified sM2HA2 and iH9N2 as the coating antigens (**Figure 1A**).

Seven days after the first immunization, the mice had relatively low sM2HA2-specific IgG levels, regardless of whether the antigens were delivered with or without an adjuvant. By contrast, 7 days after the second immunization (day 21), all mice that were injected with sM2HA2 exhibited high antigen-specific antibody levels. Moreover, anti-sM2HA2 IgG antibody titers were significantly higher in the sera of mice immunized with sM2HA2 emulsified in CAvant® WO-60 than in the sera of mice immunized with sM2HA2 alone (**Figures 1B,C**). Similar results were observed in mice immunized with iH9N2, in that second immunization boosted IgG responses to H9N2, with the highest H9N2-specific IgG titers observed in the sera of mice immunized with iH9N2 emulsified in CAvant® WO-60 (**Figures 1D,E**).

IgG isotype analysis by indirect ELISA using purified sM2HA2 protein and iH9N2 showed that antigen-specific IgG1 and IgG2a titers were balanced in the mice immunized with these antigens emulsified in CAvant® WO-60. Interestingly, antigen-specific IgG1 and IgG2a titers were higher in mice immunized with viral antigens emulsified in CAvant® WO-60 than in mice injected with antigens alone or emulsified in ISA 70 VG (**Figures 1F–I**). Thus, the results suggest that the newly introduced CAvant® WO-60 adjuvant enhanced humoral immune responses to AIV antigens.

CAvant® WO-60 Adjuvant Improved Antigen-Specific T-Cell Responses to AIV Antigens in Mice

The above-described groups of mice were sacrificed 24 days after the first immunization and their splenocytes were subjected to ELISPOT assays to quantify the numbers of antigen-specific cells that secreted interferon (IFN)-γ and interleukin (IL)-4 (**Figure 2A**). IFN-γ is a representative Th1 cytokine that is also expressed by cytotoxic T lymphocytes, whereas IL-4 is a Th2 cytokine. Thus, the splenocytes from mice immunized with sM2HA2 were stimulated with sM2HA2 or M2 or HA2 peptides. The percentages of IFN-γ and IL-4-secreting splenocytes were higher in mice immunized with sM2HA2 emulsified in CAvant® WO-60 than in mice immunized with sM2HA2 alone or with sM2HA2 emulsified in ISA 70 VG (**Figures 2B–G**). Similar results were observed when the splenocytes from mice immunized with iH9N2 were stimulated with whole H9N2 antigen. The percentages of IFN-γ and IL-4-secreting splenocytes were again higher in mice immunized with iH9N2 emulsified in CAvant® WO-60 than in mice immunized with the inactivated virus alone or with iH9N2 emulsified in ISA 70 VG (**Figures 2H,I**). These results provide evidence that immunization with sM2HA2 emulsified in CAvant® WO-60 enhanced antigen-specific T-cell immune responses to a similar or even higher degree than immunization with sM2HA2 emulsified in the control adjuvant ISA 70 VG.

CAvant® WO-60 Adjuvant Enhanced Protection Against AIV Infection in Mice

The finding that CAvant® WO-60 effectively induced both humoral and cellular immune responses to AIV antigens suggested that this adjuvant might improve the ability of the sM2HA2 or iH9N vaccine to protect mice against lethal AIV infection. Mice were therefore immunized i.m. twice at a 14 day interval with sM2HA2 or iH9N2, either alone or emulsified in CAvant® WO-60 or ISA 70 VG adjuvant. Mice immunized with sM2HA2 were challenged on day 28 with 10LD50 of A/Aquatic bird/Korea/W81/2005(H5N2) influenza virus, whereas mice immunized with iH9N2 were challenged on day 28 with 10LD50 of A/Chicken/Korea/116/2004 (H9N2) influenza virus. The efficacy of the immunization group was assessed by measuring weight loss and survival for up to 12 days post-infection (DPI) (**Figure 3A**).

