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Brief communication

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Immunogenicity and safety of a rabies-based highly pathogenic influenza A virus H5 vaccine in cattle

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The circulation of highly pathogenic H5 influenza A viruses in cattle, other mammals, and wildlife threatens animal and human health. To address this, we vaccinated heifer-calves with a deactivated rabies-virus-based H5 vaccine, which was well-tolerated and elicited neutralizing antibodies against both clade-1 and clade-2.3.4.4b H5N1 viruses, comparable to naturally H5-infected and convalescing cows. The immune responses to the vaccine platform were durable for at least 200 days and unaffected by preexisting RABV immunity.

Highly pathogenic influenza A (HPAI) H5N1 viruses infect birds, mammals, and humans, often causing severe disease and high mortality rates. In early 2024, HPAI H5N1 clade 2.3.4.4b viruses began circulating among dairy cows in the state of Texas[1], resulting in significant morbidity and substantial reduction in milk production and quality[2–4]. Despite many interventions, these and related viruses have rapidly spread to infect more than 900 farms in 16 US states[5]. The virus has also spread to other animals and is associated with severe and even lethal disease in wild mammals such as foxes, bears, seals, and sea lions; in domesticated cats and dogs; in farmed mink and foxes; and in other livestock, such as goats[6]. Thus far, at least 67 human H5N1 infections have occurred in association with this epizootic[7]. Concern is mounting that the epizootic will not be contained with traditional farm biosecurity interventions alone and cattle vaccines against H5N1 are urgently needed.

We developed a rabies virus-based H5 inactivated vaccine to address this concern and evaluated its immunogenicity in cattle. To express the H5N1 HPAI HA antigen by the rabies vector, a synthetic full-length codon-optimized HA ORF of the Influenza virus A/Viet Nam 1203/2004(H5N1) isolate was cloned into the well-established rabies vaccine vector (BNSP333)[8] between the N and P genes (Fig. 1A). The virus was entitled RABV-H5. Based on previous immunogenicity data in mice (our unpublished data), we used a vaccine harboring the clade 1 Viet Nam 1203/2004 H5 gene. We confirmed the expression of the H5 and rabies G proteins in RABV-H5-infected cells by immunofluorescence with RABV G and H5 specific antibodies (Fig. 1B). The incorporation of H5 in addition to the other structural rabies virus proteins (L, G, N, P and M) was shown by analysis of sucrose-cushion-purified vaccine-virus particles by both SYPRO®-Ruby staining and by western blot analysis (Fig. 1C, D respectively).

To assess the immunogenicity of the BNSP333-H5 vaccine in cattle, 12 heifer calves were randomly assigned to 3 vaccination groups: A- received the RABV-H5 vaccine with SEPPIC adjuvant in a prime-only regimen; B- received the vaccine without adjuvant in a prime-boost regimen; and C- received the vaccine with SEPPIC adjuvant in a prime-boost regimen (Fig. 1E). The RABV-H5 vaccine was Betapropiolactone (BPL) inactivated and a 100 µg was injected subcutaneously (SC). The 100 µg vaccine dose was selected based on previous immunogenicity studies using the RABV-based vaccine in non-human primates[9]. The SC delivery is commonly used for beef cattle vaccinations (e.g. respiratory and clostridial vaccines that are part of the vaccination program for cattle and calves on cow-calf operations in the US). The SC route is also preferred to avoid affecting carcass quality. The cattle were not euthanized, and anesthesia before vaccine delivery or blood draws is not required for large animal species.

Serum from all vaccinated animals was collected on days 0, 14, 28, 35 and 42 post-prime for virus-neutralizing antibodies (VNA). Cattle studies were completed at the USDA-ARS, Livestock Arthropod Pest Research Unit (Knipling-Bushland US Livestock Insects Research Laboratory (KBUS-LIRL), Kerrville, TX, USA), and all animal procedures were approved by the

