AJVR

Identification of 3 neutralizing linear epitopes on the VP8 outer capsid protein of group A equine rotavirus

Jill W. Skrobarczyk, PhD[1]; Patricia Flores-Ahlschwede, DVM[2]; Laurie L. Metcalfe, DVM[3]; Noah D. Cohen, VMD, MPH, PhD, DACVIM-LA[4]; Luc R. Berghman, PhD[1,5*]

[1]Texas A&M University, Department of Poultry Science, College Station, TX
[2]Rood and Riddle Equine Hospital, Saratoga Springs, NY
[3]Rood and Riddle Equine Hospital, Lexington, KY
[4]Texas A&M University, Department of Large Animal Clinical Sciences, College Station, TX
[5]Texas A&M University, Department of Veterinary Pathobiology, College Station, TX

*Corresponding author: Dr. Berghman (luc.berghman@ag.tamu.edu)

OBJECTIVE
To identify protective equine rotavirus group A (ERVA) VP8 epitopes and demonstrate that immunizing hens with synthetic peptides based on these epitopes would yield high-titered, neutralizing egg yolk antibodies for potential application in foals.

ANIMALS
26 rotavirus-positive, client-owned foals were included in the study. Five white leghorn hens were used for antibody production.

METHODS
Chicken antibodies were raised against 3 synthetic epitope peptides from the VP8 protein of the common ERVA P-type, P4[12] using CD40-targeted streptavidin-peptide complexes. Antipeptide serum- and egg yolk antibodies were subject to ELISA and in vitro virus neutralization assays to evaluate binding and neutralization activities. Lyophilized anti-VP8 egg yolk antibodies were orally administered (30 g; q 24 h for 5 days) to foals with rotaviral diarrhea. Physical examinations were performed daily. The duration of diarrhea and any adverse effects were recorded.

RESULTS
CD40-targeted vaccination of hens generated high titers of anti-VP8 serum and egg yolk antibodies after just 3 immunizations. These antibodies prevented in vitro infection of ERVA with titers of 128 in the serum and 94.5 in the yolk. Oral administration (30 g; q 24 h for 5 days) of lyophilized hyperimmune egg yolk to foals with rotaviral diarrhea did not reveal any adverse effects of the treatment.

CLINICAL RELEVANCE
This study demonstrated that antibodies raised against neutralizing epitopes of the ERVA VP8 protein could prevent ERVA infection in vitro. Based on these results and previous work in other animals, in vivo evaluation of the therapeutic efficacy of anti-VP8 egg yolk antibodies is warranted.

Keywords: egg yolk, antibodies, IgY, rotavirus, foals, synthetic peptides

Rotaviruses (RVs) are important causes of severe gastroenteritis in humans and animals.[1–6] In foals, infection results in severe diarrhea, dehydration, and, in some cases, death.[7,8] Other clinical signs include diminished suckling, depressed attitude and lethargy, fever, and colic, with younger foals generally displaying more severe signs.[7] Diarrhea usually develops within 4 days after infection.[7,8] Infections occur most often at breeding farms where contagion is high and consequently costly to manage. Seroprevalence to group A ERV is high in horses indicating infection is likely endemic and exposure to foals is likely very common.[7] The presence of ERVA particles in the feces of dams of infected foals indicates that transmission occurs through the fecal-oral route.[7]

Rotaviruses are members of the *Reoviridae* family characterized by a segmented, double-stranded

Received September 12, 2023
Accepted November 8, 2023

doi.org/10.2460/ajvr.23.08.0193

RNA genome. Classified into 9 groups (A to J), group A rotaviruses are responsible for most equine infections; however, group B rotavirus infections of foals emerged in 2021.[9,10] Within each group, 11 gene segments code for 6 structural and 5 nonstructural proteins that comprise the nonenveloped virus particles.[2,11] Six structural proteins designated as VP1 to VP4, VP6, and VP7 are arranged in layers forming the capsid which encloses the viral genome.[2] The outer capsid layer is composed of 2 structural proteins, VP4 and VP7, containing antibody neutralizing epitopes. These proteins are involved in sero- and genotyping RVs and are identified by the letters P and G, respectively.[2,4,12] VP7, denoting the G-type, is a glycoprotein forming the smooth external surface of the outer capsid shell. VP4, denoting the P-type, is protease-sensitive and is present as a series of dimeric spikes that protrude from the smooth VP7 shell.[2,12] VP4 has been characterized as the RV spike protein and has several important functions including cell attachment, penetration, virulence, and neutralization. Viral infectivity is enhanced following cleavage of the VP4 protein by trypsin into VP5 and VP8 subunits, with VP8 playing a significant role in viral infectivity and neutralization.[2,11,12] Previous studies have demonstrated that antibodies raised against the human rotavirus (HRVA) VP8 protein inhibit cell attachment and promote neutralization of the virus both in vitro and in vivo.[13,14] The neutralizing epitopes on the outer capsid HRVA VP8 protein have been mapped.[11] Five epitopes were identified and ranked according to their respective in vitro neutralization titers.[11] At the molecular level, group A human rotaviruses (HRVA) share considerable sequence homology with group A equine rotaviruses (ERVA). The 2 outer capsid, neutralizing proteins, VP4 and VP7, are approximately 75% to 80% homologous to their human counterparts. However, the neutralizing epitopes of the ERVA VP8 protein have not been mapped to date. Because of the sequence and structural homology observed between group A ERV and HRV, we hypothesized the corresponding ERVA epitopes would be protective in foals.

