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Papers

Isotype-specific antibody responses to rotavirus and virus proteins in cows inoculated with subunit vaccines composed of recombinant SA11 rotavirus core-like particles (CLP) or virus-like particles (VLP)

F.M. Fernandez*†, M.E. Conner‡§, A.V. Parwani*, D. Todhunter*, K.L. Smith*, S.E. Crawford‡, M.K. Estes‡ and L.J. Saif*§

The isotype antibody responses to bovine IND P^5, G6 and simian SA11 P^2, G3 rotavirus and SA11 rotavirus proteins (VP4, VP6 and VP7) in serum, colostrum and milk were analysed by ELISA in three groups of vaccinated cows and nonvaccinated controls. Pregnant cows were vaccinated intramuscularly and intramammarily with recombinant baculovirus-expressed SA11 rotavirus VLP (triple-layered virus-like particles containing rotavirus VP2, VP4, VP6 and VP7); CLP (double-layered core-like particles containing rotavirus VP2 and VP6); or inactivated SA11 rotavirus, respectively. Rotavirus antigen titers were highest (30–200-fold) in ELISA in the VLP vaccine compared to the inactivated SA11 vaccine. The IgG1, IgG2 and IgM geometric mean antibody titers (GMT) to rotavirus (titers to bovine rotavirus vs SA11 rotavirus did not differ significantly for any isotype or group) and the IgG2 GMT to VP6 in serum at calving in the vaccinated groups were significantly (P <0.05) higher than in the control group. In colostrum, IgG1 and IgA rotavirus antibody titers were significantly elevated for VLP (IgG1 GMT 832225; IgA GMT 16384), CLP (IgG1 GMT 660561; IgA GMT 10321) and SA11 (IgG1 GMT 131072; IgA GMT 1448) vaccinated cows compared to control cows (IgG1 GMT 11585; IgA GMT 45). The IgG1 and IgA GMT to rotavirus were significantly elevated (6–100-fold) in milk of VLP and CLP vaccinated cows compared to SA11 vaccinated or control cows. The isotype antibody responses to VP6 in serum, colostrum and milk paralleled the responses to rotavirus, but titers were ~2–10-fold lower. Only cows vaccinated with VLP had significantly enhanced serum, colostral and milk antibody titers to rotavirus VP4 and VP7. These results demonstrate that rotavirus antibody titers in serum, colostrum and milk are significantly enhanced by use of non-infectious VLP, CLP and inactivated SA11 rotavirus vaccines, but the VLP or CLP vaccines induced the highest antibody responses, corresponding to their higher rotavirus antigen titers measured by ELISA. Copyright © 1996 Elsevier Science Ltd.

Keywords: Recombinant rotavirus subunit vaccines; core-like particles; virus-like particles; bovine antibodies to rotavirus in colostrum; milk

*Food Animal Health Research Program and Animal Science Department, Ohio Agricultural Research and Development Center, The Ohio State University, Columbus, U.S.A. †Instituto de Virologia, CICV-INTA, Castelar, Argentina. ‡Division of Molecular Virology, Baylor College of Medicine, Houston, TX 77030, U.S.A. Medical Center, Houston, TX 77030, U.S.A. §To whom correspondence should be addressed. (Received 28 November 1995; revised 21 February 1996; accepted 5 March 1996)

Bovine rotavirus (BRV) is a major cause of diarrhoea in young calves[1,2]. Because infection by BRV is localized to the small intestine, "local" passive and active immunity are important to protect against infection and disease[3–8]. Two approaches have been developed to reduce BRV-associated diarrhoea in calves[3–9]. The first approach was designed to enhance active immunity by oral administration of modified live BRV to newborn calves[3,4,9]. This approach appeared to be successful in experimental

studies using gnotobiotic calves;[3,4] however, its efficacy was questionable when evaluated under field conditions, presumably due to the widespread presence of maternal antibodies that neutralized the vaccine virus[4,5,9]. The second approach for vaccine development involves maternal vaccination to enhance "lactogenic" immunity and to transfer passive protection to calves[3–8,10–18]. Investigators have shown that vaccination of cows with live or inactivated BRV vaccines significantly increased colostral antibody titers to BRV and passively protected calves from diarrhoea following experimental BRV challenge in some, but not all studies[3–8,10–12,15]. However BRV vaccine efficacy was variable in field trials[3,4,6,13,14,16–18].

Recently, using the baculovirus expression system, recombinant viral multiprotein structures have been produced, referred to as virus-like particles (VLP) or core-like particles (CLP). Such particles, reported initially by P. Roy and coworkers[19] for bluetongue virus, and by Labbe and coworkers[20] for rotavirus, represent a new generation of non-infectious, stable, antigenically authentic and highly immunogenic vaccines. Crawford *et al.*[21] described the production of triple-layered rotavirus-like particles (VLP) by the coexpression of BRV and SA11 inner (VP2 and VP6, respectively) and simian SA11 rotavirus outer (VP4 and VP7) capsid protein genes in insect cells using a baculovirus expression system (SA11 VLP). Previous studies of such VLP, including the ones produced from bluetongue virus, indicate that these VLP are antigenically authentic and immune responses induced by the VLP mimic those induced by intact virions[19–22]. Therefore our objectives were to compare the use of an inactivated SA11 rotavirus vaccine (mimic existing inactivated BRV commercial vaccine) with SA11 CLP (comprised of rotavirus VP2 and 6) and VLP (comprised rotavirus of VP2, 4, 6 and 7) subunit vaccines to immunize pregnant cows to boost antibodies to BRV in colostrum and milk for induction of passive immunity in newborn calves. For these studies we used the vaccination routes and times (intramuscular at ~9 weeks pre-portum and intramammarily at ~7 weeks pre-portum) and adjuvant (incomplete Freund's) that evoked maximal antibody responses previously[4–7,11]. Using ELISA, we investigated the isotype-specific antibody titers to rotavirus and rotavirus proteins (VP4, 6 and 7) in the serum, colostrum and milk of non-vaccinated cows, and cows vaccinated with inactivated SA11 or SA11 CLP and VLP subunit vaccines.