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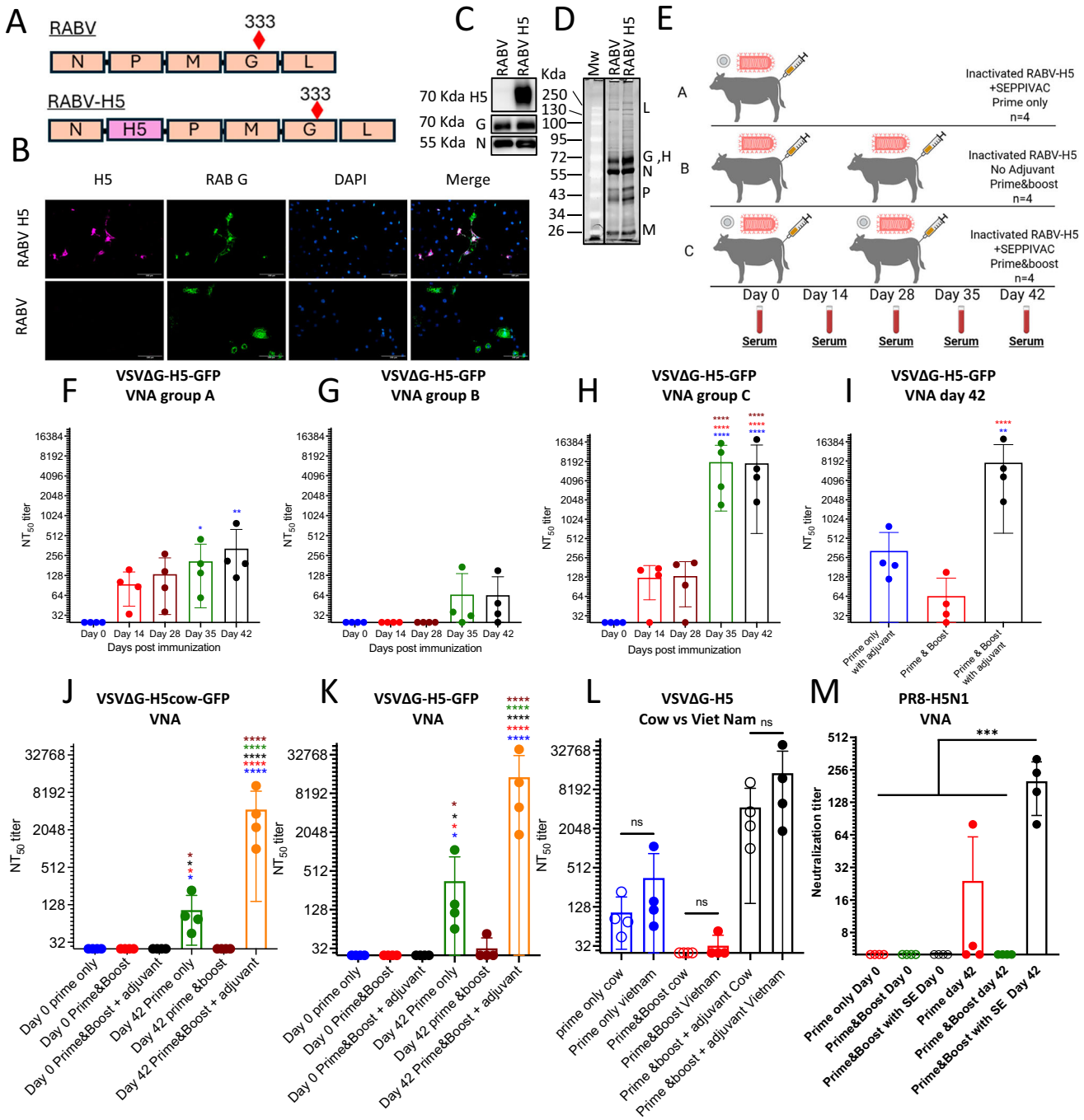


Fig. 1 | H5 immunogenicity of RABV-H5 vaccine in cattle. A Schematic genomic maps of the RABV-H5 vaccine vector and the parental RABV. The R333E-attenuating mutation in the RABV G antigen is indicated. N=nucleoprotein, P=Phosphoprotein, M=matrix protein, G=glycoprotein, L=polymerase. **B** Immunofluorescent staining of H5 and RABG in RABV-H5 and RABV-infected Vero cells. Cells were infected at MOI 0.01 and fixed after 48 h. Cells were permeabilized and stained with α-HPAI-H5N1 H5 polyclonal antibodies (purple), α-RABV-G 4C12 monoclonal antibody (green), and DAPI to visualize the nuclei (blue). In the merged images, white indicates an overlap between purple and green. Images were taken at 40X magnification. Scale bars represent 100 µm. **C, D** represent protein composition analysis of 1 µg sucrose purified virions by western blotting (**C**) and SDS-PAGE followed by SYPRO®-RUBY protein staining (**D**). Blots were either probed with α- H5 (NR-163), α-RABV-G 4C12 (middle panel) or α-RABV-N polyclonal rabbit sera (bottom panel). **E** Cattle immunization and blood draw schedule; 3 groups of 4 cattle were immunized once (group A) or twice (**B**, **C**) with 100 µg/dose of BPL-inactivated RABV-H5 vaccine, with (**A**, **C**) or without (**B**) SEPPIC-SWE™ adjuvant. Created with Biorender.com. VSVΔG-H5-GFP virus neutralization in prime only with adjuvant (**F**), prime-boost without adjuvant (**G**) and prime-boost with adjuvant (**H**). Filled circles are NT_{50} titers. Average (bars) and STDEV (error bars) are shown. Comparison of day 42 NT_{50} values between the three vaccination groups is shown (**I**). Day 0 and 42 VNA titers (NT_{50}) against VSVΔG-H5-GFP harboring the H5-cow version (**J**) or the H5- A/Viet Nam/1203/2004 version (**K**) and the comparison between them (**L**). PR8-H5N1 (**M**) and HPAI-H5N1 cow (**N**) VNAs (100% neutralization) are shown. Ordinary one-way ANOVA with Tukey's Multiple Comparison Test was used to determine statistical differences between groups at each time point. Stars indicate significant differences. Error bars indicate the mean with SD for groups of 4 cattle with samples run in duplicate. (****$p < 0.0001$; ***$p = 0.0001$; **$p \leq 0.0029$; *$p < 0.0173$; ns not significant).