Rotavirus-specific egg yolk antibodies have shown to be therapeutically effective against RV infections by significantly reducing the duration of diarrhea in a variety of host species including human infants and neonatal mice, calves, and piglets.[15] Improved disease outcomes reported include a reduction in the duration of diarrhea, fecal pathogen shedding, stool frequency, clinical signs, and the need for oral rehydration solution (ORS) administration in infants.[15–19] To date, only minimal—albeit promising—data exist for the application of hyperimmune egg yolk antibodies in horses.[20,21]

This study aimed to identify protective ERVA VP8 epitopes and demonstrate that immunizing hens with synthetic peptides based on these epitopes would yield high-titered, neutralizing egg yolk antibodies for potential application in foals. Immunization with synthetic peptides offers advantages over whole virus or subunit vaccines due to the ease of production and low cost.

Methods

All protocols and procedures for this study were approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC) and the School of Veterinary Medicine & Biomedical Sciences Clinical Research Review Committee (AUPs 2020-0113 and 2021-20247 CA) and the Texas A&M University Institutional Biosafety Committee (IBC; permit 2019-005). Hens were housed at the Texas A&M Poultry Science Research Center.

Selection of ERVA VP8 neutralizing peptides for immunization

In a previous epitope-mapping study, 5 highly neutralizing epitopes were identified for the VP8 protein of the human rotavirus Wa strain.[11] We chose to evaluate 3 of the protective HRVA epitopes. These 3 epitopes were aligned with the sequence of the predominant ERVA VP8 genotype (P4[12]) and found to be highly conserved **(Table 1)**. Any mismatched amino acids were replaced with the residue from the equine VP8 sequence. The equine-specific VP8 epitopes were then commercially synthesized as N-terminally biotinylated peptides for immunization (GenScript).

Table 1—List of peptides synthesized for immunization of laying hens.

Neutralizing peptide sequences

Peptide no.	Position	HRVA	ERVA
1	aa 1-11	MASLIYRQLL**C**	MASLIYRQLL**A**
2	aa 55-66	**I**NDSTTVEPILD	**V**NDSTTVEPILD
3	aa 223-234	LPPIQNTRNVV**P**	**G**LPPIQNTRNVV

Bolded letters denote a difference in amino acid. ERVA sequences were derived from neutralizing regions of equine rotavirus VP8 (GenBank Accession number BAA02661.1).

ERVA = Equine rotavirus group A. HRVA = Human rotavirus.

CD40-targeted immunization of laying hens

Ten 30-week-old, Single Comb White Leghorn hens housed at the Texas A&M Poultry Science Center were hyperimmunized to produce group A ERV and HRV-specific antibodies. Immunizations consisted of 50 µg of an ERVA or HRVA peptide complex in 0.1 mL PBS, pH 7.4. The complexes were formed by combining the cocktail of biotinylated ERVA or HRVA peptides (GenScript), streptavidin (VWR), and a biotinylated agonistic anti-CD40 monoclonal antibody (in-house) in a 2:1:2 molecular ratio as described in previous studies from our laboratory.[22,23] Briefly, the peptides (8 µg) were first incubated with the streptavidin (55 µg) at room temperature for 30 minutes. After the incubation, the anti-CD40 antibody (300 µg) was added to the complex and incubated

for an additional 30 minutes at room temperature. All immunizations were administered subcutaneously in the wing-web. Birds were boosted every 2 weeks and blood samples were collected after each boost to monitor ERVA and HRVA-peptide specific antibody levels by indirect ELISA. Once birds were hyperimmunized as determined by indirect ELISA, eggs were collected daily. Anti-ERVA and -HRVA IgY were prepared by ammonium sulfate precipitation and lyophilization, and used for in vitro and in vivo analysis, respectively.

Purification and lyophilization of egg yolk IgY

To precipitate IgY for in vitro analysis, frozen eggs were thawed in room-temperature deionized (DI) water and yolks were separated from the egg white. To remove lipids, yolks were pooled and emulsified in DI water before adjusting the pH to 7.0 and freezing the emulsion again at −20 °C. The yolk emulsion was thawed again the next day and spun at 3,000 X g for 20 minutes to remove the lipid fraction before precipitating total IgY with 20% (w/v) powdered ammonium sulfate.[24] The pellet containing highly enriched IgY was dissolved in and dialyzed against PBS, pH 7.4, quantified by absorbance at 280 nm and stored at −20 °C. To lyophilize egg yolk IgY for in vivo administration, yolks were separated and frozen at −20 °C. A FreeZone Triad lyophilizer (Labconco) was used to produce the egg yolk powder.

In vitro analysis of egg yolk IgY

ERVA samples and cells for virus isolation—Fecal samples (~10 g) were obtained from 1- to 3-month-old foals with diarrhea at 3 farms around Saratoga Springs, NY. Samples were identified as ERVA-positive by PCR testing at an accredited veterinary diagnostic laboratory. Fetal monkey kidney-derived cells (MA104) were acquired from a commercial source (ATCC). Cells were maintained in complete Dulbecco's essential medium (DMEM) containing 8% fetal bovine serum (FBS, Atlanta Biologicals), 1% Glutamax, and 1% antibiotic-antimycotic (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of amphotericin B; Gibco). Stool samples were processed by homogenization in 4 volumes of serum-free DMEM. The samples were spun at 10,000 X *g* for 10 minutes at 4 °C. Fecal swabs were also spun at 10,000 X *g* for 10 minutes at 4 °C. The supernatants from the fecal samples and swabs were syringe-filtered using a 0.2-µm filter before virus isolation.

