VITAMIN A EFFECTS ON ANTIBODY RESPONSES TO BOVINE CORONAVIRUS
AND ROTAVIRUS VACCINES IN FEEDLOT CALVES

DISSERTATION

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By

Junbae Jee, M.S.

* * * * *

The Ohio State University
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Dissertation Committee:
Distinguished University Professor Dr. Linda J. Saif, Adviser
Assistant Professor Dr. Armando Hoet
Assistant Professor Dr. Fred J. DeGraves
Assistant Professor Dr. Gireesh Rajashekara

Approved by

________________________
Adviser
Graduate Program in
Veterinary Preventive Medicine
Vitamin A is an essential micronutrient for immune function. Vitamin A effects on immunity to infectious agents has been demonstrated in community- and hospital-based studies, resulting in reduced morbidity and/or mortality in children supplemented with adequate dietary vitamin A. The importance of vitamin A in immune responses to and resistance against infectious agents has been studied mainly for human disease or in mouse models. The zoonotic potential of several animal infectious diseases and their association with host vitamin A status is poorly understood. Feedlot calves are subject to multiple infectious diseases, but especially diarrhea and respiratory disease. Diarrhea can be caused by multiple etiological agents including bovine coronavirus (BCoV) and bovine rotavirus (BRV). Co-infection by BCoV and BRV has been frequently observed in calves with severe gastroenteritis. The BCoV and BRV have common features such as the tissue tropism (intestinal epithelium), histopathological lesions (intestinal villous atrophy), clinical signs (diarrhea) and transmission (oral-fecal route). However, they differ morphologically and phylogenetically since BCoV is an enveloped single-stranded (ss) RNA virus and BRV, a non-enveloped double-stranded (ds) RNA virus. Furthermore, BCoV but not BRV is commonly associated with respiratory infections in feedlot cattle and may predispose feedlot cattle to severe or fatal secondary bacterial infections. Additionally, compared with BCoV infection, BRV is more rapidly resolved in calves. In feedlot cattle, dietary vitamin A restriction has been used to increase intramuscular fat, or marbling, resulting in higher quality beef production in terms of meat palatability. However, it is unclear whether vitamin A restriction which increases beef carcass value, exerts detrimental effects on the immune responses to pathogens or vaccines in cattle.
To understand the impact of vitamin A status in cattle on their immune responses, we investigated antibody (Ab) responses in feedlot calves naturally infected with BCoV as well as intramuscularly vaccinated with a commercial inactivated BCoV and BRV vaccine. These calves were fed either high or low dietary vitamin A, based on greater than or less than the 2200 international units (IU) Vitamin A/kg of dietary dry matter (DM), respectively as recommended by the National Research Council. Angus steers (n=40, average 199 days old) were randomly assigned to two groups. One group received low dietary vitamin A (LVA group; 1100 IU/kg of dietary DM, n=20) to simulate low levels in the vitamin A restricted feedlot calves and the other group received high dietary vitamin A (HVA group; 3300 IU/kg of dietary DM, n=20). Supplemental vitamin A was fed at post-arrival day (PAD) 0, then daily throughout the 140 day study period (PAD 140). Because approximately 90-112 days are required to decrease vitamin A in serum from the liver stores, all calves were vaccinated intramuscularly with an inactivated BCoV/BRV vaccine (Scourguard® 3KC, Pfizer) at PAD 112 and boosted at PAD 126 to determine the effect of vitamin A status on vaccine-induced BCoV and BRV Ab responses at PAD 140. Fecal, nasal and blood samples were obtained at PAD 0, 4, 35, 112 and 140. Real-time RT-PCR was used to detect BCoV shedding in the fecal and nasal specimens. Serum and fecal samples were assayed by ELISA for IgG1, IgG2, IgM, IgA and fecal IgA isotype Abs to BCoV and BRV. In addition, the ratios of IgG1 to IgG2 Abs were used to assess dominance of humoral immune responses (IgG1, Th2) over cell-mediated immune responses (IgG2, Th1) in cattle.