Mice injected with PBS or with sM2HA2 alone without an adjuvant showed significant loss in body weight (over 25%), with 100% dying by 8 days. By contrast, all the mice that were immunized with sM2HA2 emulsified in CAvant® WO-60 were protected against challenge, with little weight loss until day 7, followed by gradual recovery. The mice that were immunized with sM2HA2 emulsified in ISA 70 VG exhibited continuous body weight loss, with 80% surviving until day 12 (**Figures 3B,D**). Similarly, mice injected with PBS or with iH9N2 alone without an adjuvant showed significant loss in body weight

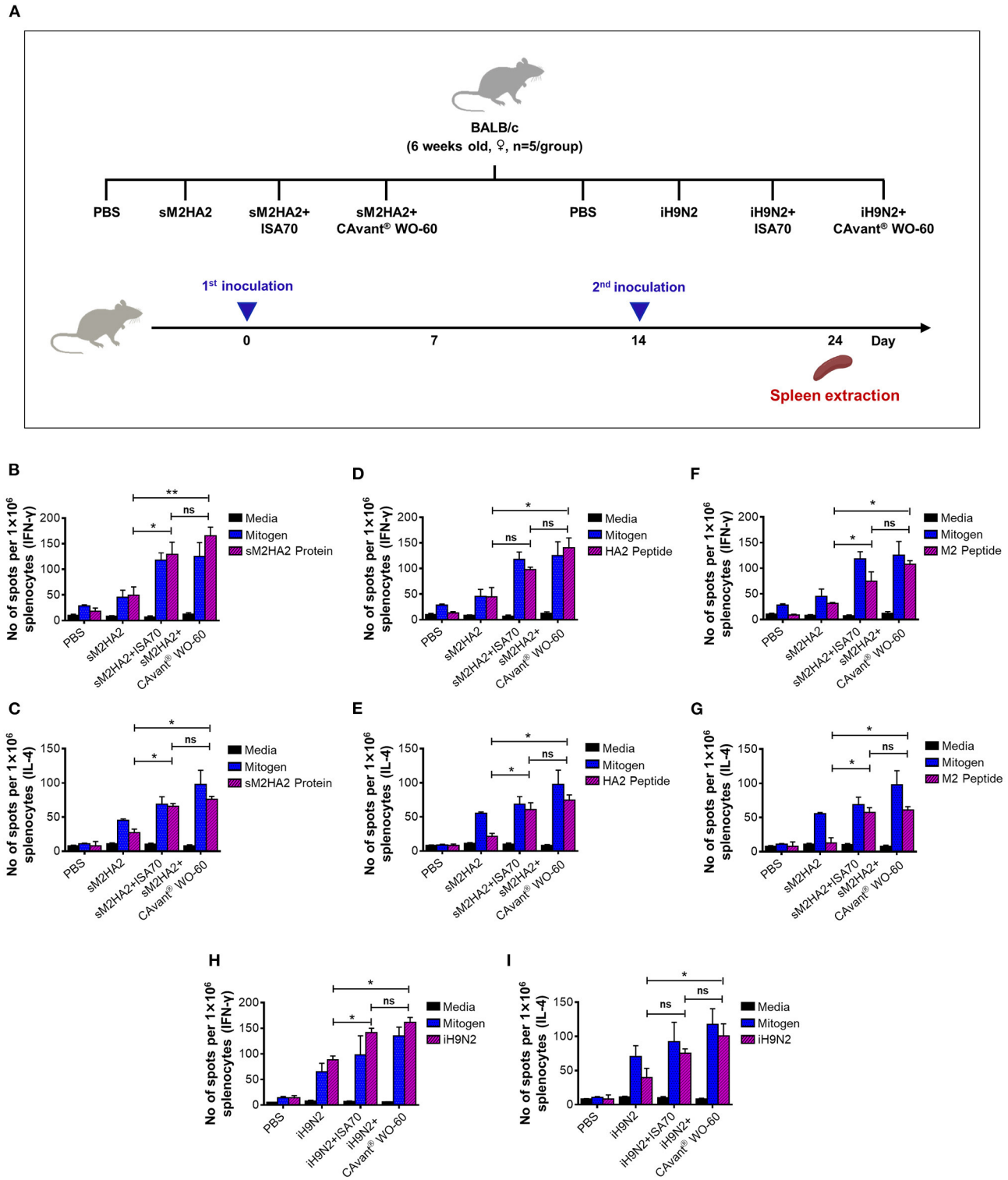

FIGURE 2 | Evaluation of the cell-mediated immune responses in mice. **(A)** Schematic depiction of mice experiment strategy. Splenocytes were harvested 10 days after the last immunization. Cells were re-stimulated *in vitro* with the **(B,C)** sM2HA2 protein, **(D,E)** HA2 peptide, **(F,G)** M2 peptide, and **(H,I)** iH9N2 and IFN-γ and IL-4 spot forming cell were determined by ELISPOT assay. The bar shows the mean ± SD of $n = 5$ samples. Data from one representative experiment of two independent experiments are shown. Comparison of groups was analyzed by ANOVA and Tukey's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$ between CAvant® WO-60 adjuvanted, ISA 70 VG adjuvanted, and antigen-only group.