Virus isolation—Confluent MA104 monolayer cultures in T25 flasks were washed 3 times with sterile Hank's balanced salt solution (HBSS) and were inoculated with 1 mL of fecal suspension or swab after activation at 37 °C for 60 minutes with trypsin at a final concentration of 15 µg/mL. After a 90-minute adsorption phase at 37 °C, the inoculum was removed and the MA104 cultures were fed with 3 mL of serum-free DMEM containing 1.5 µg/mL trypsin, 1% antibiotic-antimycotic (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of amphotericin B; Gibco), and 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), and incubated at 37 °C. One week after infection, the cultures were freeze-thawed at −80 °C 3 times, and cell lysates were inoculated into fresh cell cultures as described above.[25,26] The cell lysates were passaged until cytopathic effect (CPE) was observed, but no more than 10 times. CPE consisted of cell rounding, stretching, and eventual cell detachment from the culture flask. Virus isolates were titered using a 50% tissue culture infectious dose (TCID50) assay as described previously.[27] The highest dilution of the virus that produced CPE in 50% of the infected cells was considered the endpoint. The titer of the virus was calculated using the Karber method and expressed as $\log_{10}$ TCID50/mL.[28] One of the high-titer isolates proceeded to genotyping and virus neutralization analysis.

Genotyping ERVA isolate—CPE-positive viral isolates were subjected to PCR for characterization of the VP7 and VP4 gene sequences as the G- and P-genotype, respectively. Viral RNA was isolated using a Quick-RNA Viral Kit (Zymo Research) and used in first-strand cDNA synthesis with the qScript cDNA master mix. Gene-specific primers **(Table 2)** were synthesized for the most prevalent ERVA strains (IDT) and used to amplify regions of the G and P proteins. The PCR products were gel-purified, sequenced, and compared to the published sequences of equine G- and P-genotypes.

Table 2—Primer sequences used in the rotavirus genotyping assay.

Primer name	Sequence
G3 forward	AGA GAG AAT TTC CGT TTG GCT AGC GG
G3 reverse	AAA CGG ATC CAG TAG GCC ATC C
G14 forward	CCC AAC TGA AGC TGC AAC TCG ATG
G14 reverse	CAA CTT TAT GAT CTA CTC CAT CTA CCA CGT CCT
P12 forward	GGG GTC CTG GAG AAG TTA ATG ACT
P12 reverse	TAG CGT TTG AGT TTC TCC

Primers correspond to sequences of the outer capsid proteins from common ERVA genotypes. Nucleotide position based on GenBank Accession numbers AB046464.1 (G3), M61876.1 (G14), and D13397.1 (P12).
See Table 1 for key.

In vitro virus neutralization—The virus neutralization titer of anti-ERVA and HRVA IgY was determined using MA104 cell monolayers by methods described previously.[26,29] Briefly, 200 TCID50 units of ERVA isolate were incubated with chicken anti-ERVA or HRVA VP8 antibodies in a 2-fold serial dilution with dilution factors ranging from 2 to 2,048 at 37 °C for 1 hour. The virus plus antibody mixture was layered onto confluent MA104 cell monolayers grown in 96-well microplates (Nalge Nunc Int). After 7 days of incubation at 37 °C in a humidified 5% CO_2 atmosphere, the plates were examined for

the presence of CPE. The complete absence of CPE was scored as positive for neutralization. Antibody samples were run in triplicate and the reciprocal of the mean highest dilution of IgY was reported as the neutralization titer.

In vivo oral administration of egg yolk IgY—A pilot study using the egg yolk antibodies in 1- to 4-month-old foals with naturally occurring rotaviral diarrhea was conducted to obtain preliminary safety data associated with administration. A total of 26 diarrheic foals that were ERVA PCR-positive from Thoroughbred breeding farms in Saratoga Springs, NY (n = 15) and Lexington, KY (n = 11) were included in the study in 2021 (n = 6) and 2022 (n = 20). A majority of foals (2022) were located on farms where the G3 ERVA genotype was detected (n = 17) and the remainder were on farms where the G14 genotype was detected (n = 3). Mares of all foals were immunized 3 times according to the manufacturer's recommendation (at the 8th, 9th, and 10th month of gestation) with the commercially available ERVA vaccine (Equine Rotavirus Vaccine, Zoetis). All foals were confirmed to have adequate passive transfer of immunity within 18 hours of birth, and all foals received antigastric ulcer prophylaxis using omeprazole (Gastrogard®; 4 mg/kg; q 24 h; PO, Boehringer Ingelheim Animal Health) and sucralfate (22 mg/kg; q 24 h; PO; AbbVie). Twelve foals received standard veterinary antidiarrheal care that was individualized for each foal and consisted of 1 or more of the following medications based on the discretion of the treating veterinarian for the particular case (PFA or LLM): metronidazole (20 mg/kg; q 12 h; PO; Pfizer); commercial probiotics (Probios; 10 mL; q 24 h; PO; Vets Plus Inc; or Full Bucket Foal Probiotic; 30 mL; q 12 h; PO; Full Bucket Health); potassium penicillin (22,000 IU/kg; q 6 h; IV; AdvaCare); gentamicin (6.6 mg/kg; q 24 h; IV); or smectite powder (Biosponge®; 30 mL; q 12 h; PO; Platinum Performance) for 5 days. Fourteen diarrheic foals received 35 g of egg yolk powder mixed with water to a volume of 60 mL and administered orally once daily (q 24 h) for 5 days, but no other antidiarrheal products **(Figure 1)**. The egg yolk dose was extrapolated from previous studies in other animal species.[3,19,30,31] The duration of diarrhea and other clinical signs were recorded for all foals. Our objective for treating foals was to obtain preliminary data about the adverse effects of the product among foals with diarrhea.

Statistical analysis

The duration of diarrhea was analyzed between groups using descriptive and inferential statistical methods. Continuous data were reported in tables and summarized as medians and ranges. A comparison of the duration of diarrhea between treatment groups was made using Wilcoxon rank-sum tests for continuous variables.