At PAD 0, the calves in both LVA and HVA groups showed statistically similar background levels of BCoV shedding and serum BCoV Ab. Therefore, no sub-categorical analysis was required. Twenty calves (50% of total calves) shed BCoV either in feces or nasally at least once shortly after arrival (at PAD 0 and 4). The prevalence of BCoV shedding increased from 21.05% at PAD 0 to 41.03% at PAD 4, but with no BCoV shedding detected thereafter (at PAD 35, 112 and 140). Before the decreased
vitamin A levels in serum occurred (at PAD 0, 4 and 35), we investigated the isotype Ab responses induced at PAD 35 by natural BCoV infection at PAD 0 and 4. The BCoV fecal shedding was negatively associated with pre-existing serum IgA BCoV Ab titers at PAD 4 (based on the derived formula, probability of BCoV fecal shedding (%) = 100*[e^{(1.84–0.63*Log10IgA)} / (1+e^{(1.84–0.63*Log10IgA)})]). In addition, BCoV nasal shedding was negatively associated with pre-existing serum IgA and IgM BCoV Ab titers at PAD 4, (based on the derived formula, probability of BCoV nasal shedding (%) = 100*[e^{(2.37–1.21*Log10IgA)}/(1+e^{(2.37–1.21*Log10IgA)})] and 100*[e^{(0.25–0.61*Log10IgM)/(1+e(0.25–0.61*Log10IgM))}], respectively). Serum IgA- and IgG1-BCoV Ab seroconversion at PAD 35 were positively associated with average BCoV RNA copy numbers detected in fecal samples at PAD 0 and 4 (based on the derived formulas, probability of IgA-BCoV Ab seroconversion (%) = 100*[e^{(-5.52+1.25*average of BCoV copies) / (1+e^{(-5.52+1.25*average of BCoV copies)})}], and probability of IgG1-BCoV Ab seroconversion (%) = 100*[e^{(-7.32+1.28*average of BCoV copies)/(1+e^{(-7.32+1.28*average of BCoV copies)})}]).

After vitamin A decreased in serum (by PAD 112), we investigated BCoV and BRV Abs induced at PAD 112 and 140 by the inactivated BCoV/BRV vaccine given at PAD 112 and 126. Serum IgG1 BCoV Ab titers and the ratios of IgG1 to IgG2 Abs in the HVA group were significantly higher at PAD 140 than at PAD 112 (p < 0.005), whereas those in the LVA group did not differ significantly. At PAD 140, serum IgG1 BCoV Ab titers and the ratios of IgG1 to IgG2 Abs were significantly higher in the HVA than in the LVA group (p < 0.05). Calves naturally infected with BCoV in the LVA group (n=11) were compared to assess the impact of BCoV infection and BCoV vaccination on Ab responses before the decreased vitamin A (at PAD 0 and 35) and after the decreased vitamin A (at PAD 112 and 140) in serum. The calves naturally infected with BCoV had predominantly serum IgG1 BCoV Ab at PAD 35 (p < 0.05). However, the calves previously recovered from natural BCoV infection had a compromised serum IgG1 BCoV Ab response to an inactivated BCoV vaccine at PAD 140.
In contrast, there was no vitamin A effect on serum BRV Ab responses to the inactivated BCoV/BRV vaccine. All serum isotype BRV Ab titers were significantly higher at PAD 140 than at PAD 112, regardless of vitamin A dietary status. In addition, no statistically significant differences were observed between the HVA and LVA group at both PADs. In the LVA group, Ab responses to the inactivated BRV and BCoV differed since serum IgG1 antibody responses to the inactivated BCoV vaccine were compromised, whereas those to the inactivated BRV vaccine were significantly increased at PAD 140 compared with PAD 112.