KBUSLIRL Institutional Animal Care and Use Committee (IACUC) (Protocol #2024-05).

To analyze the vaccine-induced VNA against HPAI H5N1, we used a recombinant vesicular stomatitis virus (VSV) in which the VSV-G surface glycoprotein was deleted and replaced with HPAI H5 (Clade 1, A/Viet Nam/1203/2004(H5N1) with the intact polybasic cleavage site. In addition, the gene-encoding green fluorescent protein (GFP) was also incorporated into the genome, to generate VSVΔG-H5-GFP.

Our results presented in Fig. 1F indicate neutralizing antibodies to H5 14 days after a single immunization by the adjuvanted vaccine, and the neutralization titer was maintained throughout the study. A booster immunization with adjuvant on day 28 significantly increased the VNA titer (Fig. 1H). The unadjuvanted vaccine did not induce detectable VNAs after the prime and low VNA were detected after the boost immunization (Fig. 1G). At day 42 the highest VNA titers were measured in sera of prime-boost-immunized animals with the adjuvanted vaccine, whereas all animals that received the single immunization demonstrated a significant but lower VNA response, indicating successful priming (Fig. 1I). Prime-boost immunization without adjuvant showed VNA only in one out of four animals.

Having demonstrated that the RABV-H5 vaccine was capable of inducing H5-VNA, we asked whether the vaccine that carries a clade 1 H5 (A/Viet Nam/1203/2004(H5N1)) would induce cross VNA to the currently circulating 2.3.4.4b clade. Thus, using the above-mentioned recombinant VSV, we replaced the clade 1 H5 with the H5 gene of an HPAI from the currently circulating 2.3.4.4b clade. Indeed, sera from cattle vaccinated with the vaccine encoding the H5 clade 1 related antigen (A/Viet Nam/1203/2004(H5N1) efficiently neutralized VSVΔG-H5cow-GFP representing the circulating antigen (Clade 2.3.4.4b Cow) in both group A (Prime only with adjuvant) and group C (Prime-boost with adjuvant) (Fig. 1J). Testing the neutralization capacity of the clade 1 H5 (A/Viet Nam/1203/2004(H5N1) with parallel serum dilution revealed a similar pattern of neutralization, namely that sera from both groups A and C efficiently neutralized the VSVΔG-H5 Vietnam-GFP (Fig. 1K). Of note, the VNA titers towards clade 1 or clade 2.3.4.4b were not significantly different (Fig. 1L), indicating the potential of the RABV-H5 vaccine candidate to confer cross-clade protective immunity.

To confirm the results detected with the VSVΔG-H5-GFP, we determined the neutralizing antibody titers against PR8-H5N1, a recombinant Puerto-Rico 8 influenza A virus in which the HA and NA genomic segments have been replaced with the respective segments of H5N1(A/Viet Nam/1203/2004(H5N1) and the polybasic cleavage site has been deleted as previously reported[10]. Similar to the neutralization results obtained with VSVΔG-H5-GFP, a prime-boost vaccination with an adjuvanted vaccine (group C) induced significantly higher neutralizing antibodies compared to other vaccination regimens or to day 0 serum samples (Fig. 1M).

Analysis of the serum samples for rabies-virus-neutralizing titers revealed that already 14 days post single immunization with an adjuvanted vaccine, rabies-virus-neutralizing titers were above 0.5 IU/mL, the WHO-accepted protective antibody level against RABV (Fig. 2A). The rabies-virus-neutralizing titer remained significantly high on day 28 and then retreated but remained above the WHO-accepted protective antibody level (0.5 IU/ml) (Fig. 2A). Similarly to H5 neutralization, rabies neutralizing titers were significantly higher following prime-boost vaccination with adjuvant (Fig. 2C). Unadjuvanted vaccine required two immunizations to induce low, yet significant, neutralizing antibody response (Fig. 2B). Comparing the responses of the three vaccination groups on day 42 revealed that prime-boost with adjuvant (group C) yielded significantly higher titers than groups A and B (Fig. 2D), which was similar to the neutralization of both VSVΔG-H5-GFP (Fig. 1I) and PR-8 H5N1 viruses (Fig. 1M, N).

The safety profile of the inactivated rabies vaccine platform is well-established based on multiple studies and is currently entering a phase 1 clinical trial (ClinicalTrials.gov NCT 06546709). We used a commercially available adjuvant that is approved for use in both animals and humans (SEPIVAC SWE™) and a thorough analysis of blood samples from the vaccinated cattle revealed biochemical and hematological values as expected in healthy animals substantiating the safety profile of both our vaccine and the SEPIVAC SWE™ adjuvant (Supplementary Figs. 1–5).