Results

Selection of ERVA peptides for immunization

Three neutralizing epitopes from the human rotavirus Wa strain VP8 protein were modified to match the sequence of the VP8 protein from the most common equine P-genotype, P[12] (Table 1).[11]

Immunization of laying hens

After immunization, ERVA-specific antibody titers in hens were monitored by indirect ELISA **(Figure 2)**. Anti-HRVA IgY titers were also monitored by indirect ELISA (data not shown). Anti-ERVA IgY titers peaked after the 3rd immunization and at that stage birds were considered hyperimmunized. Antibody titers were maintained thereafter by boosters administered every 4 weeks. The response varied between biological replicates, as expected (ELISA S/N values: 9.48, 7.2, 10.4, 8.2, and 9.5).

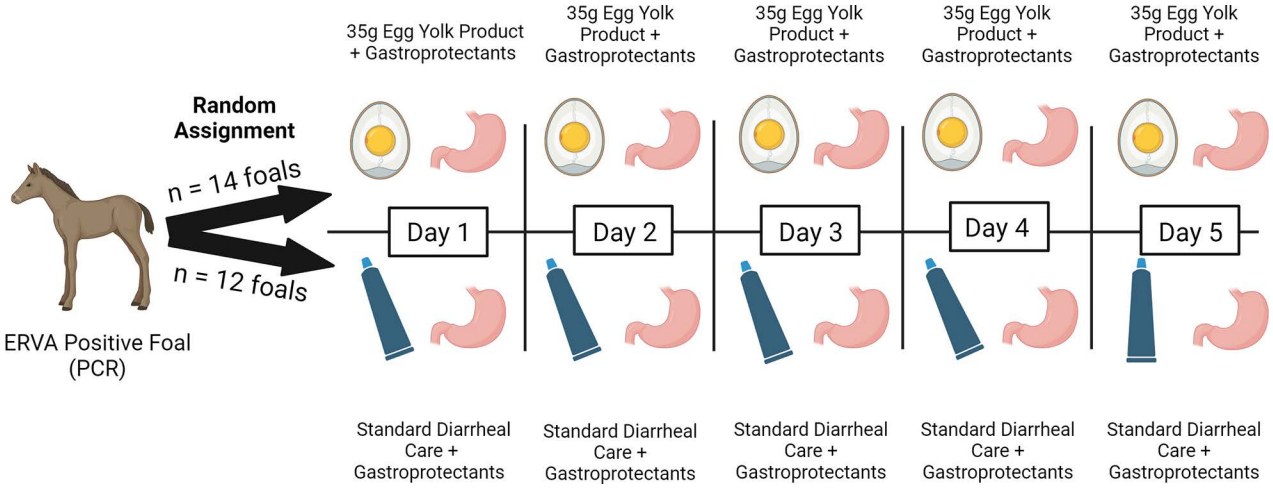

Figure 1—Treatment scheme for rotavirus-positive foals enrolled in the field studies. All study foals were treated with gastroprotectants. Foals were randomly assigned to the egg yolk IgY or standard care group before receiving treatment. Standard care consisted of gastroprotectants, antidiarrheals, antibiotics, antiulcer medication, and gastrointestinal supplements. Created with Biorender.com.

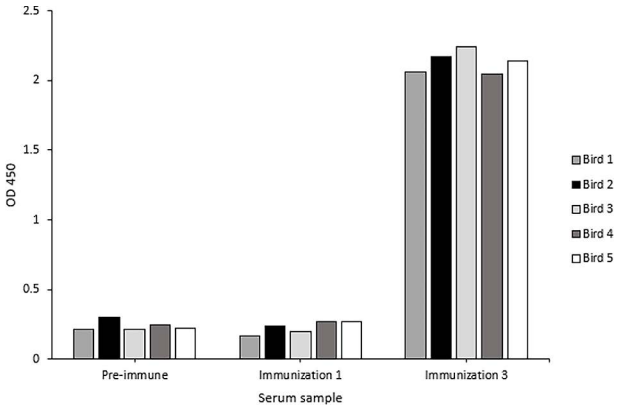

Figure 2—ELISA quantification of chicken anti-ERVA specific serum antibody level in response to immunization. An ELISA plate was coated with 5 µg/mL of a goat antibiotin + biotinylated ERVA peptide complex and blocked with 2% BSA in PBS. Pre- and hyper-immune serum antibodies served as the primary antibody (1:1,000). HRP-conjugated goat antichicken IgY served as the secondary antibody (1:3,000). After the third immunization (OD450 ~2.0) the birds were hyperimmunized. ERVA = equine rotavirus group A.

In vitro analysis of egg yolk IgY

Virus isolation and genotyping—Four fecal samples from foals in 2020 were processed for virus isolation. After 9 passages, CPE was observed in MA104 cells from 1 of the 4 samples **(Figure 3)**. The isolate capable of inducing CPE was propagated further to generate a high-titered viral stock for further in vitro analysis. After performing PCR-based genotyping, the isolate was confirmed to be of the G14 P4[12] genotype.

In vitro virus neutralization—Anti-ERVA serum neutralization titers were similar to corresponding precipitated egg yolk IgY titers, with values of 128 in the serum and 94.5 in the egg yolk **(Table 3)**. Additionally, anti-HRVA egg yolk IgY also prevented infection of the G14 P4[12] ERVA isolate in vitro with a reported virus neutralization titer of 128. Titers were reported as the reciprocal of the greatest antibody dilution capable of preventing infection. Preimmune serum and egg yolk IgY were negative for neutralization (Figure 3). These data indicate the potential for egg yolk antibodies as a therapeutic for rotaviral diarrhea in foals.

