Appendix

A. Methods

1. Design of mRNA vaccine constructs: Four alternative mRNA constructs encoding versions of the VapA protein were designed (**Figure 1**) based on the *vapa* gene (NCBI accession, #BAA04768.1). The unmodified sequence (AA1-189) was used to generate the native *VapA* construct. This sequence was codon optimized (**CO**) using an *Equus caballus* CGC codon frequency table[42] in Geneious Prime (2021.1.1, Biomatters Ltd.) to generate the *VapA-CO* construct. The transmembrane domain (**TM**) of the VapA sequence was predicted by SignalP-5.0 (https://services.healthtech.dtu.dk/services/SignalP-5.0/) and Polyphobius (https://phobius.sbc.su.se/poly.html) software. This TM region (AA 1-37) was deleted from the VapA sequence and replaced with the leader sequence from an equine immunoglobulin light chain variable region (AA 1-21; NCBI accession, #KR190600.1) to generate the *mod-VapA* construct. This construct then was codon optimized for equine tissues[42] to generate the *mod-VapA-CO* construct.

2. Culture and transfection of equine respiratory tract cells: Primary cultures were established as previously described.[36-38] Briefly, the lungs were removed en bloc within 1 hour of euthanasia and transported to the laboratory on ice. The bronchial epithelium was sharply dissected from the underlying submucosa and then minced into approximately 1-mm^2 segments. Aliquots of minced tissue were digested with 0.25% trypsin – 0.6 mM EDTA solution for 2 hours at 37°C in 5% CO_2. The suspension was filtered through a sterile cell strainer (pore size 40 µm) and then centrifuged at 250 × g for 10 minutes at 4°C. The resulting cell pellet was resuspended in warm supplemented airway culture media (Airway Epithelial Cell Growth Basal

Media and Growth Medium SupplementPack, Promocell) and then placed into a tissue-treated T75 cell culture flask and incubated for 30 minutes at 37°C in 5% CO_2 environment. The media was gently removed from the T75 flasks, allowing adhered fibroblasts to remain within the flask. Fibroblast growth media (DMEM, supplemented with 10% FBS, penicillin [200 U/mL], streptomycin [200 µg/mL], and amphotericin B [2.5 µg/mL]) was then added back to the flask for expansion of fibroblasts. The cellular suspension containing EBECs that had been removed from the T75 flask was centrifuged at 200 × g for 10 minutes at 4°C. The resulting cell pellet was resuspended in supplemented airway culture media. Cells were counted using a cell counter (CellometerAuto T4, Nexcelom Bioscience) with trypan blue for viability assessment, and then plated at a concentration of 2.5×10^5 live cells/mL on a collagen-coated tissue-culture-treated 24-well plate. Media for both cell lines was changed at 24 hours, and then every 2 days afterward. Fibroblasts were expanded and serially passaged every 3-4 days until plated at a concentration of 2.5×10^5 live cells/mL on a tissue-culture-treated 24-well plate in fibroblast media. All experiments were performed on first-passage EBECs and EBFs between 2-4 passages.

Each of the 4 mRNA constructs was formulated at a dose of 1 µg mRNA/well in a 24-well plate, with a commercially available transfection reagent (Lipofectamine MessengerMAX, ThermoFisher Scientific) according to manufacturer's instructions. Formulated mRNA was delivered to complete cell media in each well of adherent cultured cells, and plates were incubated at 37°C with 5% CO_2. Each condition was repeated in duplicate in each experiment, and the experiment was replicated 3 times. After 24 hours, supernatants and cells were harvested to assess secretion and intracellular expression of VapA. Samples were harvested, aliquoted, and stored at -80°C for subsequent analysis.

3. Western immunoblotting: Western immunoblotting was performed using supernatants and lysates of cultured EBECs and EBFs. Quantities of 30 µg protein from cell lysates and volumes of 30 µL supernatants were separated by SDS-PAGE, and then probed with a monoclonal mouse anti-VapA antibody (Santa Cruz Biotechnologies; Cat #sc-390576; 1:500 dilution) and a peroxidase-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories; Cat #115-035-062; 1:2,000 dilution). Recombinant VapA (**rVapA**) was used as a positive control. For loading control, a separate aliquot of each cell lysate during each immunoblot was probed with a monoclonal mouse anti-human GAPDH antibody (Invitrogen; Cat #43700; 1:500 dilution) and a peroxidase-conjugated goat anti-mouse secondary antibody (Abcam; Cat #6789; 1:5,000 dilution). Protein bands were developed using Radiance Plus® Femtogram HRP Substrate (Azure Biosystems) and visualized using the Bio-Rad Chemidoc Touch imaging system.