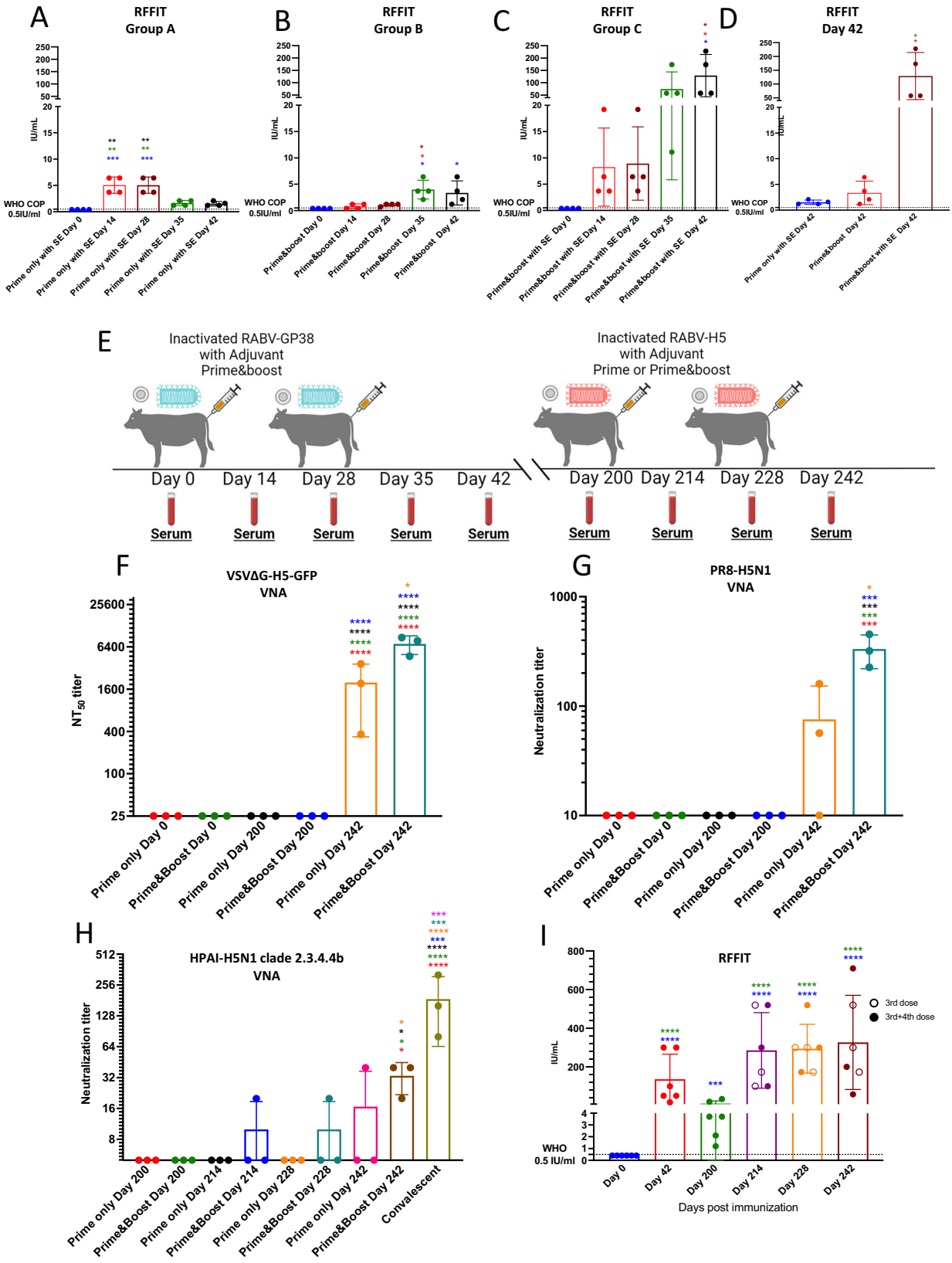
Moreover, we wanted to determine the immunogenicity of the RABV-H5 vaccine in the presence of preexisting RABV immunity. Using the SEPIVAC-SWE™ adjuvanted rabies-H5 vaccine, we vaccinated cattle ($n = 6$) at 200 days after they had been immunized (prime-boost with SEPIVAC-SWE™) with an unrelated RABV vaccine from another study (RABV-GP38[11], Fig. 2E). Blood was collected as indicated and analyzed for H5 and RABV-neutralizing antibodies (Fig. 2E).

Indeed, we found that cattle previously vaccinated with the RABV-H5 vaccine mounted a robust VSVΔG-H5-GFP and PR8-H5N1 neutralization response (Fig. 2F, G, respectively), similar to the response of vaccinated animals without preexisting immunity to the RABV vaccine vector (Fig. 1H, M, respectively). For neutralization of both VSVΔG-H5-GFP and PR8-H5N1 viruses, a prime-boost of the H5 vaccine was significantly better than a prime-only regimen (Fig. 2F, G). We further tested the capacity of the same cattle sera to neutralize the circulating HPAI virus (A/cattle/Texas/56283/2024 (H5N1))[12]. As early as 14 days after the first H5 vaccination (day 214) one animal already had detectable neutralizing antibodies. By day 42 post H5 vaccination (day 242), all prime-boost vaccinated animals and one animal that received only a single dose ("prime only") had neutralizing antibodies (Fig. 2H). Importantly, the day 242 neutralizing titers following a prime-boost regimen were not significantly different from the titers of naturally H5N1 infected and convalescent cows, yet additional samples of convalescent sera are needed to substantiate these results. Analysis of rabies-virus-neutralizing antibodies revealed that after 200 days, rabies-neutralizing titers were lower than day 42, yet were above the WHO protective value (0.5 IU/ml). Despite preexisting immunity to rabies, vaccination with an adjuvanted rabies-H5 vaccine induced a robust rabies-virus-neutralization immune response (Fig. 2I), similar to the H5 VNAs (Fig. 2F, G). A single boost (third dose of the RABV antigens) on day 200 was sufficient to induce robust RABV VNAs despite preexisting immunity, and an additional vaccine dose (fourth dose) had no significant benefit (Fig. 2I, compare open and closed circles).