In vivo administration of anti-VP8 egg yolk IgY—Based on the observation that IgY against the 3 rotavirus peptides could neutralize an ERVA isolate in vitro, we conducted an in vivo pilot study to collect preliminary data about the ease of delivery and safety of the anti-ERVA egg yolk antibodies. No adverse effects such as worsening of diarrhea were observed for the 14 foals treated with egg yolk: no significant difference (P = .5567) in the duration of diarrhea was observed between the 14 foals receiving the egg yolk antibodies (median 3 days; range = 1 to 6 days) and the 12 foals receiving standard antidiarrheal care (median, 4 days; range = 2 to 7 days).

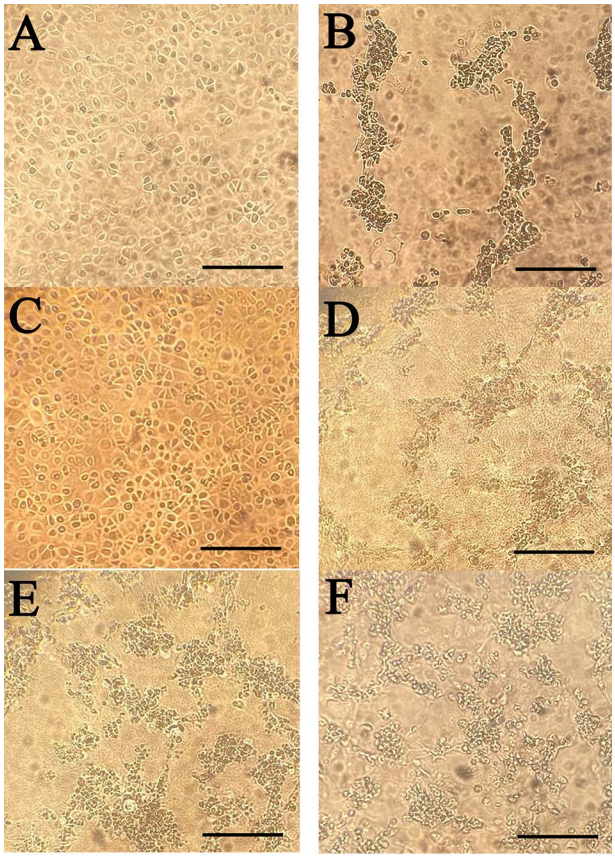

Figure 3—Detection of cytopathic effect from virus isolation and anti-VP8 serum neutralization assays. Images depict an uninfected, confluent MA104 monolayer (A) and an infected MA104 monolayer after isolation (B). After 9 passages, the virus had adapted to cell culture and was deemed infectious. In virus neutralization assays, wells were scored as negative for CPE indicating the absence of infection (C), or positive indicating cells were infected with ERVA (D, E, F). Serum dilutions were reported as 1:128 (C), 1:2,048 (D), 1:1,024 (E), and 1:512 (F). Bar = 50 µm.

Table 3—List of calculated virus neutralization titers from in vitro antibody assay.

Antibody sample	VN titer
Anti-ERVA peptide chicken serum	128
Anti-ERVA peptide egg yolk IgY	~94.5
Anti-HRVA peptide egg yolk IgY	128

Titers were recorded as the reciprocal of the greatest antibody dilution capable of preventing infection or cytopathic effect. Preimmune serum and egg yolk IgY were negative for virus neutralization.

See Table 1 for key.

Discussion

Rotavirus is commonly identified as a pathogen in the feces of foals with diarrhea.[7] The commercialization of the Zoetis ERVA G3 vaccine appears to have reduced the occurrence of ERVA diarrhea in neonatal foals born to vaccinated dams.[32,33] Once maternal

immunity wanes, however, foals are more susceptible to ERVA infection particularly those infected with the G14 genotype.[34] Foals included in this study that had diarrhea attributed to ERVA that were born to vaccinated mares developed clinical signs between 1 and 4 months of age. Thus, the development of a cross-protective vaccine against multiple ERVA strains and highly effective treatments are needed to improve foal health.[7,34] Egg yolk antibodies (IgY) have an established record of providing protection against enteric pathogens such as RVs in multiple host species.[35–38] Previous work has demonstrated that antibodies raised against the HRVA VP8 protein protect against group A rotaviral infections.[13,14] The neutralizing epitopes of the HRVA (G1P[8]) VP8 protein have been mapped.[11] These epitopes are highly conserved between group A human and equine RVs differing by just a single amino acid between species for any given peptide sequence. Because of the homology of sequence and structure observed between human and equine RVs, especially in the region of the VP8 epitopes, we sought to evaluate the ability of egg yolk from hens immunized with these epitopes to neutralize group A ERV in vitro as a potential treatment option for foals with ERVA diarrhea.

Single-comb White Leghorn hens were hyperimmunized with 3 ERVA peptides corresponding to the selected neutralizing VP8 epitopes. After just 3 immunizations, the anti-ERVA peptide titers peaked indicating that the birds were hyperimmune and antibodies were precipitated from egg yolks. The rapid antibody response can be attributed to the CD40-targeted immunization strategy and the fact that chickens can be boosted more frequently than mammals.[22,23] Additionally, vaccination with synthetic peptides, as opposed to intact virus or recombinant proteins, offers practicality given the low cost and short time to production. Both the hyperimmune anti-ERVA chicken serum and egg yolk antibodies prevented infection of an ERVA isolate in vitro. The comparable neutralization titers obtained with egg yolk and serum IgY demonstrate the efficiency of egg yolk antibody production. Egg yolk antibodies raised against the HRVA VP8 peptides also prevented infection of ERVA in vitro. These data reinforce the conservation of the neutralizing VP8 epitopes between human and equine strains and provide implications for cross-protection. The rotavirus VP8 P-type sequence used to convert the neutralizing human VP8 epitopes to equine RV counterparts was reported as P4[12]. To date, 6 P-types have been identified in group A ERV infections, but P4[12] is most prevalent.[7] The dominant P-type suggests that antibodies raised against the VP8 protein could provide cross-protection against multiple equine RV strains with variable G- or even P-types. Six G-types have been identified in equine infections with G3 and G14 detected most often. The observed results of in vitro virus neutralization data support future evaluation of egg yolk antibody safety and efficacy in vivo.