4. Formulation of mRNA vaccine in lipid nanoparticles: Based on *in vitro* expression results, the *mod-VapA* construct was selected as a vaccine candidate and *in vitro* transcription was repeated in larger scale under identical conditions (TriLink BioTechnologies). Lipid nanoparticles (**LNPs**) were synthesized using the microfluidic benchtop system NanoAssemblr (Precision Nanosystems). The composition of the LNPs included DLin-MC3-DMA (an ionizable lipid for mRNA conjugation), DSPC, cholesterol, and DMG-PEG2000, with molar ratios of 50:10.5:38:1.5, respectively. To achieve a total lipid concentration of 10 mM, these components were dissolved in ethanol. VapA mRNA was dissolved in the aqueous phase, which consisted of

a 50 mM sodium acetate buffer (pH=4). The mass ratio of lipids to mRNA was maintained at 1:20.

Prior to synthesis, the organic phase was heated for 3 minutes at 45°C, while the aqueous phase was briefly warmed up to 42°C for less than 60 seconds. The synthesis process utilized a flow ratio (organic phase to aqueous phase) of 1:3, with a total flow rate of 2 mL/min for dispensing the formulation. Following synthesis, the samples were dialyzed against 1× PPBS (overnight, 4°C), followed by sterile filtration before characterization (0.22μm filters). Fresh samples were prepared for each vaccination time point and kept at 4°C until vaccination.
The RNA concentration in the LNP formulation was determined using the RiboGreen RNA Assay Kit (Cat #R11490, Invitrogen) with 1× TE buffer, both with and without Triton X-100 detergent. The encapsulation efficiency of the RNA exceeded 85% for all batches. The average size and polydispersity index (**PDI**) of the LNPs were evaluated using dynamic light scattering (**DLS**) with a Malvern Zetasizer. The VapA LNPs exhibited an average diameter of 86.3 nm (lot #1, prime vaccination) and 77.7 nm (lot #2, boost vaccination) based on DLS analysis. The polydispersity (PDI) was less than 0.12 for both lots.

5. Bronchoalveolar lavage (BAL): BAL was performed at age 2 days in lateral recumbency under general anesthesia, using xylazine for sedation (0.5 mg/kg), followed by ketamine (2.2 mg/kg) and midazolam (0.04 mg/kg) for induction. At ages 22 and 35 days, the BAL was performed with standing sedation using xylazine (0.5 mg/kg) and butorphanol (0.04 mg/kg). Briefly, a BAL catheter (Jorgensen Laboratories) was passed down the trachea and lodged into a bronchus, and the air cuff was inflated. A volume of 50 mL sterile saline solution (0.9% NaCl) was infused through the catheter and immediately aspirated.

6. Anti-VapA antibody testing via ELISA

Indirect ELISA was used to determine anti-VapA IgG_1, $IgG_{4/7}$, and IgA antibody activities in serum and BALF. Immunoassay plates (Nunc Maxisorp immunoassay plate, Thermo Fisher Scientific) were coated with rVapA diluted in sensitization buffer (0.04 M PO_4, pH 7.2) at a concentration of 1 µg/mL for serum testing and 2 µg/mL for BALF testing, and then incubated overnight at 4°C. Plates were washed with PBS with 0.1% Tween-20 and then blocked with 1% non-fat milk in PBS at 37°C for 1 hour. Serum from a horse hyperimmunized against *R equi* was added to the plate in duplicate for positive control at dilutions of 1:640 for IgG_1, 1:40,960 for $IgG_{4/7}$, and 1:160 for IgA testing. Foal serum samples were diluted in blocking buffer (1:20 for IgG_1, 1:80 for $IgG_{4/7}$, and 1:40 for IgA) and added to the plate in duplicate. BALF samples were added to the plate undiluted in duplicate. Following washes, the subisotype-specific peroxidase-conjugated detection antibody for IgG_1 (Bethyl Laboratories; Cat #A70-124P; 1:40,000 dilution), $IgG_{4/7}$ (Bethyl Laboratories; Cat #A70-123P; 1:80,000 dilution), or IgA (Bethyl Laboratories; Cat #A70-123P; 1:2,500 dilution) was added to the plate, then incubated at room temperature for 1 hour.