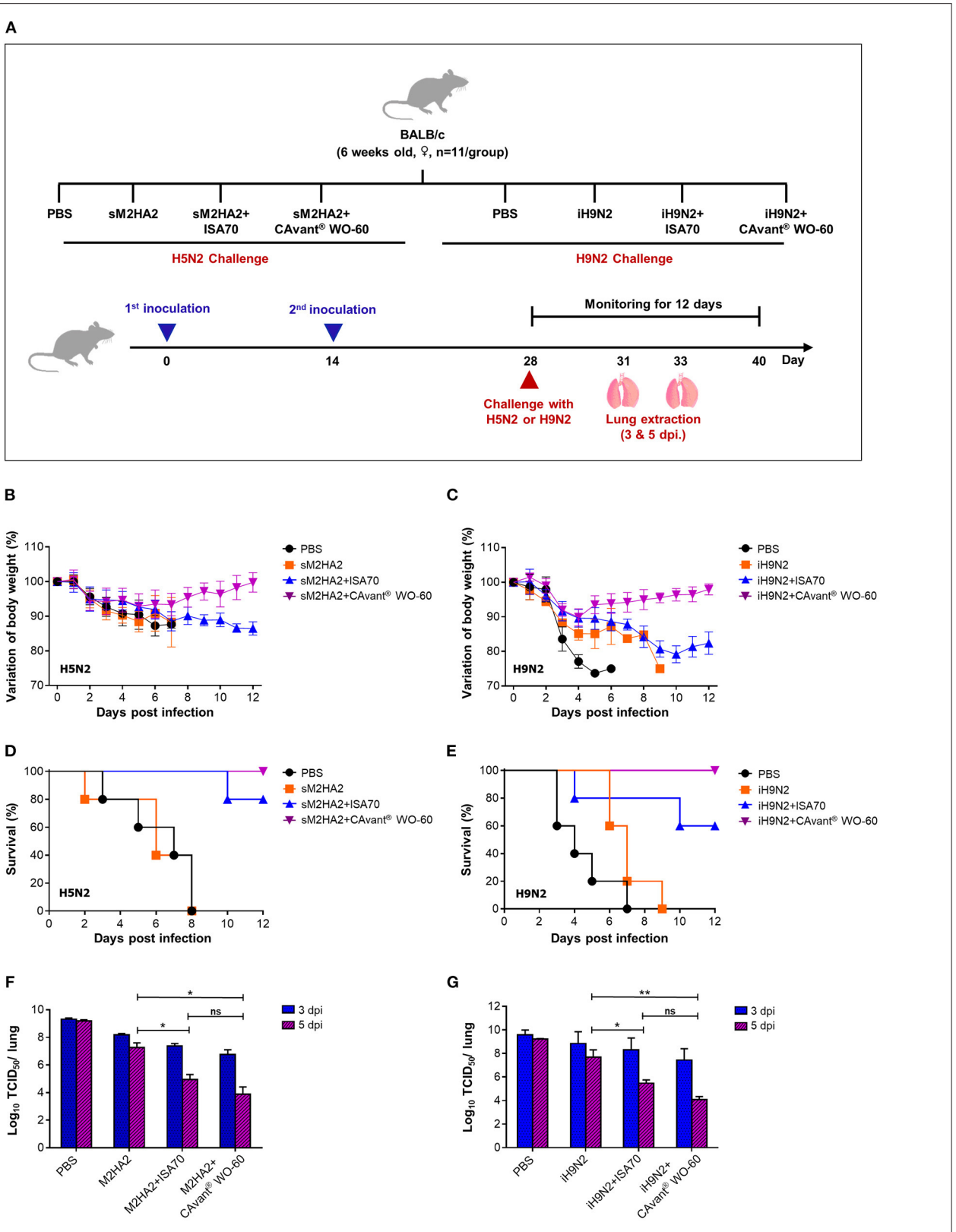

FIGURE 3 | Protective efficacy against lethal influenza virus infection in mice. **(A)** Schematic depiction of mice experiment strategy. BALB/c mice were intramuscularly administrated twice every other week. Mice were intranasally challenged with 10LD50 of mouse-adapted A/Aquatic bird/Korea/W81/2005(H5N2) and A/Chicken/Korea/01310/2001(H9N2) at 2 weeks after the second immunization. **(B,C)** The changes in body weight and **(D,E)** survival rate after performing lethal challenges

(Continued)

FIGURE 3 | with H5N2 and H9N2, respectively, were monitored for 12 days. **(F,G)** Virus titers in the lung tissues were investigated at 3 and 5 DPI by TCID50 in the MDCK cell following infection with A/Aquatic bird/Korea/W81/2005(H5N2) and A/Chicken/Korea/01310/2001(H9N2), respectively. The bar shows the mean ± SD of $n = 5$ or $n = 3$ samples. Data from one representative experiment of two independent experiments are shown. Comparison of groups was analyzed by ANOVA and Tukey's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$ between CAvant® WO-60 adjuvanted, ISA 70 VG adjuvanted, and antigen-only group.

after the challenge, with all dying on days 7–9. By contrast, mice that were immunized with iH9N2 emulsified in CAvant® WO-60 exhibited little weight loss, with all surviving for 12 days after the challenge. Body weight loss was significantly greater in mice immunized with iH9N2 emulsified in ISA 70 VG than in mice immunized with iH9N2 emulsified in CAvant® WO-60, with 40% of the former dying following challenge with the virus (**Figures 3C,E**). Taken together, these results suggest that immunization using the CAvant® WO-60 adjuvant can improve the protective efficacy of vaccines against influenza virus infection.

CAvant® WO-60 Adjuvant Augmented the Ability of AIV Vaccines to Control Lung Virus Titers After Challenge