Newborn foals absorb intact antibodies via pinocytosis during approximately the first 36 hours of life.[39,40] Nonetheless, neonatal pigs can absorb IgY from the intestinal tract with similar efficiency as colostral antibodies and the IgY had a serum half-life of <2 days.[41] Thus, newborn foals might absorb some of IgY administered orally; however, because IgY does not bind FcRn receptors, it would not interfere with colostral absorption of maternal antibodies and would likely be rapidly eliminated.[42,43] Delaying administration of IgY products to newborn foals to age >36 hours would be expected to restrict the IgY to the intestinal tract.[41]

The in vivo findings from this study have important limitations. The small number of foals included was the result of an outbreak of group B rotaviral diarrhea in 2021 and 2022 that prevented us from enrolling a larger number of foals with ERVA diarrhea as planned. We did not include a nonimmune egg yolk IgY control group that prevented analysis of the potential therapeutic effect provided by crude egg yolk documented by previous studies.[44,45] Evidence exists, however, that egg yolk with pathogen-specific IgY is superior to nonimmune egg yolk, including studies with rotavirus infections.[3,20,30,31,46] Nevertheless, including a nonimmune egg yolk comparison group would improve the design of future studies. We also did not include an untreated control group; however, participating veterinarians and farms were unwilling to withhold standard antidiarrheal treatment from foals.

Diarrheic foals may be concurrently infected with multiple enteropathogens, complicating diagnosis.[47] Not all foals in the study were tested for other enteropathogens because of farm history of ERVA infection, evidence of ERVA in feces of affected foals at the participating farms, and absence of evidence of other enteropathogens by fecal PCR in some foals from the participating farms that were tested.

The egg yolk dose (35 g) and dosing interval (q 24 h) were determined empirically by extrapolating from other species of animals. Systematic evaluation of the dose and dose interval is needed before the efficacy of an anti-ERVA egg yolk product can be evaluated in randomized, controlled trials. Higher doses and more frequent dosing might enhance the efficacy of egg yolk anti-ERVA antibodies to neutralize enteric ERVA. Thus, our in vivo data merely provide evidence that the ERVA hyperimmune egg yolk product was safe to administer to foals with diarrhea because clinical signs were similar in foals treated with the egg yolk product and standard antidiarrheal treatment. This information, however, is noteworthy for future studies to evaluate the dose, interval, and clinical efficacy of ERVA hyperimmune egg yolk antibodies in foals.

This study describes the identification of protective group A ERV epitopes and subsequent neutralizing antibody production. Based on the homology between group A HRV and ERV, converting protective epitopes from human to equine sequences enabled us to design peptides that neutralized group A ERVs. A high titer of ERVA-specific egg yolk IgY was attained, and these antibodies also prevented infection of an ERVA isolate in vitro. These data provide a tool for the fast and versatile

development of therapeutic antibodies to help combat the threat of rotavirus infection in foals. Raising antibodies against the VP8 protein could provide cross-protection against multiple equine strains with variable G-types, including the 2 most common strains, G3P[12] and G14P[12]. No adverse effects were observed for the foals treated with gastroprotectants and egg yolk relative to diarrheic foals from the same farms managed with gastroprotectants and standard antidiarrheal care. Considering the efficacy of egg yolk antibodies used in the prevention of rotaviral diarrhea in other animal species (including humans infected with closely related strains), the identification of conserved neutralizing ERVA epitopes reported here, and the absence of adverse effects and similar duration of diarrhea relative to standard of care from our pilot safety study, additional studies are warranted to optimize the dose and frequency of the egg yolk antibody administration to further evaluate the efficacy of anti-ERVA egg yolk antibody treatment in foals.

Acknowledgments

The authors thank the Rood & Riddle Equine Hospital in Lexington, KY, and Saratoga Springs, NY, for their participation in the in vivo study.

Material from this manuscript is scheduled for publication in Jill Skrobarczyk's PhD dissertation, *Generation of Unique Chicken Antibodies for Therapeutic Use* (Texas A&M University; 2025).

Disclosures

JWS and LRB received a gift from Mg Biologics, Inc that was used to support this work. The funders had no role in the design, conduct, analysis, interpretation, or reporting of the results. No AI-assisted technologies were used in the generation of this manuscript.

Funding

This work was supported by funding from a gift to the Berghman laboratory by Mg Biologics, Inc. Further support was provided by Dr. Berghman's research funds, the Department of Poultry Science, the Link Equine Research Endowment, Texas A&M University, and the Glenn P. Blodgett Chair in Equine Studies.