The ability of vaccines to confer cross clade protection against the circulating HPAI-H5N1 is important. While mouse study by Hawman and collaborators[13] using a replicating RNA vaccine indicated that a clade 2.3.4.4b, but not clade 1 HA-based vaccine, protects against H5N1 (cattle) challenge in mice, our cattle study and importantly human studies demonstrated the ability of clade 1 H5N1 vaccine to mount clade 2 immunity. Others demonstrated the induction of clade 2.3.4.4b-cow antibodies (neutralizing and acting through ADCC) in humans vaccinated with an A/Viet Nam/1203/2004(H5N1) vaccine[14,15]. Additionally, the observed milder disease in aged immune individuals and the protective role of vaccine history in animal models[16,17] further support our results, demonstrating cross-clade virus neutralization in immunized cattle.

In the current study and in light with previous publications[18,19], we show that using VSVΔG-H5-GFP (clade 1 and clade 2.3.4.4b) for virus neutralization assays (Figs. 1 and 2) gave a similar pattern of neutralization when using the same samples with either PR8-H5N1 or HPAI-H5N1 (A/cattle/Texas/56283/2024). Besides ease of performance for multiple samples and no safety concerns, pseudoviruses allow for precise evaluation of VNAs towards H5, reducing the possible responses to other influenza viruses from previous natural exposures. Despite the differences between the different pseudoviruses used here and in previous studies[18,19], and the fact that we applied cattle rather than human sera, we similarly observed higher neutralization titers with the recombinant VSV. Unravelling the reasons for these differences is however beyond the scope of this manuscript.

Our study has several limitations. First, beef cattle were used instead of dairy cows. Although the immune response should also be tested in milk-producing cows, we believe that this cattle vaccination study supports the further development of the RABV-H5 vaccine to control the virus spread and mitigate the pandemic risk. Second, to expedite the study, we focused on



neutralizing antibody titers and did not study other mechanisms of protection such as ADCC. Third, we could not include a virus challenge step in the current study.

We show that the vaccine induced VNAs against HPAI-H5N1 (A/cattle/Texas/56283/2024) were not significantly different from convalescent samples (Fig. 2H). Neutralizing antibodies play an important role in protection against influenza in general and particularly against HPAI-H5N1[20–22]. Unfortunately, correlates of protection in the serum of cattle against the circulating clade are not known. Convalescence following infections confer at least partial protection from infection with the

Fig. 2 | Vaccine immunogenicity in the presence and absence of preexisting RABV immunity. Rabies virus (strain CVS-11) VNAs (IU/ml) in cattle sera were determined by Rapid fluorescent focus inhibition test (RFFIT) in prime only with adjuvant (**A**), prime- boost without adjuvant (**B**) and prime-boost with adjuvant (**C**). Comparison of day 42 VNA titers is shown (**D**). Cattle immunization and blood draw schedule (**E**). Six cattle were immunized twice with 100 µg/dose of BPL-inactivated, SEPPIC-SWE™ adjuvanted RABV-GP38 vaccine. On Day 200, the cattle were vaccinated with the same dose of RABV-H5 with SEPPIC-SWE; and on day 2283 cattle received another dose of the adjuvanted RABV-H5 vaccine. Syringes represent immunizations, and blood tubes indicate the days blood was drawn. Created with Biorender.com. VSVΔG-H5-GFP virus-neutralization titers (NT_{50}) (**F**), PR8-H5N1 virus-neutralization titers (100% neutralization) (**G**), A/cattle/Texas/56283/2024 (H5N1) virus-neutralization titers (100% neutralization) (**H**) and Rabies virus-neutralization titers (IU/ml) (**I**) are shown. Open circles in (**I**) refer to cows receiving 1 dose of the H5N1 vaccine 3 total doses of the RABV vector while closed circles refer to 2 H5 doses and 4 total RABV doses. Bars represent the mean values, and error bars indicate standard deviation. Ordinary one-way ANOVA with Tukey's Multiple Comparison Test was used to determine statistical differences between groups (****$p < 0.0001$; ***$p \le 0.0008$; **$p \le 0.0024$; *$p \le 0.047$; ns not significant). The dotted line indicates 0.5IU/mL, the WHO-accepted protective antibody level against RABV.

homologous clade[20]. Demonstrating that VNAs in vaccinated and convalescent sera were not significantly different, allows us to suggest that the vaccine induces protective neutralizing antibodies titers.