To our knowledge, this is the first study to analyze the impacts of micronutrients, i.e., vitamin A on Ab responses to a BCoV and BRV vaccine in feedlot calves. The vitamin A status in feedlot calves had a significant effect on Ab responses to an inactivated BCoV vaccine. Serum IgG1 BCoV Abs were induced predominantly under the high vitamin A dietary regimen, whereas they were compromised under the low vitamin A dietary regimen, suggesting that the low vitamin A diet suppresses the Th2 associated Ab (IgG1) responses. However, the vitamin A dietary regimens did not affect Ab responses to inactivated BRV vaccine. This study suggests that vitamin A effects on antibody responses may be vaccine antigen-dependent. Additional studies are needed to assess the effects of low vitamin A on systemic and mucosal immune responses to other vaccines and on vaccine-induced protection in feedlot calves. In addition, this study should be investigated with various other infectious agents responsible for diarrhea as well as the intestinal microbiota to confirm the pathogen-specific vitamin A effects on antibody responses.
Dedicated to my parents who made all of this possible,
for their endless encouragement and patience.

And also to
my lovely wife
for staying with me.

이 모든 과정을 마칠 수 있도록 저를 끝까지 믿어주고 물심양면으로 도와주신
부모님과 가족에게 감사드립니다.

그리고 또한

같이 있어준것 만으로도

큰 힘이 되어준 내 아내에게도

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March 18, 1974.......................Born – Seoul, South Korea

1993 – 2000.........................B.S. Biology
......................................Department of Biology
......................................Inha University, South Korea

2000 – 2004.........................M.S. Biomedical Science
......................................Department of Pathobiology
......................................College of Veterinary Medicine
......................................Auburn University, Alabama

2004 – 2005.........................Research Associate I
......................................Department of Horticulture
......................................Auburn University, Alabama

2006 – present......................Graduate Research Associate
......................................Food Animal Health Research Program
......................................Department of Veterinary Preventive Medicine
......................................Ohio Agricultural Research and Development Center
......................................The Ohio State University, Wooster, Ohio
PUBLICATIONS


FIELDS OF STUDY

Major Field: Veterinary Preventive Medicine

Studies in Epidemiology and Immunology
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CHAPTER 1