MATERIALS AND METHODS

Viruses and vaccine preparation

The SA11 strain of simian rotavirus P^2, G3 or the IND strain of BRV P^5, G6 were cultivated in fetal rhesus monkey kidney (MA104) cells in the presence of trypsin as previously described[11,23]. For use in ELISA (SA11 and IND BRV), or as a vaccine (SA11), the rotavirus-infected MA104 cells were subjected to two cycles of freezing and thawing, the suspensions centrifuged to remove cellular debris (800*g* for 15 min) and the supernatant lysates stored at −70°C. The rotavirus titers (SA11 or IND) in the supernatant lysates were approximately 10^7 FFU/ml[11]. For ELISA, rotavirus in the supernatant fluids was semi-purified by centrifugation through 40% sucrose cushions (96500*g* for 2 h at 4°C)[11]. The viral pellets were resuspended to ~10× the original volume in 0.05 M Tris buffer (pH 7.5) containing 0.001 M $CaCl_2$ (Tris–$CaCl_2$), aliquoted and stored at −20°C. Control antigen was prepared from mock-infected cells, treated and stored in an identical manner[11].

Recombinant triple-layered VLP or double-layered CLP were generated by the coexpression in baculovirus of BRV VP2 and SA11 rotavirus VP4, VP6 and VP7; or BRV VP2 and SA11 rotavirus VP6, respectively, and purified on $CsCl_2$ gradients as previously described[21]. Western blots were used to confirm the presence of the respective rotavirus proteins in VLP and CLP vaccines as described[21]. The size and morphology of CLP and VLP were examined using electron microscopy with ammonium molybdate (pH 5.5) used as the negative stain[21]. The SA11 rotavirus vaccine (supernatant of infected cell culture lysates) was inactivated with 10% 2-bromoethyleneamine (BEI) for 18 h at 37°C on a mechanical shaker[4,6]. Residual BEI was inactivated with sodium thiosulfate to a final concentration of 10% and rotavirus inactivation was verified by loss of infectivity for MA104 cells. The vaccines were stored at 4°C.

Immunization of cows, sample collection and preparation

Seventeen pregnant Holstein cows from the Ohio Agricultural Research and Development Center dairy research herd were randomly assigned to four experimental groups. All cows had completed at least one lactation and had not been previously vaccinated with rotavirus, but were seropositive for rotavirus antibodies due to natural field exposure. At each inoculation, VLP vaccine cows received 250 μg of VLP (n=4) or 100 μg of VLP (n=2); CLP vaccine cows received 250 μg of CLP (n=3); SA11 vaccine cows received BEI-inactivated SA11 rotavirus (titer of 1×10^7 PFU/ml prior to inactivation) (n=4); and the remaining four cows were uninoculated controls. Vaccinated cows were inoculated by intramuscular (IM) injection into the gluteal region (5 ml in each hip), one week before drying off (approximately 9 weeks prior to anticipated calving date) with 5 ml of VLP, CLP or inactivated SA11 rotavirus, emulsified (immediately prior to vaccination) in an equal volume of incomplete Freund's adjuvant (IFA). Two weeks after the first IM injection, cows were vaccinated intramammarily (IMm) with similar volumes of VLP, CLP or inactivated SA11 in IFA. The inoculum was infused into each mammary quarter (2.5 ml/quarter) using a sterile teat infusion canula. Before infusion, teat ends were cleaned by scrubbing with cotton pads soaked in 75% alcohol.

Blood samples were collected from all groups at 1 week before drying off (time of initial injection), at calving (post-partum day, PPD 0) and at PPD 7, and the serum extracted[11]. Mammary secretion samples (colostrum and milk) were obtained at PPD 0 and 7. The entire first milking colostrum was obtained from all cows at calving. Milk samples were collected from all cows by manual expression of an equal volume from each quarter. Colostrum and milk samples were centrifuged at 800*g* for 45 min at 4°C. The supernatant was removed, heat inactivated (56°C, 30 min) and centrifuged at 96500*g* for 60 min at 17°C to obtain a clear whey, which was filtered (0.45 nm). The samples were stored frozen in aliquots at −20°C.