To better understand the superior protection provided by the AIV vaccines when they were emulsified in CAvant® WO-60 adjuvant, randomly selected challenged mice from the above groups were sacrificed 3 or 5 days after infection, and the virus titers in their lungs were measured using the $TCID_{50}$ method. Compared with mice injected with PBS or with sM2HA2 alone without adjuvant, the mice that were vaccinated with sM2HA2 emulsified in CAvant® WO-60 had significantly lower virus titers in the lungs 5 days after challenge. These titers were also lower than in the lungs of mice immunized with sM2HA2 emulsified in ISA 70 VG (**Figure 3F**).

Similar results were obtained when the iH9N2-immunized mice were examined, with virus titers being significantly lower in the lungs of mice immunized with iH9N2 emulsified in CAvant® WO-60 adjuvant than in the lungs of mice injected with PBS or with iH9N2 alone without an adjuvant. Virus titers were also lower in the lungs of mice immunized with iH9N2 emulsified in CAvant® WO-60 than in the lungs of mice immunized with iH9N2 emulsified in ISA 70 VG (**Figure 3G**). These results suggest that CAvant® WO-60 can significantly improve the ability of AIV vaccines to inhibit viral replication in the lungs, thereby providing superior protection from infection with the influenza virus.

The CAvant® WO-60 Adjuvant Improves Antigen-Specific Seroconversion Responses of The NDV-H9N2 Bivalent Vaccine

To further assess the effect of the CAvant® WO-60 adjuvant on host animals, chickens were immunized i.m. once (priming) or twice (boosting) with a bivalent vaccine consisting of iNDV plus iH9N2 emulsified in CAvant® WO-60 or ISA 70 VG at a ratio of 3:7 (v/v). Control chickens were injected with PBS, and serum samples were collected from individual birds to determine their homologous HI titers (**Figures 4A,B**).