LITERATURE REVIEW

1.1. Vitamin A

1.1.1. Absorption and transport of vitamin A

Provitamin A carotenoids from vegetables and retinyl esters from animal sources are the main dietary source of vitamin A (24, 214). Carotenoids and retinyl esters are converted into retinol in the intestinal lumen, followed by the retinol being absorbed in the enterocytes via facilitated diffusion (24, 214). However, some carotenoids are partially absorbed and converted into retinol in the enterocytes (24, 214). It was demonstrated that pancreatic lipase and brush-border membrane enzymes are responsible for hydrolyzing retinyl esters to retinol in the intestinal lumen (24, 214). The majority of retinols are converted into retinyl esters in the enterocytes via two enzymes; acyl CoA:retinol acyltransferase (ARAT) and lecithin:retinol acyltransferase (LRAT) (24, 214). Some retinols in enterocytes are tethered by cellular retinol binding protein (CRBP) II, followed by being converted to retinyl esters via LRAT, whereas untethered retinols located in membranes are esterified by ARAT (24, 214). Under physiological concentrations of retinol, LRAT is responsible for esterification of retinol, whereas ARAT is much more expressed than LRAT in the presence of excess amounts of retinol, resulting in the saturation of CRBP II with retinols (24, 214). The retinyl esters
associated with chylomicrons leave the enterocytes via the lymphatic system and are transported from the intestine to various tissues (24, 214). Once the chylomicrons containing retinyl esters reach the liver parenchymal cells, the up-take is mediated by low-density-lipoprotein (LDL) receptors. The chylomicron remnants are cleared and the retinyl esters are hydrolyzed to retinol at plasma membranes or in endosomes by retinyl ester hydrolyses (24, 214). The converted retinol compounds are transferred to the endoplasmic reticulum and are subjected to interactions with retinol binding proteins (RBPs) highly expressed in the endoplasmic reticulum (24, 214). The retinols bound to RBPs are secreted through the Golgi compartment (24, 214). Most retinols in the liver parenchymal cells are transferred to hepatic stellate cells for storage (24, 116, 214). In addition, it was reported that the transfer of retinols from parenchymal to stellate cells in liver is mediated by RBPs (24, 116, 214). Esterification of retinol seems to be essential, because the most stored form of vitamin A is the retinyl esters. Like CRBP II in the intestinal enterocytes, CRBP I in stellate cells also plays an important role in esterification of retinol by LRAT, resulting in accumulation of retinyl esters in large cytoplasmic lipid droplets (24, 214). The control of storage in the liver stellate cells and secretion into the circulation is dependent upon retinol concentrations in blood plasma (214). To maintain constant retinol concentration, i.e., 1.7~2µmol/L in human plasma, retinol-RBP complex from liver stellate or parenchymal cells are released into the circulatory system (214). Most of the retinol-RBP released from the liver are non-covalently associated with transthyretin (TTR), one of the plasma proteins (24, 279). The mechanism of transporting the retinol-RBP from the circulatory system to cells still remains unclear; however, it has been explained mainly by cell surface receptors for
plasma RBPs (270). The receptor for plasma RBP has been studied in various tissues, e.g., placenta, retina pigment epithelial cells, bone marrow, kidneys, small intestine, spleen, liver, lung, testis, cerebral cortex, etc. (270).

1.1.2. Gene expression regulated by retinoic acid

Retinol taken up by cells can be oxidized into retinoic acid, resulting in retinoic acid-dependent gene expression. Approximately, 132 genes have been suggested to be directly regulated by retinoic acid (15, 24). The predominant mechanism in retinoic acid dependent transcription is composed of cellular retinoic acid binding protein (CRABP), nuclear receptors, i.e., retinoic acid receptor (RAR, three isotypes, i.e., α, β and γ), retinoid X receptor (RXR, three isotypes, i.e., α, β and γ) and retinoic acid response elements (RAREs) in regulatory regions of numerous genes (15, 24). Gene transcription by retinoic acid signals is initially mediated by the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), which form heterodimers (RXR/RAR) (18, 24). The RARs and RXRs contain a DNA binding domain (DBD) which recognizes specific DNA sequences (18). The DBD shows highly conserved functional domains among nuclear receptor isotypes (18). Another conserved region, ligand binding domain (LBD) is responsible for retinoic acid binding, dimerization and retinoic acid-dependent transcription (AF-2) (18). In addition, retinoic acid-independent transcriptional activation function (AF-1) is found in N-terminal of nuclear receptors (18). The AF-1 and 2 domains contain phosphorylation sites (18). Mitogen-activated protein kinase (MAPK), cycline-dependent kinase (CDK) and JNK (jun amino-terminal kinase) are major kinases which phosphorylate specific residues located in AF-1 and 2 domains (18).
First of all, the nuclear receptors bind to responsive elements located in the regulatory sequences of target genes independent of retinoic acids (18). The dimerized nuclear receptor, i.e., RAR/RXR binds to specific DNA sequence called retinoic acid response elements (RAREs) composed of repeated PuG(G/T)TCA (18). The majority of RAREs have been found in the promoters of genes regulated by retinoic acid (18). In addition, the RAREs are classified into three groups according to the number of nucleotides between the repeated RAREs, i.e., 5bp spaced direct repeat (DR5), 1bp spaced direct repeat (DR1) and 2bp spaced direct repeat (DR2) (18). Normally, RAR/RXR heterodimers bind to all three RAREs; however, RXR/RXR homodimers bind to only DR1 RAREs (18). The location of nuclear receptor in heterodimerization is different according to the types of direct repeat; RXR/RAR heterodimers bind to DR2 and DR5 RAREs where RXR DBD binds to upstream and the RAR DBD binds to downstream of RAREs (5’-RXR/RAR-3’) whereas RAR/RXR heterodimers bind to DR1 RAREs in the fashion of 5’-RAR/RXR-3’ (18).