Determination of viral antigen concentration of vaccines by ELISA

A modification of an indirect ELISA described previously[24] was used to assess the relative concentrations of rotavirus antigens (VP4, VP6 and VP7) in each vaccine preparation. Separate plates* were coated with MAbs (ascites) 60[25] (broadly reactive VP7-specific), 2G4[26] (VP4-specific), 255/60 (VP6 subgroup specific) or SP2/0 (negative control)[24]. Other plates were coated with antiserum (goat) against double-layered SA11 rotavirus particles (verified to react with VP2 and VP6 by Western blot), and pre-immune and hyperimmune serum (verified to react with VP2, 4, 6 and 7 in Western blot) against semi-purified BRV produced in gnotobiotic pigs. After overnight incubation at 4°C, 10% nonfat dry milk in phosphate-buffered saline solution (pH 7.4) containing 0.05% Tween 20 (PBS-T) was applied as a blocking reagent for 2 h at 37°C. The vaccine samples were diluted, added to duplicate wells and incubated for 2 h. A guinea pig hyperimmune antiserum against group A BRV (NCDV–Cody strain) was added as the secondary antibody (diluted 1:2000), followed by sheep anti-guinea pig IgG conjugated to horseradish peroxidase† (diluted 1:5000). The substrate, 2,2,-azino-di-3-ethylbenzthiazoline sulfonic acid (ABTS) with a final concentration of 0.03% hydrogen peroxide was added. The plates were read and the cut-off value was calculated as previously described[24]. Antigen end point titers were expressed as the reciprocal of the highest sample dilution with an absorbance 450 nm of 3 SD above the absorbance on SP2/0 control wells.

Isotype-specific antibody ELISA

Indirect isotype antibody ELISAs were used to quantitate the isotype antibody titers to IND BRV and SA11 rotavirus and SA11 rotavirus VP4, 6 and 7 in serum, colostrum and milk. Optimal volumes and reagent dilutions were determined by checkerboard titration, using rotavirus antibody positive and negative standards. Plates were washed using PBS-T prior to addition of standard volumes of 0.1 ml/well of each reagent (diluted in PBS-T) followed by 2 h incubations of each reagent at 37°C in a humid CO_2 atmosphere. ELISA tests were modified for the detection of antibodies to rotavirus or VP4, VP6 and VP7 as described below.

Rotavirus antibody assay. Microtiter, 96-well Nunc plates‡ were coated with 2 μg/ml (in 0.06 M bicarbonate buffer, pH 9.6) of hyperimmune serum against BRV produced in gnotobiotic pigs (capture antibody). Plates were stored for 2–7 days at 4°C, washed twice and IND BRV, SA11 rotavirus or mock-infected MA104 cell culture control fluids were added. Test reagents were added in the following sequence: serial 4-fold dilutions of each test sample (starting at 1:16) added to antigen-coated and control (mock infected) wells; secondary antibodies consisting of MAb§ (from ascites) to bovine IgG1, IgG2, IgM (dilution 1:10000) and IgA (dilution 1:500) added to separate plates; the indicator antibody, goat anti-mouse IgG (H + L), F(ab') conjugated to alkaline phosphatase¶ (diluted 1:10000); and the substrate, p-nitrophenyl phosphate in 10% diethanolamine buffer (DEA) (pH 9.6).

VP6 and VP7 antibody assay. Nunc plates‖ were coated with antiserum (goat) against double layered SA11 rotavirus particles (VP6 and VP2-specific) or MAb 60 (VP7-specific) to capture VP6 and VP7, respectively and blocked with PBS-T-fetal bovine serum (3%) for 2 h at 37°C. Supernatant fluids from lysates of SF-9 insect cells infected with recombinant baculoviruses expressing SA11 rotavirus VP6 (diluted 1:1000) or VP7 (diluted 1:10) or infected with wild-type (WT) baculovirus (diluted 1:1000 or 1:10, respectively) were added. Serially diluted serum, colostrum and milk samples were added to each plate followed by secondary antibodies consisting of biotinylated MAb to bovine IgG1; IgG2; and IgM (diluted 1:1000–1:2000). The indicator antibody was peroxidase-conjugated streptavidin** (diluted 1:10000) and the substrate was ABTS with 0.03% H_2O_2. For detection of IgA antibodies in the above system, a MAb conjugated to alkaline phosphatase (diluted 1:500) was used followed by the substrate p-nitrophenol phosphate in 10% DEA buffer.

VP4 antibody assay. Immulon I plates†† were coated directly with VP4 from supernatant fluids of lysates of SF-9 cells infected with a recombinant baculovirus expressing SA11 rotavirus VP4 (diluted 1:25) or with lysates of WT baculovirus-infected cells (control) (diluted 1:25). Samples were added and the remainder of the assay was conducted as described under (ii). The absorbance for all assays, was determined in an ELISA reader‡‡ at 405 nm (alkaline phosphatase system) or 450 nm (peroxidase system). The ELISA antibody titers were expressed as the reciprocal of the highest sample dilution which had an absorbance of 3 SD above the background control (sample in mock-infected fluids or WT control wells). Each test included a positive and negative control serum. Geometric mean titers (GMT) and 95% confidence intervals (CI) were calculated for each vaccine group as described previously[11].

Statistical analysis

Statistical analyses were performed on the log transformation of the reciprocal antibody titers. An analysis of variance was performed, and the least significant difference test (LSD) and 95% CI were used to test for significant differences ($P < 0.05$) among the GMT.

RESULTS

Antigens and antigen concentrations in the vaccines

Analysis by Western blot revealed the presence of VP2 and VP6 in the CLP and the presence of VP2, VP4, VP6

*Maxisorp Immunoplate, Nunc, Naperville, IL.

†Boehringer Mannheim Corporation, Indianapolis IN.

‡Maxisorp Immunoplate, Nunc, Naperville, IL.

§Hybridomas secreting MAb to bovine IgG1, IgG2, IgA and IgM were kindly provided by Dr Srikumaran, Univ NE, Lincoln, NE and Dr Goldsby, Amherst Univ., Amherst MA.

¶Boehringer Mannheim Corporation, Indianapolis IN.

‖Maxisorp Immunoplate, Nunc, Naperville, IL.