For serum IgG subisotype ELISAs, plates were treated with SureBlue Reserve TMB Peroxidase Substrate (SeraCare) and incubated 20 minutes at room temperature in the dark. The reaction was stopped by adding sulfuric acid solution (0.18 M H_2SO_4) and optical densities (ODs) were determined at 450 nm using a microplate reader. For serum IgA and all BALF ELISAs, plates were treated with QuantaRed Enhanced Chemifluorescent Peroxidase Substrate (Cat #15159, ThermoFisher Scientific) and incubated 15 minutes at room temperature in the dark. The reaction was stopped by adding QuantaRed stop solution and ODs were determined at

570 nm using a microplate reader. An endpoint OD titer was calculated by dividing the experimental OD values with that achieved by the positive control on the same plate.

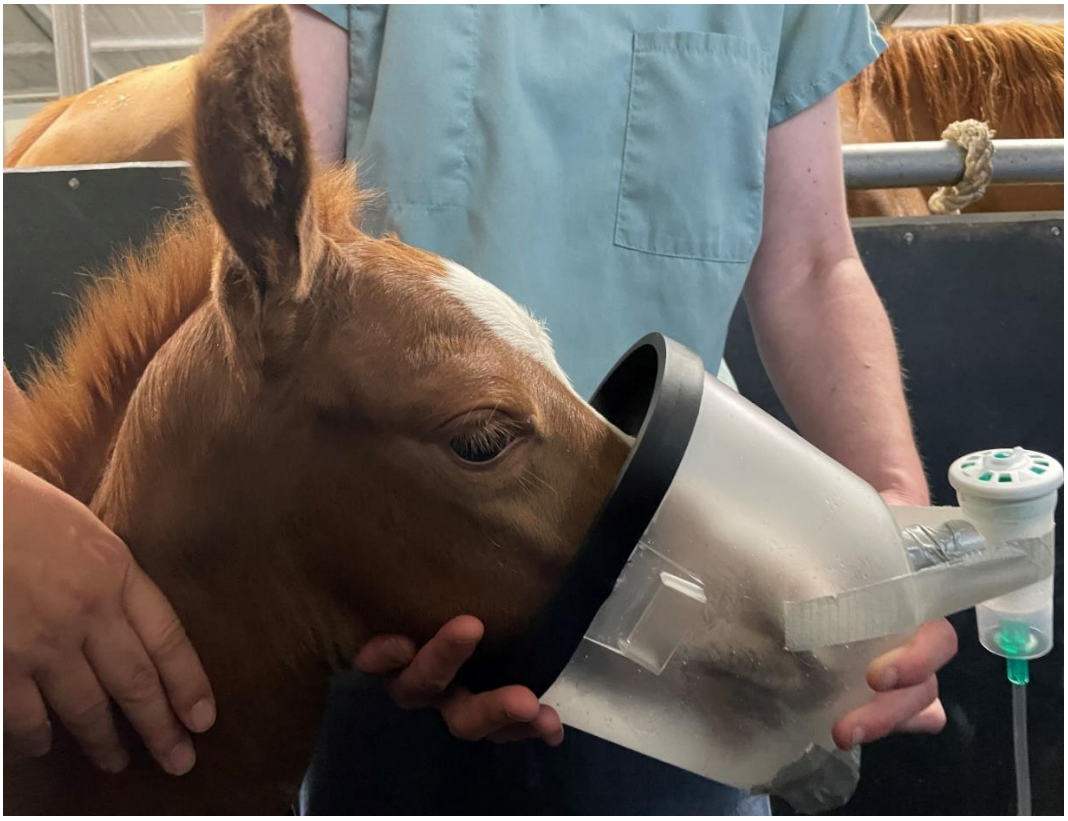
7. ELISpot Testing

The CMI response to vaccination was assessed by measuring interferon gamma (IFN-γ) production from isolated foal PBMCs stimulated with a lysate of the virulent *R equi* (strain ATCC 33701) using a commercially available ELISpot kit (Cat #3117-2A, Mabtech) performed according to the manufacturer's directions. A 96-well PVDF filter plate (Cat #S2EM004M99, MilliporeSigma) was treated with the mouse anti-bovine IFN-γ capture antibody (diluted to 15 µg/mL in PBS), then incubated overnight at 4°C. PBMCs were isolated using a Ficoll-Paque gradient separation (Histopaque-1077, GE Healthcare) and resuspended in RPMI-1640 media with L-glutamine, 15% FBS, and 1.5% penicillin-streptomycin. The PBMCs were added to the ELISpot plate at 100,000 cells/well in triplicate per condition, with 3 conditions: 