Once the nuclear receptors bind to RAREs without retinoic acid ligation, transcription is repressed by recruitment of corepressors, i.e., nuclear receptor corepressor (NCoR) and silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) which bind to LBDs of both nuclear receptors, consequently resulting in formation of histone deacetylase-containing complex (HDAC) (18). Under the influence of retinoic acid, ligand-binding to LBD induces the conformational change of the heterodimerized nuclear receptor, resulting in dissociation of corepressor (18). Upon dissociation with HDAC, coactivators are recruited with histone acetyltransferase (HAT) complex, followed by decondensation of local chromatin structure and location of the
transcriptional machinery at the promoter (18).

Three isomers of retinoic acid, i.e., *all-trans*-retinoic acid, *13-cis*-retinoic acid and *9-cis*-retinoic acid were identified *in vivo*. The three isotypes of RAR family bind the three isomers of retinoic acid, while those of RXR family bind only *9-cis*-retinoic acid (124). It was demonstrated *in vivo* that combination of retinoic acids and nuclear receptors differently regulates the immune response. Th2 development has been explained with *9-cis*-retinoic acid and synthetic RXR agonists (282). The naïve Th0 cells selected from mice lymph nodes were cultured with *9-cis*-retinoic acid under stimulative condition, i.e., splenic antigen presenting cells and ovalbumin, resulting in increased IL-4 concentration (282). Additional experiments with synthetic RXR agonist AGN194204 significantly enhanced Th2 development resulting from strongly increased IL-4, IL-5 concentration, and Th2 transcription factors, i.e., GATA-3 and e-maf as well as decreased IFN-γ, IL-12 concentration and Th1 transcription factor, T-bet, compared with control cultures (276, 282). In addition, the concentration of IL-4 stimulated with synthetic RXR agonist AGN194204 was greater than that with synthetic RAR agonist TTNPB (282). Memory CD4+ T lymphocytes (CD62LlowCD3+CD4+) from Rxra gene knockout mice were investigated by stimulation with anti-CD3 and anti-CD28 antibodies in which Th1 lymphocytes were predominantly produced, resulting in significantly higher ratio of IFN-γ to IL-4 in Rxra gene knockout mice (280).

The effect of RXR nuclear receptor on proliferation and apoptosis of lymphocytes was investigated with Rxra gene knockout mice. After stimulation with anti-CD3 and anti-CD28 antibodies, the cell division of CD4+ and CD8+ T lymphocytes *ex vivo* was significantly lower, and apoptosis assayed by Annexin A5 staining method in T
lymphocytes tended to increase in Rxra gene knockout mice (280). Similar results were seen in rat kidney mesangial cells pre-treated with RAR pan-antagonist AGN193109 or RXR pan-antagonist HX531, resulting in an attenuated anti-apoptotic effect of all-trans-retinoic acid, e.g., shrinkage of the cytoplasm, membrane blebbing, nuclear condensation (pyknosis) and fragmentation (karyorrhexis); otherwise, it strongly induced an anti-apoptotic effect in H2O2 stimulated cells (172). Retinoic acid has been reported to have an effect on maturation stages of thymocytes. Thymus tissues isolated from young children (0~5 years old) were cultured in vitro with all-trans-retinoic acid, resulting in significantly higher percentage of double positive (CD4+CD8+) T cells and single positive CD4+ T cells, compared with control cultures. In contrast, all-trans-retinoic acid treatment inhibited the differentiation of thymocytes from double positive (CD4+CD8+) T cells into single positive CD8+ T cells (345). The regulation of thymocyte development by all-trans-retinoic acid corresponded with the changes of expression level of RARα in thymus (345). In contrast to RARα mediated CD4+ T cell development, RARγ has been shown to play an important role in CD8+ T cell development (94, 124). It was demonstrated with RARγ-deficient mice that RARγ is not necessary for the proliferation of T and B lymphocytes, polarization of CD4+ T cells to Th1 or Th2 lineage, and antibody production; however, IFN-γ secreted by antigen-specific CD8+ cell was significantly decreased in response to primary and secondary Listeria infection (94, 124).