**Boehringer Mannheim Corporation, Indianapolis IN.

††Immulon I, Dynatech Laboratories Inc, Alexandria.

‡‡Titer Multiskan (MC/340), Flow Laboratories, McLean, VA

Table 1 Relative viral antigen titers in VLP, CLP and SA11 inactivated rotavirus vaccines assayed in ELISA using monoclonal and polyclonal antibodies to rotavirus

Vaccines	Monoclonal antibodies 2G4[a] anti-VP4	C60[a] anti-VP7	255/60[a] anti-VP6	Polyclonal antibodies Goat anti-DC RV[b]	Pig anti-DC + TC RV[c]
VLP	1600[d]	1600	6400	25600	25600
CLP	<8	<8	3200	12800	12800
SA11 BEI-Inactivated	8	8	50	800	800
Control SA11 (1×10^7 PFU/ml)	8	8	50	800	800

[a]MAbs kindly provided by H. Greenberg[25,26]
[b]Hyperimmune serum against double capsid rotavirus particles (DC RV) was produced in goats (VP2 and VP6) reactivity were verified by Western blot)
[c]Hyperimmune serum against semipurified group A BRV (Lincoln strain) containing both DC RV and triple capsid rotavirus (TC RV) was produced in a gnotobiotic pig (VP4 and VP7) reactivity were verified by Western blot)
[d]Antigen titers (expressed as reciprocal of final positive dilution)

Table 2 Geometric mean isotype antibody titers and 95% CI to IND BRV in the serum of cows vaccinated with VLP, CLP, or inactivated SA11 rotavirus vaccines or control cows

Vaccines	Pre-vaccination (pre-partum day 60) IgG1	IgG2	IgA	IgM	Post-vaccination (post-partum day 0) IgG1	IgG2	IgA	IgM
VLP	812^{A*} (272–2430)†	322^{A} (59–1767)	4^{A} (4–4)	51^{A} (17–152)	10321^{A} (4869–21878)	52015^{A} (28721–94206)	4^{A} (4–4)	5160^{A} (1234–21573)
CLP	1625^{A} (223–11871)	406^{A} (56–2969)	4^{A} (4–4)	40^{A} (6–294)	10321^{A} (1413–75573)	41285^{A} (5653–301494)	4^{A} (4–4)	10321^{A} (1413–75573)
SA11 inactivated	724^{A} (240–2182)	512^{A} (143–1830)	4^{A} (4–4)	90^{A} (30–273)	4096^{A} (676–24807)	23170^{A} (2804–191497)	4^{A} (4–4)	4096^{A} (676–24807)
Control	1448^{A} (481–4363)	512^{A} (143–1830)	4^{A} (4–4)	128^{A} (36–457)	724^{B} (240–2182)	512^{B} (143–1830)	4^{A} (4–4)	128^{B} (36–457)

*Means followed by the same letter are not significantly different ($P>0.05$); those followed by different letters are significantly different ($P<0.05$)
†Values in parentheses represent 95% confidence interval (CI)

and VP7 in the VLP as reported previously[21] (data not shown). Triple-layered particles averaging ~70–75 nm in diameter with dark staining cores were evident in the VLP preparations and double-layered particles averaging ~65–70 nm in diameter were observed in the CLP vaccine.

The rotavirus antigen titers and the relative titers of rotavirus VP4, VP6 and VP7 antigens in each vaccine preparation were estimated (*Table 1*). The relative antigen titers of the VLP, inactivated SA11 and untreated control (1×10^7 PFU/ml) SA11 rotavirus reactive with MAbs to either VP4 (2G4) or VP7 (C60) in ELISA were 1600, 8 and 8, respectively. The CLP rotavirus vaccine (VP2+ VP6) did not react with the MAb to rotavirus VP4 or VP7 in ELISA (<8). The vaccine antigen titers with the VP6 MAb (255/60) in ELISA were: 6400 (VLP); 3200 (CLP); and 50 (SA11-inactivated or control). The vaccine antigen titers reactive with polyclonal antibodies (either goat anti-DC or pig anti-DC + TC) in ELISA were 25600 (VLP), 12800 (CLP), and 800 (SA11-inactivated or live). No differences were seen in the titers of the untreated control versus inactivated SA11 rotavirus against any of the antibody reagents.

Geometric mean isotype antibody titers (GMT) to rotavirus in serum

There were no significant differences in serum, colostrum and milk antibody titers to SA11 versus IND rotavirus strains in ELISA; hence, the GMT summarized (*Table 2Table 3*) are against IND BRV. Since there were no significant differences in antibody titers in cows given 100 vs 250 μg of VLP, the GMT was computed for all six cows. The GMT and 95% CI of isotype rotavirus antibodies in serum from vaccinated and control cows are summarized (*Table 2*). At initial vaccination (prepartum day ~60), all cows were seropositive to BRV, indicative of previous field exposure and no significant differences were observed between isotype-specific GMT among groups.

The isotype rotavirus antibody GMT in post-vaccine serum at calving (PPD 0) are summarized (*Table 2*). Cows vaccinated with VLP, CLP and inactivated SA11, developed significantly enhanced antibody GMT associated with IgG1, IgG2 and IgM compared to control cows. The highest GMT to BRV in postvaccine serum at calving were associated with IgG2 in VLP (52015), CLP (41285) and SA11 (23170) vaccinated cows and were elevated at least 45-fold above the IgG2 GMT in the control cows (512). The IgG1 antibody GMT in serum in vaccinated cows were significantly increased (at least 6-fold) above the IgG1 GMT in the control cows. No increases in serologic IgA GMT occurred in any groups after vaccination.