References

1. Bines JE, Kirkwood CD. Conquering rotavirus: from discovery to global vaccine implementation. *J Paediatr Child Health.* 2015;51(1):34–39. doi:10.1111/jpc.12815
2. Estes MK, Cohen J. Rotavirus gene structure and function. *Microbiol Rev.* 1989;53(4):410–449. doi:10.1128/mmbr.53.4.410-449.1989
3. Hammarström L. Passive immunity against rotavirus in infants. *Acta Paediatr Suppl.* 1999;88(430):127–132. doi:10.1111/j.1651-2227.1999.tb01311.x
4. Hoshino Y, Kapikian AZ. Rotavirus serotypes: classification and importance in epidemiology, immunity, and vaccine development. *J Health Popul Nutr.* 2000;18(1):5–14. doi:10.3329/jhpn.v18i1.33
5. Lieberman JM. Rotavirus and other viral causes of gastroenteritis. *Pediatr Ann.* 1994;23(10):529–532. doi:10.3928/0090-4481-19941001-06
6. Parashar UD, Hummelman EG, Bresee JS, Miller MA, Glass RI. Global illness and deaths caused by rotavirus disease in children. *Emerg Infect Dis.* 2003;9(5):565–572. doi:10.3201/eid0905.020562
7. Bailey KE, Gilkerson JR, Browning GF. Equine rotaviruses-current understanding and continuing challenges. *Vet Microbiol.* 2013;167(1–2):135–144. doi:10.1016/j.vetmic.2013.07.010
8. Nemoto M, Matsumura T. Equine rotavirus infection. *J Equine Sci.* 2021;32(1):1–9. doi: 10.1294/jes.32.1
9. Yandle Z, Coughlan S, Dean J, Tuite G, Conroy A, De Gascun CF. Group A rotavirus detection and genotype distribution before and after introduction of a national immunisation programme in Ireland: 2015–2019. *Pathogens.* 2020;9(6):1–15. doi:10.3390/pathogens9060449
10. Uprety T, Sreenivasan CC, Hause BM, et al. Identification of a ruminant origin group B rotavirus associated with diarrhea outbreaks in foals. *Viruses.* 2021;13(7):1330. doi:10.3390/v13071330
11. Kovacs-Nolan J, Yoo D, Mine Y. Fine mapping of sequential neutralization epitopes on the subunit protein VP8 of human rotavirus. *Biochem J.* 2003;376(Pt 1):269–275. doi: 10.1042/BJ20021969
12. Hoshino Y, Kapikian AZ. Classification of rotavirus VP4 and VP7 serotypes. *Arch Virol Suppl.* 1996;12:99–111. doi:10.1007/978-3-7091-6553-9_12
13. Ruggeri FM, Greenberg' HB. Antibodies to the trypsin cleavage peptide VP8* neutralize rotavirus by inhibiting binding of virions to target cells in culture. *J Virol.* 1991;65(5):2211–2219. doi:10.1128/JVI.65.5.2211-2219.1991
14. Matsui SM, Offit PA, Vo PT, et al. Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to the heterotypic neutralization domain of VP7 and the VP8 fragment of VP4. *J Clin Microbiol.* 1989;27(4):780–782. doi:10.1128/jcm.27.4.780-782.1989
15. Thu HM, Myat TW, Win MM, et al. Chicken egg yolk antibodies (IgY) for prophylaxis and treatment of rotavirus diarrhea in human and animal neonates: a concise review. *Korean J Food Sci Anim Resour.* 2017;37(1):1–9. doi:10.5851/kosfa.2017.37.1.1
16. Sarker SA, Casswall TH, Juneja LR, et al. Randomized, placebo-controlled, clinical trial of hyperimmunized chicken egg yolk immunoglobulin in children with rotavirus diarrhea. *J Pediatr Gastroenterol Nutr.* 2001;32(1):19–25. doi:10.1097/00005176-200101000-00009
17. Pant N, Marcotte H, Brüssow H, Svensson L, Hammarström L. Effective prophylaxis against rotavirus diarrhea using a combination of *Lactobacillus rhamnosus* GG and antibodies. *BMC Microbiol.* 2007;7:86. doi:10.1186/1471-2180-7-86
18. Xie YM, Gao S, Wang LY, Wang ZL. Therapeutic effect of probiotics and oral IgY as supplementary drugs in the treatment of pediatric rotavirus enteritis: a comparative study. *Chinese J Contemp Pediatr.* 2013;15(11):1000–1005. doi:10.7499/j.issn.1008-8830.2013.11.018
19. Vega C, Bok M, Saif L, Fernandez F, Parreño V. Egg yolk IgY antibodies: a therapeutic intervention against group A rotavirus in calves. *Res Vet Sci.* 2015;103:1–10. doi:10.1016/j.rvsc.2015.09.005
20. Bennett-Steward K. Neonatal foals and hyperimmunized egg protein- a viable therapy. *J Equine Vet Sci.* 2002;22(3):105–107. doi:10.1016/S0737-0806(02)70120-2
21. Bustos CP, Leiva CL, Gambarotta M, Guida N, Chacana PA. In vitro inhibitory activity of IgY antibodies against *Salmonella* ser. Newport isolated from horses. *J Equine Vet Sci.* 2021;103:103657. doi:10.1016/j.jevs.2021.103657
22. Chou WK, Chen CH, Vuong CN, et al. Significant mucosal sIgA production after a single oral or parenteral administration using in vivo CD40 targeting in the chicken. *Res Vet Sci.* 2016;108:112–115. doi:10.1016/j.rvsc.2016.08.013
23. Chen CH, Abi-Ghanem D, Waghela SD, et al. Immunization of chickens with an agonistic monoclonal anti-chicken