Twenty-one days after a single injection, the serum geometric mean titers (GMT) of anti-HI antibodies specific for NDV LaSota (1:50.2) and H9N2 (1:81.5) viruses from birds immunized with iNDV plus iH9N2 emulsified in CAvant® WO-60 surpassed the minimum protective titers (≥1:40). By contrast, titers in birds immunized with these viruses emulsified in ISA 70 VG were close to, but did not reach, the minimum protective titers. None of the control chickens was seropositive (≥1:4) against either virus (**Figures 4C,E**).

The kinetics of antigen-specific antibody induction and persistence were determined in immune sera collected at different times from chickens that received both priming and boosting immunizations. Fourteen days after the priming immunization, the mean HI titer for NDV LaSota virus was seroconverted in chickens from both adjuvant groups. The mean HI titers were further increased after the booster immunization, reached their maximum (>1:32) 14 days after the booster immunization, and declined gradually thereafter. Mean HI titers for NDV LaSota virus throughout the examination period were comparable in chickens immunized with antigens emulsified in CAvant® WO-60 and ISA 70 VG adjuvants (**Figure 4D**).

Seven days after the first immunization, the mean GMT HI titer for the H9N2 virus was seroconverted in both adjuvant groups. These titers were further increased after the booster immunization, maximizing at >1:128 14 days after the booster immunization. Similar to the NDV LaSota HI titer kinetics, H9N2 HI titer also decreased over time, with mean HI titers for H9N2 virus comparable throughout the examination period in chickens immunized with antigens emulsified in CAvant® WO-60 and ISA 70 VG adjuvants (**Figure 4F**). Taken together, these results suggest that, compared with the reference adjuvant ISA 70 VG, CAvant® WO-60 provides comparable or even superior immune responses to avian antigens and protection against avian viruses.

DISCUSSION

Poultry accounts for a significant share of worldwide food production (24). Effective protection of the global poultry industry against costly infectious diseases requires proper monitoring and active health management of the birds. Currently, active immunization using various types of infectious agents is a routine practice (22). Adjuvants contribute to the effectiveness of vaccines by enhancing the immunogenicity of antigens (17, 18). Because hundreds of poultry vaccines are produced annually, even small improvements in vaccine efficacy will have enormous dividends. Adjuvants can enhance vaccine efficacy in several ways, including by reducing the number of