Geometric mean isotype antibody titers to rotavirus in colostrum and milk

The IgG1 and IgA GMT to BRV in colostrum were significantly elevated in vaccinated cows compared to the control cows (*Table 3*). Moreover, the IgG1 and IgA GMT were significantly higher in colostrum of VLP and

Table 3 Geometric mean isotype antibody titers and 95% CI to IND BRV in the colostrum and milk of cows vaccinated with VLP, CLP, or inactivated SA11 rotavirus vaccines or control cows

Vaccines	Colostrum (post-partum day 0) IgG1	IgG2	IgA	IgM	Milk (post-partum day 7) IgG1	IgG2	IgA	IgM
VLP	832225^{A*} (459532–1507291)†	16^{A} (16–16)	16384^{A} (3329–80639)	13^{A} (3–53)	10321^{A} (3147–33854)	16^{AB} (4–59)	812^{A} (272–2430)	32^{A} (9–108)
CLP	660561^{A} (90454–4823900)	16^{A} (1–501)	10321^{A} (1413–75373)	6^{A} (1–46)	6501^{A} (890–47482)	25^{A} (2–1354)	645^{A} (88–4719)	40^{A} (2–7763)
SA11 inact.	131072^{B} (36678–468402)	4^{B} (4–4)	1448^{B} (175–11969)	6^{A} (2–17)	1024^{B} (1024–1024)	11^{AB} (1–93)	64^{B} (5–817)	16^{B} (3–97)
Control	11585^{C} (3845–34907)	4^{B} (4–4)	45^{C} (5–374)	4^{A} (4–4)	64^{C} (64–64)	4^{B} 4–4)	4^{C} (4–4)	4^{C} (4–4)

*Means followed by the same letter are not significantly different ($P>0.05$); those followed by different letters are significantly different ($P<0.05$)
†Values in parentheses represent 95% confidence interval (CI)

CLP cows than in the other two groups. The rotavirus antibody isotype GMT in milk (PPD 7) for each group are summarized (*Table 3*). Although, lower GMT were detected in milk compared to colostrum of vaccinated cows, the relative distribution of BRV antibodies among the different isotypes was similar. The GMT to rotavirus associated with IgG1, IgA and IgM in milk were significantly higher in vaccinated cows than in the control cows. The GMT of all BRV antibody isotypes (except IgG2) were significantly higher in VLP and CLP vaccinated cows than in SA11 vaccinated cows, with IgG1 the predominant isotype followed by IgA.

Geometric mean isotype antibody titers (GMT) to SA11 rotavirus proteins (VP4, VP6 and VP7) in serum and mammary secretions

The GMT and 95% CI of isotype antibodies to rotavirus VP4, VP6 and VP7 in serum, colostrum and milk from vaccinated and control cows are summarized (*Table 4, 5*). There were no significant differences in antibody isotype titers in serum among the four groups to any rotavirus protein (VP6, VP4 or VP7) at vaccination (Pre-partum day 60) (*Table 4*). At calving (PPD 0), significantly enhanced antibody GMT against VP6 in serum were associated with IgG2 and IgM in all vaccinated groups and also with IgG1 in VLP and CLP vaccinated cows compared with the control group. The highest GMT to VP6 in serum were detected in VLP and CLP vaccinated cows (IgG2 GMT of 8192 and 6502, respectively) and were at least 51-fold above the IgG2 GMT in the control cows. Significantly elevated GMT to VP4 and VP7 rotavirus proteins in serum at calving were associated with IgG1, IgG2 and IgM in the VLP vaccinated cows. Significantly increased isotype antibody titers were detected for VP4 (associated with IgM) and for VP7 (associated with IgG2) in SA11 vaccinated cows compared with control cows. In serum at calving no significant differences in GMT to VP4 and VP7 for any isotypes were detected between CLP vaccinated cows and control cows.

In colostrum and milk, IgG1 and IgA GMT against VP6 were significantly elevated in vaccinated groups compared to controls, and were significantly higher in VLP and CLP than in SA11 vaccinated cows (*Table 5*). Significantly higher GMT to VP4 and VP7 in colostrum and milk were detected only in VLP vaccinated cows associated with IgG1 and IgA. In colostrum these titers were at least 6-fold higher than in the control cows.

DISCUSSION

Rotavirus is a major etiologic agent of bovine neonatal diarrhoea[1,2]. Various vaccination strategies have been used for prevention of rotaviral diarrhoea[3–14,16–18]. Use of live attenuated oral rotavirus vaccines in calves to stimulate active immunity has shown variable results under field conditions[4,9]. Vaccine failures were largely attributed to the presence of maternal antibodies to rotavirus that neutralized the vaccine virus in the gut of the neonate[4,9]. The widespread presence of maternal antibodies and the degree of attenuation needed to insure safety of live vaccines in the neonates limit the feasibility of active immunization.