1) media only as negative control; 2) the mitogen concanavalin A as positive control (Sigma-Aldrich; Cat #C5278; 2.5 µg/mL), or 3) *R equi* lysate (2.5 µg/mL). PBMCs were cultured for 48 hours at 37°C in 5% CO_2. Plates were washed to remove unbound cells, treated with a biotinylated mouse anti-bovine IFN-γ monoclonal detection antibody (diluted to 0.5 µg/mL in PBS and 5% FBS), and incubated 2 hours at room temperature. Plates were washed and treated with streptavidin-alkaline phosphatase (diluted to 1:1,000 in PBS and 5% FBS) for 1 hour at room temperature. The plate was washed, then treated with BCIP/NBT substrate solution and developed for 10 minutes in the dark. Development was stopped by washing the plate thoroughly with DI water, then the plate was dried overnight in the dark. Spots were quantified using an ELISpot plate reader (AID Plate Reader, Advanced Imaging Devices GmbH). For each condition, the number

of spot-forming cells was quantified by subtracting the background spots from a foal's negative control condition (media only) from that foal's PBMCs stimulated with the virulent *R equi* lysate (containing VapA).

8. Jet Nebulizer Setup

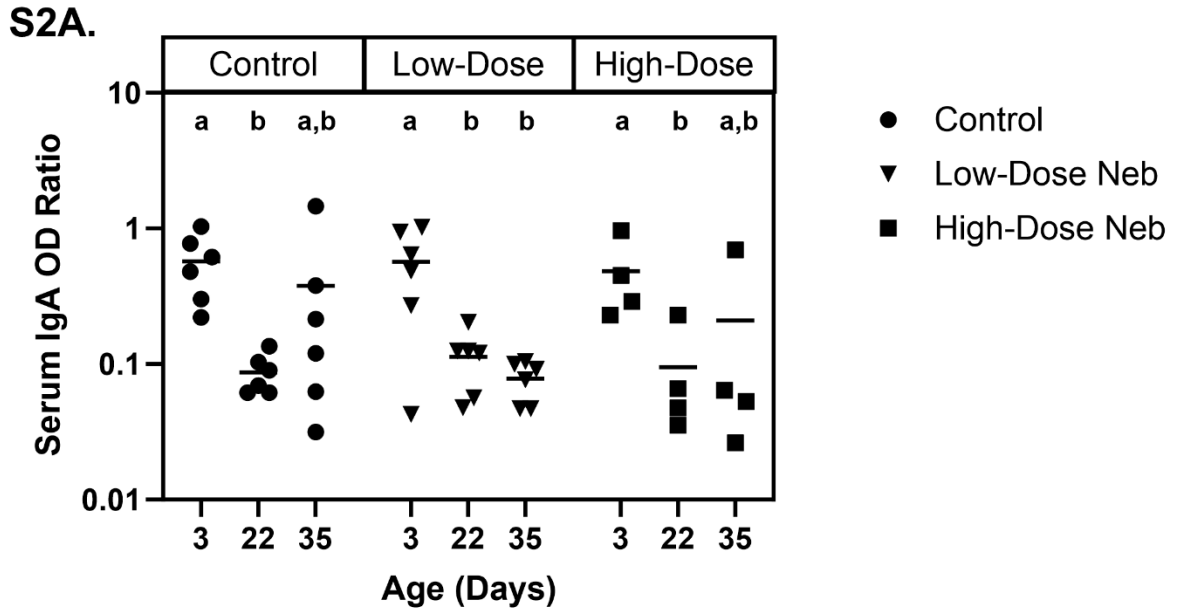
Jet nebulizer administration set-up for aerosol delivery of mRNA vaccine to neonatal foals is demonstrated in the figure below. A custom-made mask with rubber flange gasket around the muzzle was directly connected to a jet nebulizer cup (AeroEclipse-II Breath Actuated Nebulizer, Monaghan Medical)[40] in front of the nostrils with a commercially available equine jet nebulizer (Elite Equine Nebulizer System, Equi-Resp) at a running pressure of 35 psi.