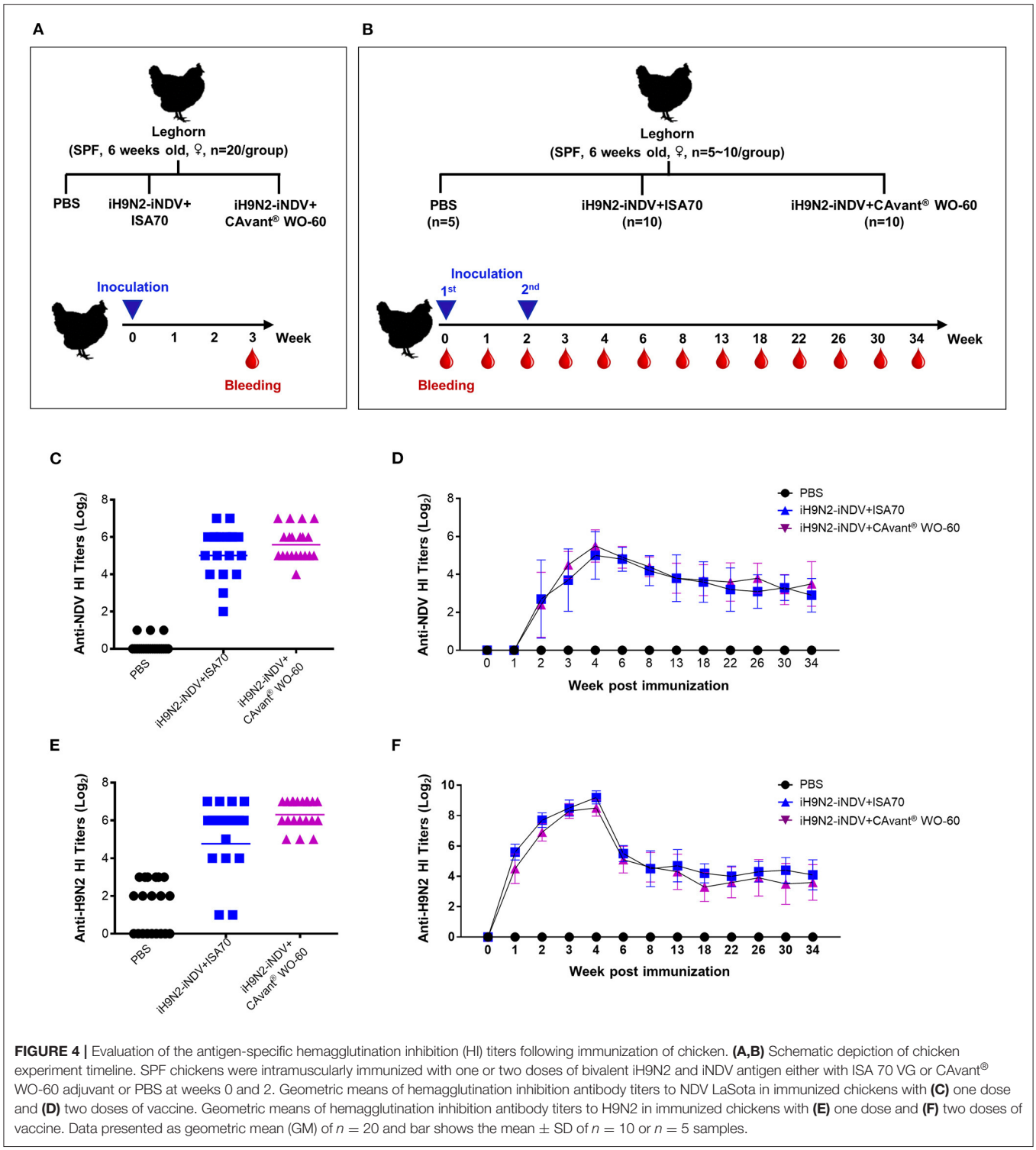

FIGURE 4 | Evaluation of the antigen-specific hemagglutination inhibition (HI) titers following immunization of chicken. **(A,B)** Schematic depiction of chicken experiment timeline. SPF chickens were intramuscularly immunized with one or two doses of bivalent iH9N2 and iNDV antigen either with ISA 70 VG or CAvant® WO-60 adjuvant or PBS at weeks 0 and 2. Geometric means of hemagglutination inhibition antibody titers to NDV LaSota in immunized chickens with **(C)** one dose and **(D)** two doses of vaccine. Geometric means of hemagglutination inhibition antibody titers to H9N2 in immunized chickens with **(E)** one dose and **(F)** two doses of vaccine. Data presented as geometric mean (GM) of $n = 20$ and bar shows the mean ± SD of $n = 10$ or $n = 5$ samples.

booster injections, reducing the amount of antigen per injection, broadening immune responses to antigenic variants, increasing vaccine availability, and reducing vaccine price.

Oil-based adjuvants have been shown to be superior to alternative formulations in vaccinating poultry. In particular, the W/O adjuvants ISA 70VG and ISA 71VG, which perform similarly, were reported to be for poultry vaccines (25). ISA 70VG-based vaccines against inactivated AIV and NDV induced higher protective antibody titers than Al (OH) 3/mineral oil-based vaccines (26, 27). ISA 71VG is effective

at eliciting humoral and cellular immune responses with the potential to generate protective immunity against Eimeria and NDV (28–30).

CAvant® WO-60 is a novel W/O emulsion type adjuvant containing a high-grade injectable mineral oil, refined non-ionic hydrophilic and non-ionic lipophilic surfactant system. The small average diameter and low viscosity of CAvant® WO-60 facilitate syringeability and injectability. *In vivo* safety profiles in chicken demonstrated no abnormal clinical sign or bodyweight reduction after vaccination (**Supplementary Table 1** and **Supplementary Figure 1**). The CAvant® WO-60 emulsion can have a variety of effects on vaccine biological activity by modulating antigen delivery to APCs or modulating a slow release of antigen to continue the stimulation of the immune system or having an intrinsic adjuvant effect through direct stimulation of immune cells. In addition, CAvant® WO-60 can have immunomodulatory properties by directing balance immune responses toward T helper (TH) 1 and TH2 response (18, 31).