Young calves are highly susceptible to enteric viral infections, and therefore transfer of effective passive immunity via colostrum and milk plays a pivotal role for conferring protection immediately after birth. Several studies have highlighted the importance of immunization of pregnant dams during the last third of the gestation period to confer effective passive immunity for the prevention of BRV associated diarrhoea[3–8,11–18]. This strategy is associated with the feeding of colostrum and milk containing high levels of specific antibody and the presence of these antibodies in the intestinal lumen in colostrum-fed calves[4–8]. Studies in calves have suggested that IgG1 antibodies absorbed from colostrum are transudated from the serum back into the intestine,[15] thereby complementing the role of milk antibodies in passive immunity[4–8]. Increased antibody titers to BRV in mammary secretions following parenteral vaccination of cows using an inactivated BRV vaccine were described by Mebus *et al.*[3] and Snodgrass *et al.*,[12] but in the latter report, calves born to vaccinated dams were only partially protected against homotypic BRV challenge. Probable explanations for this lack of protection were: (1) high challenge dose of virus; (2) milk antibody titers below the protective threshold; and (3) destruction of important epitopes by formalin inactivation. A subsequent study of the U.K. vaccine under farm conditions in a single herd, demonstrated reduced BRV shedding and diarrhoea in calves[16]. Other investigators have described variable results in experimental and field BRV vaccine trials dependent upon the vaccine strain titer, dose, adjuvant, inactivating agent and route of vaccination,[3–6,10,18] documenting a need for improved BRV vaccines in the field.

Saif *et al.*[4–6,11] described optimal methods for vaccination of pregnant cows with BRV to boost antibody

Table 4 Geometric mean isotype antibody titers and 95% CI to VP4, VP6 and VP7 in serum of cows vaccinated with VLP, CLP, or inactivated SA11 rotavirus vaccines or control cows

Vaccines	IgG1 VP6	VP4	VP7	IgG2 VP6	VP4	VP7	IgA VP6	VP4	VP7	IgM VP6	VP4	VP7
Pre-vaccination (pre-partum day 60)												
VLP	203^{A*} (68–607)†	25^{A} (4–148)	16^{A} (4–59)	40^{A} (17–152)	20^{A} (7–60)	4^{A} (4–4)	4^{A} (4–4)	4^{A} (4–4)	5^{A} (3–9)	32^{A} (14–71)	25^{A} (12–54)	8^{A} (4–18)
CLP	256^{A} (8–8013)	161^{A} (22–1178)	32^{A} (3–185)	56^{A} (2–2150)	40^{A} (6–294)	4^{A} (4–4)	4^{A} (4–4)	4^{A} (4–4)	4^{A} (4–4)	64^{A} (64–64)	25^{A} (3–185)	16^{A} (16–16)
SA11	181^{A} (22–1496)	32^{A} (9–114)	16^{A} (3–97)	64^{A} (5–817)	32^{A} (9–114)	8^{A} (4–4)	4^{A} (4–4)	4^{A} (4–4)	4^{A} (4–4)	45^{A} (15–136)	32^{A} (9–114)	16^{A} (16–16)
Control	256^{A} (256–256)	32^{A} (9–114)	32^{A} (9–114)	128^{A} (36–457)	45^{A} (15–136)	4^{A} (4–4)	4^{A} (4–4)	4^{A} (4–4)	4^{A} (4–4)	45^{A} (15–136)	32^{A} (9–114)	16^{A} (16–16)
Post-vaccination (post-partum day 0)												
VLP	2048^{A} (923–4543)	32^{A} (9–108)	50^{A} (17–152)	8192^{A} (3693–18174)	322^{A} (178–584)	512^{A} (231–1136)	16^{A} (16–16)	4^{A} (4–4)	13^{A} (4–38)	2048^{A} (606–6918)	128^{A} (58–284)	161^{A} (49–529)
CLP	1625^{A} (223–11871)	10^{AB} (1–538)	6^{B} (1–46)	6502^{A} (890–47482)	25^{B} (3–185)	6^{C} (4–4)	16^{A} (16–16)	4^{A} (4–4)	4^{A} (4–4)	6502^{A} (890–47482)	25^{BC} (3–185)	16^{B} (2–74)
SA11	90^{B} (30–273)	8^{AB} (2–29)	8^{B} (2–29)	4096^{A} (676–24807)	45^{B} (15–136)	32^{B} (2–552)	8^{B} (2–29)	4^{A} (4–4)	4^{A} (4–4)	362^{B} (120–1091)	45^{B} (15–136)	11^{B} (4–34)
Control	32^{B} (9–114)	6^{B} (2–17)	4^{B} (4–4)	128^{B} (36–457)	16^{B} (3–97)	4^{C} (4–4)	4^{B} (4–4)	4^{A} (4–4)	4^{A} (4–4)	64^{C} (64–64)	16^{C} (16–16)	4^{B} (2–17)

*Means followed by the same letter are not significantly different ($P>0.05$); those followed by different letters are significantly different ($P<0.05$)
†Values in parentheses represent 95% confidence interval (CI)

Table 5 Geometric mean isotype antibody titers and 95% CI to VP4, P6 and VP7 in the colostrum and milk of cows vaccinated with VLP, CLP, or inactivated SA11 rotavirus vaccines or control cows