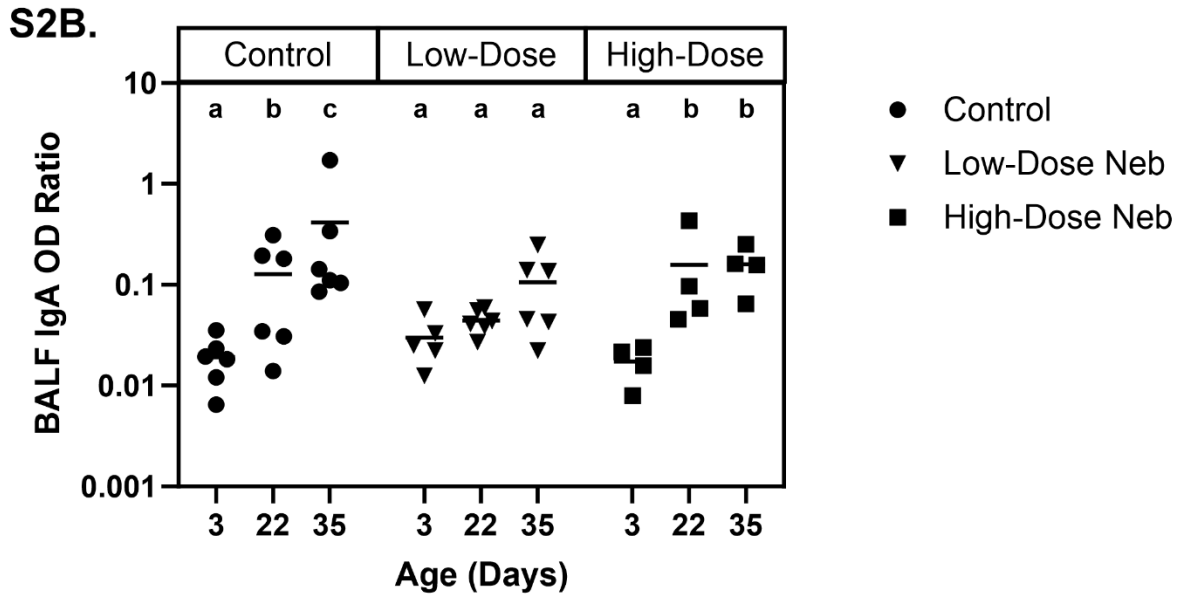


Supplemental Figure 1. Jet nebulizer delivering aerosolized mRNA to foal age 2 days.

B. Results

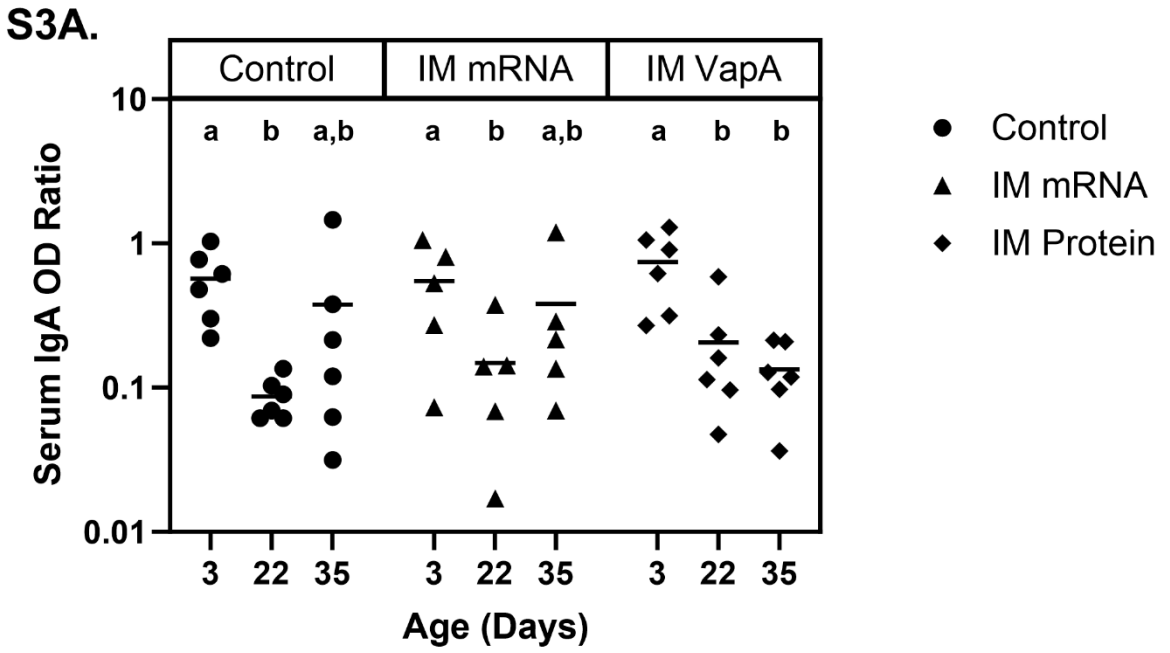
1. Anti-VapA IgA Activities in Serum and BALF