Besides the effect of retinoic acid on T lymphocytes, retinoic acids play an important role in B lymphocyte maturation. All-trans-retinoic acid accelerates the maturation of human tonsillar B cells into plasma cells, accompanied by high expression
level of three isotype RARs, RXRα, and CD38, suggesting that all-trans-retinoic acid may differentiate resting B cells into antibody secreting cells through CD38 signaling (202).

Dendritic cells (DCs) are responsible for linking innate and adaptive immune responses to infectious agents since immature DCs sample, phagocytose and degrade pathogens, and upon maturation present the degraded proteins on their surface using MHC molecules to activate T lymphocytes. It was demonstrated in vitro that vitamin A effects on dendritic cells are dependent upon their differentiation stage (114). Supraphysiological levels of all-trans- or 9-cis-retinoic acid induced apoptotic cell death in immature dendritic cells through RARα-RXR heterodimerized nuclear receptor, but not in mature dendritic cells. However, under influence of an inflammatory cytokine milieu, e.g., TNFα and IL-1β, the apoptotic effect of retinoic acid on immature dendritic cells was inhibited (114). In addition, retinoic acid has effects on differentiation of dendritic cells. Immature dendritic cells were pulsed with tetanus toxin, and cultured with TNFα and all-trans-retinoic acid, resulting in more proliferative responses of T lymphocytes as compared to immature dendritic cells pulsed with medium, treated with TNFα alone or all-trans-retinoic acid alone. Likewise, retinoic acid and inflammatory cytokines are signals to activate immature dendritic cells and induce their antigen-presenting ability (114).

Macrophage is one of the phagocytic cells in an early defense line against invading bacteria. However, some bacteria have evolved to avoid the bactericidal mechanisms after engulfment. It was demonstrated that treatment with retinoic acid has rescued the macrophages from maturation arrest, resulting in degradation of the pathogen
Once phagosomes containing *Mycobacterium tuberculosis* are pinched off from the plasma membrane, tryptophan-asparate-containing coat (TACO) protein surrounds the phagosomes. The TACO proteins should be removed before the phagosomes are fused with a lysosome; however, the removal of TACO protein from the phagosomes is inhibited, resulting in no fusion with lysosomes and intracellular survival of *M. tuberculosis*. Retinoic acids have been known to be involved in the down-regulation of TACO gene and degradation of pathogens within lysosomes (7). The THP-1 (human acute monocytic leukemia) treated with vitamin D3 and retinoic acid or chenodeoxycholic acid (CDCA) and retinoic acid showed down-regulation of TACO gene through heterodimerization of nuclear receptors, e.g., vitamin D receptor (VDR)-RXR or farnesoid-X-receptor (FXR)-RXR, respectively, resulting in inhibition of *M. tuberculosis* entry and survival within macrophages (6, 7).