Vaccines	IgG1 VP6	VP4	VP7	IgG2 VP6	VP4	VP7	IgA VP6	VP4	VP7	IgM VP6	VP4	VP7
Colostrum (post-partum day 0)												
VLP	52015^{A*} (28720–94205)† 104032^{A}	645^{A} (196–2116) 101^{B}	1290^{A} (432–3857) 161^{B}	4^{A} (4–4) 4^{A}	8^{A} (4–18) 4^{B}	4^{A} (4–4) 4^{A}	3250^{A} (1087–9718) 2580^{A}	256^{A} (102–645) 64^{B}	203^{A} (48–849) 6^{B}	81^{A} (27–241) 102^{A}	16^{A} (3–33) 4^{A}	32^{A} (14–71) 10^{B}
CLP	(14246–759717) 16384^{B}	(14–742) 128^{B}	(22–1178) 256^{B}	(4–4) 4^{A}	(4–4) 11^{A}	(4–4) 4^{A}	(9353–18843) 128^{B}	(64–64) 64^{B}	(1–46) 16^{B}	(14–742) 22^{AB}	(4–4) 11^{A}	(1–74) 22^{AB}
SA11	(270–99228)	(36–457) 256^{B}	(256–256) 181^{B}	(4–4) 4^{A}	(4–34)	(4–4) 4^{A}	(36–457) 4^{C}	(11–388) 64^{B}	(3–97) 4^{B}	(3–187) 11^{B}	(1–93) 4^{A}	(8–68) 11^{B}
Control	1448^{C}(480–4363)	(256–256)	(60–645)	(4–4)	6^{AB}(2–17)	(4–4)	(4–4)	(64–64)	(4–4)	(2–93)	(4–4)	(4–34)
Milk (post-partum day 7)												
VLP	1024^{A}(163–6449) 2580^{A}	25^{A} (8–83) 6^{B}	50^{A} (12–212) 4^{B}	4^{A} (4–4) 10^{A}	4^{A}0 (4–4) 4^{A}	4^{A} (4–4) 4^{A}	322^{A} (179–584) 256^{A}	32^{A} (14–71) 4^{B}	32^{A} (14–71) 4^{B}	20^{AB} (5–84) 64^{A}	4^{A} (4–4) 4^{A}	8^{A} (4–18) 4^{A}
CLP	(353–18843) 90^{B}	(1–46) 4^{B}	(4–4) 4^{B}	(1–538) 4^{A}	(4–4) 4^{A}	(4–4) 4^{A}	(256–256) 23^{B}	(4–4) 4^{B}	(4–4) 4^{B}	(2–2003) 4^{B}	(4–4) 4^{A}	(4–4) 4^{A}
SA11	(30–273) 6^{C}	(4–4) 4^{B}	(4–4) 4^{B}	(4–4) 4^{A}	(4–4) 4^{A}	(4–4) 4^{A}	(8–68) 4^{C}	(4–4) 4^{B}	(4–4) 4^{B}	(4–4) 4^{B}	(4–4) 4^{A}	(4–4)
Control	(2–17)	(4–4)	(4–4)	(4–4)	(4–4)	(4–4)	(4–4)	(4–4)	(4–4)	(4–4)	(4–4)	4^{A}(4–4)

*Means followed by the same letter are not significantly different ($P>0.05$); those followed by different letters are significantly different ($P<0.05$)
†Values in parentheses represent 95% confidence interval (CI)

titers in colostrum and milk. Using a combination of IM and IMm routes, pregnant cows were vaccinated with modified live or BEI inactivated BRV in IFA. Significantly increased colostral and milk antibody titers were mainly associated with IgG1, followed by IgG2, IgA and IgM. Saif and coworkers[4,5,67] also reported that significantly increased virus neutralizing and IgG1 BRV antibody titers in colostrum following immunization correlated with passive protection of the newborn calf against a homotypic BRV challenge.

Several approaches have been developed to produce safe, non-replicating, effective rotavirus vaccines.[21,22,27–29] In earlier studies, immunization of a single cow with a VP7-containing empty BRV capsid (purified from BRV-infected cell lysates) induced a 10–30-fold increase in neutralizing and ELISA antibody titers to SA11 rotavirus in serum[27]. No substantial neutralizing antibody or ELISA titer increases were evident in individual cows immunized with purified VP7 or with a VP7 peptide, suggesting that VP7 efficiently boosted antibody responses in cows only when it was presented in the context of the empty capsid. Similarly, baculovirus-expressed VP7 failed to induce neutralizing antibodies or passive protection of mice[28,29]. In other studies, it was shown that conjugation of VP4 or VP7 peptides (corresponding to highly conserved regions) to rotavirus VP6, but not to an unrelated carrier molecule, induced ELISA antibody titers to BRV in mice dams[28,29]. A mixture of both VP4 and VP7 peptides coupled to VP6 gave optimal passive protection in neonatal mice challenged with various rotavirus strains, whereas VP6 alone induced no neutralizing antibodies and only partial passive protection[29].

The recent production of double (CLP), or triple-layered (VLP) particles that mimic authentic virions but lack nucleic acid offers a new approach to develop both safe and highly immunogenic vaccines[19–22]. P. Roy *et al.*[19] reported high antibody responses and protection against bluetongue virus in sheep vaccinated with oil adjuvanted bluetongue VLP derived from multiple baculovirus expression vectors. The antibody responses to SA11 rotavirus VLP derived by the coexpression of individual recombinant SA11 VP2, VP4, VP6 and VP7 genes in a baculovirus expression system were evaluated in mice and rabbits[22]. Animals immunized twice IM with low doses (10 or 20 μg) of SA11 VLP in IFA developed neutralizing and ELISA antibodies, and immunized rabbits were totally or partially protected from virus shedding after homotypic rotavirus challenge.

In the present study, we demonstrated that ELISA antibody titers in serum, colostrum and milk of cows were significantly enhanced by immunization with non-infectious VLP, CLP or inactivated SA11 rotavirus vaccines administrated by the IM + IMm routes. At calving, antibody titers to BRV were significantly elevated in the serum of vaccinated cows, and IgG1 was the predominant antibody isotype followed by IgG1. Other investigators also reported significantly elevated, but higher IgG1 and IgG2 antibody titers to BRV in serum of cows vaccinated with a live attenuated homologous BRV vaccine[11]. It is unclear whether these higher titers are attributable to the use of live virus of bovine origin. Comparative studies of VLP generated using BRV VP4 and VP7 should help resolve this question.