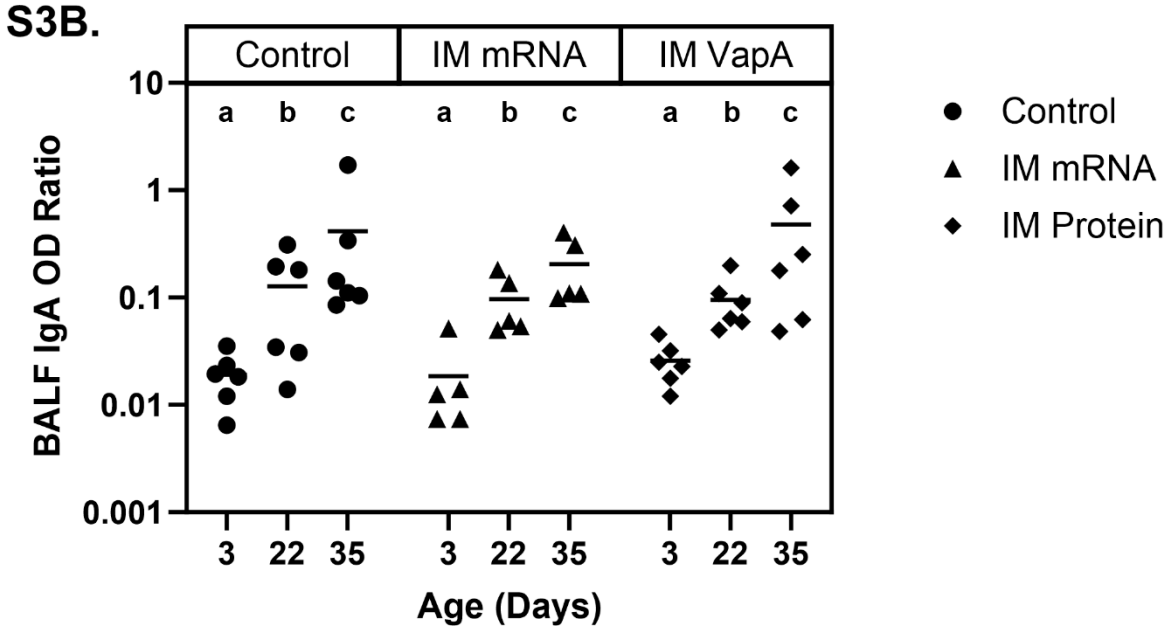




Supplemental Figure 2. IgA responses to nebulized mRNA vaccine in neonatal foals.

Supplemental Figure 2. IgA responses to nebulized mRNA vaccine in neonatal foals. Effects of group (controls nebulized with saline or principals nebulized with either a low or higher dose of mRNA encoding VapA), age, and their interaction on anti-VapA IgA activity in serum (**Fig. S2A**) and BALF (**Fig. S2B**) were examined. Serum IgA activities decreased significantly from age 3 days to 22 days in foals of all groups (P=0.0004 for controls, P=0.0483 for low-dose, and P=0.0263 for high-dose). Although values at age 35 days appeared lower than those at age 3, a significant difference was noted for the low dose mRNA group (P=0.0090) but not for the controls (P=0.2668) or the high dose group (P=0.0899). Within age (i.e., 3, 22, or 35 days), no significant differences between groups were detected. Values of IgA activity in BALF increased significantly between ages 3 and 22 days for the control group (P=0.0090) and for the high dose nebulized group (P=0.0050) but not the low dose nebulized group (P=0.9474). Similarly, IgA activities were significantly higher at age 35 days than 3 days for foals in the control group (P=0.0005) and the high dose group (P=0.0009) but not the low dose group (P=0.0533). Different letters denote significant differences between groups ($P<0.05$) within specific ages, and there were no pairwise differences between groups within specific ages. Black bars are means.