Simultaneous enhancement of antibody and cell-mediated immune responses remains the prime objective of vaccination. Apart from virus neutralization, antibody-induced host effector mechanisms that aid in the clearance of virus are important properties of influenza vaccines (32–34). Vaccines consisting of viral antigens emulsified in CAvant® WO-60 induced better but not significantly higher level of antigen-specific IgG1 and IgG2a responses than vaccines consisting of the same antigens emulsified in ISA 70VG. Generally, Th1 cells improve the production of IgG2a, which effectively neutralizes and clears viruses, whereas Th2 cells induce IgG1 Abs, which effectively neutralize viruses (34). These results indicate that the CAvant® WO-60 adjuvant mediates the Th1/Th2 cell balance, providing effective cellular and humoral immune responses to antigens contained in these vaccines.

This study also investigated the splenic recall of Th1 and Th2 cell signature cytokine responses to the vaccine antigens by ELISPOT. Under these experimental conditions, antigens emulsified in CAvant® WO-60 increased but not at a significantly higher level of the frequencies of antigen-specific IFN-γ- and IL-4-secreting T cells compared to the antigens emulsified in ISA 70VG; the results showed good agreement with the IgG1 and IgG2a antibody titers. Furthermore, injection of antigens emulsified in CAvant® WO-60 completely protected mice from lethal challenges with A/Aquatic bird/Korea/W81/2005 (H5N2) and A/Chicken/Korea/116/2004 (H9N2) viruses, suggesting that CAvant® WO-60 can be an effective adjuvant for vaccines that target different influenza A virus subtypes. To further assess the potency of this adjuvant, the effects of CAvant® WO-60 on lung virus titer was assessed following lethal challenge with A/Aquatic bird/Korea/W81/2005 (H5N2) and A/Chicken/Korea/116/2004 (H9N2) viruses. Immunization with antigens emulsified in CAvant® WO-60 reduced the virus titers in the lungs after lethal challenges with these influenza A subtypes.

Although CAvant® WO-60 showed better adjuvant efficacy than ISA 70 VG in mice immunization experiments, the two adjuvants showed similar efficacy in chickens. Chickens were immunized with bivalent inactivated NDV and AIV emulsified in CAvant® WO-60 or ISA 70 VG, and their HI antibody titers, which correlate positively with protection in chickens, were evaluated (35). Chickens subjected to a single immunization with inactivated viruses in CAvant® WO-60 were found to have seroprotective levels of GM HI antibody titers 3 weeks after immunization, 1:50.2 against NDV and 1:81.5 against H9N2. Seroprotective GMT against these antigens was not achieved in chickens immunized with inactivated viruses emulsified in ISA 70 VG. Chickens subjected to both primary and booster immunizations were monitored for HI antibody responses for up to 32 weeks after the initial immunization. HI antibody kinetics were found to be comparable in chickens that received two injections of antigens emulsified in CAvant® WO-60 or ISA 70 VG adjuvant, with titers against both NDV and H9N2 peaking 4 weeks after the initial immunization and these antibody titers remaining seropositive for at least 32 weeks. Taken together, the results suggested that the two adjuvants were similarly effective in generating HI antibody responses.

In conclusion, this study demonstrated that the novel water-in-oil emulsion CAvant® WO-60 adjuvant was capable of inducing strong mixed humoral and cellular immune responses against viral antigens. These responses were capable of protecting mice against lethal influenza challenge, as well as enhancing the seroprotective antibody responses against the NDV and AIV bivalent antigen in chickens. The adjuvant efficacy of CAvant® WO-60 was comparable to that of the poultry vaccine adjuvant ISA 70VG. These findings suggest that CAvant® WO-60 adjuvant would be a promising candidate for the development of an effective poultry vaccine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Chungnam National University, Daejeon, Republic of Korea (Reference number CNU-00952) and ChoongAng Vaccine Laboratories Co., Ltd., Daejeon, Republic of Korea (Reference number CAVAC 18-009).

AUTHOR CONTRIBUTIONS

J-SL and S-SY: conceived and designed the experiments. E-SL, Y-JS, WC, and Y-HA: performed the experiment. E-SL, Y-JS, I-JY, J-SL, and S-SY: analyzed the data and wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.730700/full#supplementary-material

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Conflict of Interest: Y-JS, Y-HA, I-JY, and S-SY were employed by company Choong Ang Vaccine Laboratories Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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