In summary, we demonstrated the safety and immunogenicity of this RABV-H5 vaccine in cattle. Despite the limitations of this study, the increasing awareness of the virus spillover to other animal species and humans warrants protective measures. The RABV vaccine platform has demonstrated long-term performance in multiple hosts, including mice, hamsters, nonhuman primates, and humans. Having a rabies based HPAI-H5N1 dual vaccine is valuable in certain world regions where rabies is endemic and transmitted to cattle from bats[23,24]. This further aligns with the one health approach to protect animals and humans from zoonotic infections (rabies and HPAI-H5N1). Thus, we believe the results obtained with the rabies-H5 vaccine candidate support its further development as a vaccine candidate for HPAI H5N1.

Methods

Animals

All animal procedures were approved by the KBUSLIRL IACUC (Protocol #2024-05). The cattle used in this study were not euthanized, and anesthesia before vaccine delivery or blood draws is not required for large animal species. Guidelines for the welfare of livestock from which blood is harvested for commercial and research purposes. New Zealand Government. March 2009. https://www.mpi.govt.nz/dmsdocument/1475-guidelines-forthe-welfare-of-livestock-from-which-blood-is-harvested-for-commercial-andresearch-purposes (Accessed January 22, 2025).

For the RABV-H5 vaccine study, Angus heifers (approx. 200–275 kg, 12–18 months old at the time of the study) were randomly distributed across 3 treatment groups ($n = 4$ cattle per group). To evaluate RABV-H5 vaccine immunogenicity in cattle previously immunized with a RABV-based vaccine, Angus heifers (approx. 300–370 kg at time of treatment) were randomly distributed into 2 treatment groups ($n = 3$ cattle per group). Study randomization was done through Study Randomizer, a web-based randomization service[25]. Black Angus cattle were used in this study because they are the most popular beef cattle breed in the United States, thus study results would translate to a large number of cattle production systems. The RABV-H5 was adjuvanted using the water-in-oil SEPPIC SWE™ adjuvant (SEPPIC, Inc; Fairfield, NJ) in a 50:50 immunogen:adjuvant ratio. All immunizations were delivered SQ to the neck region in a 1 ml volume using an 18-gauge, 3.8 cm needle. Alternate sides of the neck were injected if a booster immunization was required. Blood was drawn by jugular venipuncture using EDTA or plain Vacutainer tubes and blood collection needles. Approx 5 ml of whole blood (EDTA) was drawn per animal, and these were shipped overnight to the Galveston National Laboratory for analysis. Separately, approx. 20 mls of blood were collected in plain tubes, allowed to clot, and centrifuged at 1500 *x* g, 10 minutes for serum collection. Serum was transferred to labeled, sterile cryovials and stored at −80 °C until shipped to TJU for analyses. The Galveston National Lab and TJU were blinded to the study groups during the RABV-H5 vaccine study.

Cells, viruses and vaccine

Vero (ATCC CCL81), BSR (available from the Schnell laboratory), and MDCK (ATCC®CRL-9609™) cells were cultured using DMEM (Corning®) with 5% fetal bovine serum (FBS) (Atlanta-Biologicals®) and 1% Penicillin-Streptomycin (P/S) (Gibco®). All cells were stored in incubators with 5% CO_2 at 37 °C for standard cell culture or 34 °C for virus infected cells.

RABV strain CVS-11 (GenBank: GQ918139.1) was produced and titrated on BSR cells and is available upon request.

The codon-optimized HPAI H5 gene (Clade 1, A/Viet Nam/1203/2004(H5N1)) was synthesized (GenScript) and amplified using the following primers:

Forward primer: AAACTAACACCCCTCCGTACGCCGCCACCATGGAGAAGATTGTTCTGCTC and reverse primer: TTAGTTTTTTTCATGGCTAGCTCAGATGCAAATTCGGCATTGCAGG. The amplified product was cloned using infusion cloning (Takara) between the N and P genes and the plasmid was designated BNSP333-H5 (GeneBank Submission ID: 2976311). Vaccine recovery, production purification, titration and inactivation were performed as previously described[26]. 1 µg of the vaccine and of the parental rabies vector were analyzed by SDS-PAGE followed by either Sypro®-Rubi staining for total protein composition or by Western blot analysis using antibodies to H5 (NR-163, BEI), rabies G (mAb 4C12) or rabies N. RABV-GP38 vaccine has been previously described[11]. VSVΔG-H5-GFP encoding either the codon optimized full length H5 gene of either clade 1 (A/Viet Nam/1203/2004(H5N1) or the circulating clade 2.3.4.4b (A/cattle/Texas/24-009028-002/2024) was generated as described[27]. Both H5 genes include the polybasic cleavage site.

Influenza virus PR8-H5N1 was kindly provided by Adolfo-Garcia Sastre and Randy Albrecht (Ichan Institute, Mount Sinai School of Medicine, NY, USA)[10]. Work with PR8-H5N1 at TJUH was performed in compliance with the Institutional biosafety safety guidelines. PR8-H5N1 was propagated in MDCK cells in OptiMEM (Invitrogen) in the presence of 2 µg/ml Trypsin-TPCK. For titration of PR8-H5N1, 4 replicates were serially diluted in OptiMEM and incubated with MDCK monolayers for 1 h followed by 96 h of incubation in the presence of 2 µg/ml Trypsin-TPCK and then stained with 4% Crystal Violet in 20% Ethanol solution.