CD40 antibody-hapten complex: rapid and robust IgG response induced by a single subcutaneous injection. *J Immunol Methods.* 2012;378(1–2):116–120. doi:10.1016/j.jim.2012.02.006
24. Deignan T, Kelly J, Alwan A, O'Farrelly C. Comparative analysis of methods of purification of egg yolk immunoglobulin. *Food Agric Immunol.* 2000;12(1):77–85. doi:10.1080/09540100099643
25. Ward RL, Knowlton DR, Pierce MJ. Efficiency of human rotavirus propagation in cell culture. *J Clin Microbiol.* 1984;19(6):748–753. doi:10.1128/jcm.19.6.748-753.1984
26. Arnold M, Patton JT, McDonald SM. Culturing, storage, and quantification of rotaviruses. *Curr Protoc Microbiol.* 2012;1–29. doi:10.1002/9780471729259.mc15c03s15
27. Smither SJ, Lear-Rooney C, Biggins J, Pettitt J, Lever MS, Olinger GG. Comparison of the plaque assay and 50% tissue culture infectious dose assay as methods for measuring filovirus infectivity. *J Virol Methods.* 2013;193(2): 565–571. doi:10.1016/j.jviromet.2013.05.015
28. Karber G. 50% End point calculation. *Arch Exp Path Pharmokol.* 1931;162:480–483. doi:10.1007/BF01863914
29. Gauger PC, Vincent AL. Serum virus neutralization assay for detection and quantitation of serum-neutralizing antibodies to influenza a virus in swine. *Methods Mol Biol.* 2020;2123:321–333. doi:10.1007/978-1-4939-0758-8_26
30. Vega C, Bok M, Vlasova A, et al. IgY antibodies protect against human rotavirus induced diarrhea in the neonatal gnotobiotin piglet disease model. *PLoS ONE.* 2012;7(8):e42788. doi:10.1371/journal.pone.0042788
31. Wang Z, Li J, Li J, et al. Protective effect of chicken egg yolk immunoglobulins (IgY) against enterotoxigenic *Escherichia coli* K88 adhesion in weaned piglets. *BMC Vet Res.* 2019;15(1):234. doi:10.1186/s12917-019-1958-x
32. Barrandeguy M, Parreño V, Lagos Mármol M, et al. Prevention of rotavirus diarrhoea in foals by parenteral vaccination of the mares: field trial. *Dev Bio Stand.* 1998;92:253–257.
33. Powell DG, Dwyer RM, Traub-Dargatz JL, et al. Field study of the safety, immunogenicity, and efficacy of an inactivated equine rotavirus vaccine. *J Am Vet Med Assoc.* 1997;211(2):193–198.
34. Carossino M, Barrandeguy M, Li Y, et al. Detection, molecular characterization and phylogenetic analysis of G3P[12] and G14P[12] equine rotavirus strains co-circulating in central Kentucky. *Virus Res.* 2018;255: 39–54. doi:10.1016/j.virusres.2018.05.025
35. Mine Y, Kovacs-Nolan J. Chicken egg yolk antibodies as therapeutics in enteric infectious disease: a review. *J Med Food.* 2002;5(3):159–169. doi:10.1089/ 10966200260398198
36. Pereira EPV, van Tilburg MF, Florean EOPT, Guedes MIF. Egg yolk antibodies (IgY) and their applications in human and veterinary health: a review. *Int Immunopharmacol.* 2019;73:293–303. doi:10.1016/j.intimp.2019.05.015
37. Barati B. Egg yolk antibodies for disease prevention. *J Bacteriol Mycol Open Access.* 2016;3(2):219–222. doi:10.15406/jbmoa.2016.03.00058
38. Yolken RH, Leister F, Wee SB, Miskuff R, Vonderfecht S. Antibodies to rotaviruses in chickens' eggs: a potential source of antiviral immunoglobulins suitable for human consumption. *Pediatrics.* 1988;81(2):291–295. doi:10.1542/ peds.81.2.291
39. Jeffcott LB. Studies on passive immunity in the foal. 1. gamma-globulin and antibody variations associated with the maternal transfer of immunity and the onset of active immunity. *J Comp Pathol.* 1974;84(1):93–101. doi:10.1016/0021-9975(74)90031-0
40. Bauer JE, Harvey JW, Asquith RL, McNulty PK, Kivipelto J. Serum protein reference values in foals during the first year of life: comparison of chemical and electrophoretic methods. *Vet Clin Pathol.* 1985;14(1):14–22. doi:10.1111/ j.1939-165x.1985.tb00841.x
41. Yokoyama H, Peralta RC, Sendo S, Ikemori Y, Kodama Y. Detection of passage and absorption of chicken egg yolk immunoglobulins in the gastrointestinal tract of pigs by use of enzyme-linked immunosorbent assay and fluorescent antibody testing. *Am J Vet Res.* 1993;54(6):867–872.
42. Israel EJ, Wilsker DF, Hayes KC, Schoenfeld D, Simister NE. Increased clearance of IgG in mice that lack beta 2-microglobulin: possible protective role of FcRn. *Immunology.* 1996;89(4):573–578. doi:10.1046/j.1365-2567.1996.d01-775.x
43. Leiva CL, Geoghegan P, Lammer M, et al. In vivo neutralization of bee venom lethality by IgY antibodies. *Mol Immunol.* 2021;135:183–190. doi:10.1016/ j.molimm.2021.04.015
44. Agunos A, Silphaduang U, Mine Y. Effects of nonimmunized egg yolk powder-supplemented feed on *Salmonella enteritidis* prevention and elimination in broilers. *Avian Dis.* 2006;50:366–373. doi:10.1637/7443-092205R.1
45. Kassaify ZG, Mine Y. Nonimmunized egg yolk powder can suppress the colonization of *Salmonella typhimurium*, *Escherichia coli* O157:H7, and *Campylobacter jejuni* in laying hens. *Poult Sci.* 2004;1497–1506. doi:10.1093/ ps/83.9.1497
46. Vega C, Bok M, Chacana P, Saif L, Fernandez F, Parreno V. Egg Yolk IgY- protection against rotavirus induced diarrhea modulatory effect on the systemic and mucosal antibody responses in newborn calves. *Vet Immunol Immunopathol.* 2011;142:156–169. doi:10.1016/j.vetimm.2011.05.003
47. Olivo G, Lucas TM, Borges AS, et al. Enteric pathogens and coinfections in foals with and without diarrhea. *Biomed Res Int.* 2016: Article ID 1512690. doi:10.1155/2016/1512690