Supplemental Figure 3. IgA responses to intramuscular VapA vaccine in neonatal foals.

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Effects of group (controls nebulized with saline or foals immunized IM with either mRNA encoding VapA or VapA protein), age, and their interaction on anti-VapA IgA activity in serum (**Fig. S3A**) and BALF (**Fig. S3B**) were examined. Serum IgA activities decreased significantly from age 3 days to 22 days in control and IM VapA protein groups (P=0.0010 and 0.0028, respectively) but not foals in the IM mRNA group (P=0.0601). At age 35 days, serum IgA was not significantly lower than at age 3 days for control foals (P=0.2337) or IM mRNA foals (P=0.0899); however, activities of IgA for the IM VapA protein foals were significantly (P=0.0021) lower at age 35 days than at age 3 days. Serum IgA concentrations at day 22 and 35 did not differ within groups for controls (P=0.7973), IM VapA (P=0.9995), or IM mRNA (P=0.6146). Within age, there were no significant differences between groups. No significant effects of group or group by age interaction were detected for BALF anti-VapA IgA activity. Accounting for effects of group, anti-VapA IgA activity was significantly higher at ages 22 (P<0.0001) and 35 (P<0.0001) days relative to age 3 days, and values on day 35 were significantly (P=0.0003) higher than those on day 22. Different letters denote significant differences between groups (P<0.05) within specific ages, and there were no pairwise differences between groups within specific ages. Black bars are means.

2. Clinical response of foals: The maximum temperatures among the foals are tabulated in the table below. The maximum temperatures in the 3 IM VapA protein were 39.6°C, 39.9°C, and 40.4ºC (103.2ºF, 103.9ºF, and 104.8ºF). The maximum temperatures of the 2 foals in the nebulized mRNA were 39.4ºC and 39.5ºC (103.0ºF and 103.1ºF). The maximum temperature of the foal in the IM mRNA group that developed fever was 39.6ºC (103.2ºF).

	Control		**Low-Dose**		**High-Dose**		**IM mRNA**		**IM Protein**	
Foal	**P**	**B**	**P**	**B**	**P**	**B**	**P**	**B**	**P**	**B**
1	102.8 (39.3)	102.8 (39.3)	102.2 (39.0)	102.4 (39.1)	102.4 (39.1)	102.1 (38.9)	101.7 (38.7)	103.2 (39.6)	102.2* (39.0)	104.8* (40.4)
2	102.8 (39.3)	102.1 (38.9)	101.4 (38.6)	101.9 (38.8)	101.9 (38.8)	102.2 (39.0)	101.7 (38.7)	102.2 (39.0)	102.1 (38.9)	103.9* (39.9)
3	102 (38.9)	101.8 (38.8)	102.4 (39.1)	101.4 (38.6)	102.3 (39.1)	101.3 (38.5)	102.4 (39.1)	101.6 (38.7)	101.7 (38.7)	103.2 (39.6)
4	101.9 (38.8)	101.8 (38.8)	103.0 (39.4)	102.5 (39.2)	102.0 (38.9)	102.4 (39.1)	102.0 (38.9)	101.3 (38.5)	102.7 (39.3)	102.4 (39.1)
5	102.7 (39.3)	102.5 (39.2)	103.1 (39.5)	102.0 (38.9)			102.7 (39.3)	101.3 (38.5)	102.0 (38.9)	102.2 (39.0)
6	102.1 (38.9)	102.0 (38.9)	102.7 (39.3)	101.4 (38.6)					102.4 (39.1)	101.8 (38.8)

Supplemental Table 1. Maximum temperatures in foals following immunizations. Foals were examined by veterinarians twice daily. Maximum temperatures following prime (P) and boost (B) are denoted in degrees Fahrenheit (Celsius). Asterisk denotes foals that were treated with non-steroidal anti-inflammatory drug (**NSAID**) to treat inflammation post-immunization.