HPAI-H5N1 isolated from a cow (A/cattle/Texas/56283/2024 (H5N1)) (Genbank accession #PP600140) was isolated and characterized by I.S. and G.C.G of the University of Texas Medical Branch at Galveston's and shared via the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA). The virus was isolated from dairy cattle displaying clinical signs such as fever, cough, depression and nasal discharge. The virus was propagated in MDCK cells in Minimum Essential Medium Eagle (EMEM, Sigma-Aldrich) in the presence of 2 µg/ml Trypsin-TPCK and the viral titer was determined by fifty percent tissue culture infectious dose (TCID50).

Work with the virus was performed in an enhanced BSL3 laboratory at the University of Texas Medical Branch (UTMB), Galveston, TX, in compliance with the Institutional safety guidelines.

Immunofluorescence

3E5 Vero cells were seeded on glass coverslips in a 12-well plate and infected the next day at an MOI of 0.01 with the respective viruses. After 72 h (RABV viruses), cells were washed in 1X DPBS and fixed for 10 mins in 2% paraformaldehyde (PFA) in 1X DPBS, then washed with DPBS and permeabilized for 2 mins with 2% PFA and 0.5% Triton™ X-100 (Sigma-Aldrich®) diluted in DPBS and then washed 3 times with DPBS. Subsequently, cells

were blocked in 1XDPBS with 5% FBS for 1 h at room temperature. Cells were then probed overnight at 4 °C with polyclonal goat anti-HA (H5N1) (1:500, NR163- BEI) diluted in 1X DPBS with 1% FBS, specifically. Cells were washed 3 times with 1X DPBS and incubated overnight at 4 °C with anti-RABV-G 4C12 (provided by Scott Dessain, Lankenau Institute for Medical Research, Wynnewood, PA) conjugated with AlexaFluor (AF) 488 at 4 µg/mL 2.5 µg/mL of anti-goat AF647 (ThermoFisher, A-11004) in 1X DPBS with 1% FBS containing 1 µg/mL 4',6- diamidino-2-phenylindole (DAPI, Invitrogen) for 45 min at room temperature. Cells were then washed 5 times with 1X DPBS, mounted onto slides using mounting media (ProLong™ Glass Antifade Mountant, Invitrogen™ catalog number: P36984), and stored O.N. at room temperature in the dark. Slides were visualized the next day using a Nikon Ti-E microscope with Nikon A1R Laser Scanning confocal A1R-HD with the Plan Fluor 40x/1.3 objective lens on the NIS-Elements C software for multi-dimensional experiment acquisition and analysis at 23 °C. Color channels were processed (channels separated for individual images and merged for merged images) using ImageJ software (OSS NIH).

SDS PAGE protein gel and western blot

Sucrose purified virus particles were denatured with Laemmli Sample Buffer (Bio-Rad#1610737) supplemented with 2-mercaptoethanol (CAS No. 60–24–2, Millipore Sigma®) and boiling at 95 °C for 10 min. 1 µg of samples for total protein analysis were resolved on a 10% SDS PAGE and stained O.N. with SYPRO™ Ruby Protein Gel Stain (ThermoFisher Scientific). 1 µg of samples for western blot analysis were resolved on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane in Towbin buffer (192 mM glycine, 25 mM Tris, 20% methanol). Blots were then blocked in 5% milk dissolved in PBS-T (0.05% Tween® 20 [MilliporeSigma®]) at room temperature for 1 h. Next, membranes were incubated with primary antibody O.N. at 4 °C. Antibodies were made in a solution of 5% bovine serum albumin (BSA) in PBS. Goat Anti-HA H5N1 polyclonal sera (BEI resources, NR-163) was used at a dilution of 1:1000, anti- rabies N polyclonal sera (Schnell lab) was used at a dilution of 1:1000, and anti-RABV-G 4C12 (provided by Scott Dessain, Lankenau Institute for Medical Research, Wynnewood, PA) was used at 2 µg/mL dilution. The next day the blots were washed with PBS-T and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti goat (Jackson ImmunoResearch, 115–035–146) or human IgG (SouthernBiotech, 2040–05) at 1:20,000 dilution in PBST. Proteins were detected with SuperSignal West Dura Chemiluminescent substrate (Pierce®) and imaged on the FluorChem R system (proteinsimple®).

Determination of VSVΔG-H5-GFP neutralization titer

All serum samples were heat inactivated (30 min at 56 °C). VSVΔG-H5-GFP neutralization was determined by serial dilution of the heat-inactivated sera (in duplicates) in OptiMEM in U-shaped 96-well plates, followed by addition of a dose of VSVΔG-H5-GFP that was previously diluted in OptiMEM+2 µg/ml Trypsin TPCK and incubated for 1 h in 34 °C. The virus dose was calculated to result in about 300–1200 GFP positive cells within 20 h of incubation. After 2 h of incubation of the serum dilutions with the virus, the mixture was added to monolayers of Vero cells plated on µclear 96-well black plates (Greiner) and incubated for 20 h at 34 °C. The number of GFP-positive cells was determined using Agilent's BioTek Cytation5 automated microscope at 4X magnification. The Neutralization titer was determined as the EC50 compared to samples in the absence of serum.