In contrast, in the present study, IgG1 antibody titers to BRV were significantly elevated in the colostrum and milk of vaccinated cows, and were the predominant antibody isotype in mammary secretions of all cows, followed by IgA. This agrees with previous observations from our laboratory or by others using experimental BRV vaccines[4–6,11,12]. The IgG1 and IgA titers were significantly higher in the colostrum and milk of the VLP- and CLP-vaccinated cows compared to the SA11-vaccinated or control cows. The lower IgG1 antibody titer to BRV in serum at calving compared to other times and its predominance in colostrum probably reflects the active transport of IgG1 from serum to colostrum prior to parturition in ruminants[30].

Although the antibody GMT to VP6 were lower overall compared to antibody GMT against whole virus, the antibody responses to VP6 were greater than responses to VP4 or VP7 and had similar isotype distribution patterns as described for BRV. These results confirm that the antibody response to VLP, CLP and inactivated rotavirus was mainly associated with the major structural protein (VP6) which accounts for about 50% of the total virion mass, with lower antibody GMT detected to VP4 and VP7 (for VLP and SA11 vaccinated cows only) which account for only 1.5% and 30%, respectively of the virion mass. Also, only in cows that received the VLP vaccine (containing VP4+ VP7) were significantly increased antibody responses detected in colostrum and milk to these two outer capsid proteins. A probable explanation for the differences observed in response to the VLP, CLP and inactivated SA11 rotavirus vaccines may be the differences in antigen concentrations in each vaccine preparation as measured by ELISA using a panel of MAb and polyclonal antibodies (*Table 2*). The VP4, VP6 and VP7 antigen concentrations were higher for the VLP vaccine (at least 30–200-fold, respectively than the SA11 vaccine). Although, no differences were detected in VP6 antigen concentration between CLP and VLP vaccines, as expected the VP4 and VP7 proteins were undetected in the CLP vaccine which was comprised of only VP2 and VP6. The BEI-inactivated SA11 vaccine did not show a loss of reactivity in ELISA after inactivation compared with the live virus, confirming a previous report[31]. Therefore, the inactivation procedure per se did not account for the lower antigenicity of this vaccine in ELISA or the lower antibody responses elicited *in vivo*. In the present work, we did not detect differences in the antibody response of cows vaccinated with 100 versus 250 μg of VLP; however, the lowest quantity of VLP that could induce a significantly elevated antibody response was not determinated. Analysis of virus neutralizing (VN) antibody responses to SA11 in the vaccinated cows confirmed the protein specific antibody responses[32,33]. The VN antibody titers were significantly elevated and highest in serum, colostrum and milk of the VLP vaccinated cows (reflecting the presence of VP4 and VP7 in the VLP vaccines which induce VN antibodies); VN titers were not significantly elevated in the CLP-vaccinated cows compared to controls[33].

There were no differences in rotavirus antibody titers using the vaccine SA11 rotavirus (P^2, G3) or the serotypically unrelated IND BRV (P^5, G6) as capture antigen in ELISA, which probably reflects the predominance of cross-reactive antibodies induced to VP6, the major rotavirus structural protein. However cross-reactive, non-serotype specific epitopes are also present on the outer capsid proteins, VP4 and VP7 and it is established

that heterologous strains of rotavirus can enhance heterologous and homologous antibody responses in seropositive animals[4,34,35]. It is possible that the ELISA antibody responses to SA11 VP4 which often were similar in magnitude to the responses to SA11 VP7 in the VLP-inoculated cows may reflect a greater tendency of VP4 to induce broadly cross-reactive antibodies as one might expect during booster responses in rotavirus seropositive cows. However a direct comparison of the antibody titers to VP4 versus VP7 is difficult since different ELISA test systems (direct coating versus antibody-capture) had to be used to develop reliable assays. Additional studies are needed to determine if the protein specific immune responses differ using heterotypic (to the vaccine) G and P type proteins.

Another important variable in regard to maternal vaccines for BRV is the route of vaccination. Previous reports indicated that vaccination with inactivated or live BRV via IM + IMm routes induced higher antibody responses compared with other parenteral routes[11]. However because other parenteral routes (IM or subcutaneous) are more practical in the field (although in dairy cattle the IMm immunizations are analogous to dry cow antibiotic treatments), future studies will analyse the immunogenicity of the VLP or CLP vaccines comparing these routes.

Our results confirm that BRV antibody titers in serum, colostrum and milk are significantly enhanced by the use of non-infectious heterologous VLP, CLP and inactivated SA11 vaccines in rotavirus seropositive cows, but significantly higher antibody responses were observed in the VLP and CLP vaccinated cows. The VLP vaccines offer advantages over conventional existing modified live or inactivated rotavirus vaccines including: exclusion of adventitious agents and extraneous antigens potentially associated with live vaccines; the consistent production of the outer capsid proteins, VP4 and VP7 in an immunogenic form (possibly altered or destroyed in inactivated vaccines); and the use of SA11 CLP (VP2+ VP6) to assemble VP4 and VP7 outer capsid proteins from existing or new BRV strains that arise in the cattle population, therefore creating a novel approach for constantly updating vaccines efficacious for boosting lactogenic immunity to BRV in cattle. Studies are currently underway to test the efficacy of colostrum from the inactivated SA11, VLP and CLP-vaccinated cows to passively protect calves against BRV challenge[23].

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