Determination of influenza virus neutralization titer

For PR8-H5N1 and HPAI-H5N1, cow serum samples were RDE treated (Denka Seiken, Tokyo, Japan) for 24 h according to the manufacturer's instructions. One volume of a serum was added to three volumes of reconstituted RDE and incubated for 24 h at 37 °C. Then, PBS was added to a final dilution of 1:10 and the RDE treated serum was heat inactivated (56 °C for 30 min). Virus-neutralizing antibody titer was determined by a modified microneutralization assay[28]. Serum samples were serially diluted in 4 replicates in OptiMEM, a 100 TCID50 dose of PR8-H5N1 was added, and the mixtures were incubated for 1 h at 34 °C. Then, the virus-serum mixtures were added to MDCK monolayers that were pre-washed with PBS followed by incubation for 1 h at 34 °C. Then the mixture was aspirated, and the cells were overlayed with OptiMEM supplemented with 2 µg/ml Trypsin-TPCK and incubated for 96 h at 34 °C, followed by fixation and staining with 4% Crystal Violet in a 20% Ethanol solution. The virus-neutralization titer was determined as the last dilution where CPE was prevented. All wells infected with virus without sera should have CPE.

To determine the neutralization titer against the circulating HPAI-H5N1 virus, RDE-treated and heat-inactivated serum samples, including a known positive cattle serum control from convalescent cows, serially diluted (2-fold, in triplicates) in EMEM. 100 TCID 50 of H5N1 (A/cattle/Texas/56283/2024 (H5N1)) was added and the virus-serum mixtures were incubated for 1 h at room temperature (RT) on a rocking shaker at 100 rpm. The mixture and 2 µg/ml Trypsin-TPCK was then added to MDCK cells, and the plates were placed in an incubator at 37 °C, 5% CO2 for 48 h. The cells were then fixed and stained with a solution of crystal violet in 10% neutral buffered formalin. The virus neutralization titer was determined as described above.

Determination of Rabies virus neutralization titer

Determination of rabies-virus-neutralization titer was performed by Rapid Fluorescent Focus Inhibition Test (RFFIT) as described[26]. Briefly, serum was heat inactivated at 56 °C for 30 min. BSR cells were seeded at 3E4 cells per well in a 96-well plate; 24 h later, serum samples were diluted in a 3-fold dilution series in Opti-MEM in 96-well plates at a starting dilution of 1:50. The WHO standard rabies immune globulin was used at a starting dilution of 2 IU/mL. A dose of CVS-11 previously determined to produce 90% infection was added to each well and incubated for 1 h at 34 °C. The media in the plates with the BSR cells was then replaced by the sera/virus mixture, incubated for 22 h at 34 °C, aspirated, and the monolayers were fixed with 80% acetone and stained with a 1:200 dilution of FITC-conjugated anti-RABV-N antibody in Evans-blue for at least 4 h. Based on the WHO standard, the calculated 50% endpoint titers were converted to international units (IU) per milliliter.

Blood chemistry and hematological profiling

Quantitative blood chemistry analyses were done using the large animal profile kit from Abaxis (#500-0023).100 µl sera from each cow was added to the rotor and samples were analyzed using a Vetscan VS2® chemistry analyzer (Zoetis).

Blood was collected in EDTA tubes (BD Vacutainer, #366450) and blood counts were determined on a Vetscan® HM5 (Zoetis).

Statistical analyses

All animals were included in the analyses and no data were excluded.

All statistical analyses were performed using GraphPad Prism10 on log10 transformed data with an ordinary one-way ANOVA with a post-Hoc analysis using Tukey's Multiple Comparison Test with a 95% confidence interval.

Data availability

All data are available upon request to the lead contact author. No proprietary software was used in the data analysis.

Materials availability

Upon request, further information, resources, and reagents are available from the authors pending an executed MTA as well as biosafety approval of the requesting institutions(s).

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Author contributions

N.P.: conceptualization, formal analysis, investigation, methodology, visualization, writing, original draft. C.W. investigation, methodology, visualization, writing, review and editing, C.O. investigation, methodology, formal analysis, A.T. methodology, K.H.L. investigation, methodology, visualization, S.S.C- writing, review and editing, D.K- investigation, methodology, visualization, writing, review and editing, I.S. and G.C.G. isolated, characterized, and shared live A/cattle/Texas/56283/2024 (H5N1), screened and shared selected cattle sera with neutralization elevated antibody against HPAI H5N1, drafting and editing the manuscript; D.A.B.: conceptualization, funding acquisition, investigation, supervision, writing, review and editing P.O.: conceptualization, funding acquisition, investigation, supervision, writing, review and editing, M.J.S.: conceptualization, funding acquisition, project administration, supervision, writing, review and editing.

Competing interests

M.J.S, N.P and C.W are inventors of a pending patent application.

Additional information

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