1.1.3. Vitamin A effect on immunity

1.1.3.1. Mucosal epithelial barriers

Vitamin A deficiency significantly impairs regeneration of mucosal epithelial barriers such as the respiratory and gastrointestinal tracts. Reduced numbers of goblet cells is the main factor compromising mucosal epithelial barriers (3). Mucus secreted from the goblet cells plays an important role in trapping and removing various pathogens from hosts. In addition, secreted IgA (sIgA) associated with the mucus builds a pathogen specific barrier, resulting in resistance to infectious agents. The change in the epithelial integrity caused by vitamin A deficiency was demonstrated using animal models. Weanling male rats fed a vitamin A deficient diet showed reduced proliferating cells in
crypts, decreased villus height and lower numbers of mucous secreting goblet cells, compared with rats fed the same diet supplemented with vitamin A (241). Rats subjected to partial small bowel resections have been used to investigate the mechanisms responsible for intestinal adaptation associated with an effect of vitamin A (289). It was reported that after partial small bowel resection, vitamin A deficient rat groups showed an inhibited intestinal adaptation characterized by the reduction of crypt cell proliferation, enhancement of early crypt cell apoptosis, and reduction of enterocyte migration rates (241, 289).

The activity of brush border enzymes, e.g., disaccharidases, peptidases and alkaline phosphatase, has been used as an indicator of intestinal integrity status. The activity of these enzymes was markedly lower in the vitamin A deficient diet group compared to the vitamin A supplemented group, reflecting the direct effects of vitamin A on the rate of proliferation and differentiation of the epithelial tissue (241). Germ-free rats monocolonized with non-pathogenic *Escherichia coli* (*E. coli*) O6 K13 pOmp 21 strain under a vitamin A free diet exhibited reduced brush border enzyme activities, i.e., lactase, sucrase, gamma-glutamyltranspeptidase (GGT) and dipeptidyl peptidase IV (DPP IV), indicating a severe functional disturbance of the enterocytes. In a chicken model, the expression levels of alkaline phosphatase, sucrose-isomaltase and aminopeptidase were lower in vitamin A deficient chickens than controls, indicating that intestinal maturation was less in vitamin A deficiency (310).

Disturbance of the epithelial barrier caused by vitamin A deficiency paralleled the increased risk of infection. The small intestine as well as the colon in vitamin A-deficient rats was heavily colonized with a non-pathogenic *Escherichia coli* (*E. coli*) O6 K13
Moreover, *E. coli* O6 K13 pOmp 21 strain was detected in the mesenteric lymph nodes and kidneys in vitamin A-deficient rats, indicating that vitamin A deficiency increases translocation of intestinal bacteria to extraintestinal sites (173, 331). Besides the intestinal tract, vitamin A deficiency also compromises the mucosal epithelial barrier against bacteria penetration in respiratory epithelial cells (41). As evidenced by a significantly higher number of *Klebsiella pneumoniae* adhering to nasopharyngeal epithelial cells in vitamin A deficient children, this study suggests that in vitamin A deficiency, bacteria are not restricted to only colonization of the respiratory epithelial surfaces, but they can penetrate the mucosal barrier, leading to systemic infection.

1.1.3.2. Neutrophils

Neutrophil function has been reported to be significantly altered in vitamin A deficiency (308). Phagocytic activity and generation of active oxidative molecules in neutrophils from vitamin A deficient rats were significantly lower than those from vitamin A replenished or those fed a vitamin A complete diet. Moreover, in vitamin A deficient rats, administration of vitamin A restored the phagocytic activity and generation of active oxidative molecules of neutrophils, comparable to rats fed a vitamin A complete diet. The altered abilities of neutrophils under vitamin A deficiency may contribute to systemic infection (217). Vitamin A deficient rats with impaired neutrophil phagocytosis were markedly susceptible to endogenous bacterial infection, resulting in spontaneously increased bacteremia during late stage of vitamin A deficiency. In addition, leukopenia was observed in vitamin A deficient rats in which numbers of lymphocytes decreased and
numbers of neutrophils increased (211). The neutrophilia was probably attributed to decreased neutrophil apoptosis under vitamin A deficiency (174).

1.1.3.3. Macrophages

The effect of vitamin A deficiency on macrophage numbers and function has been studied. The total number of macrophages in secondary organs and peripheral blood was increased in vitamin A deficient rats (274, 334). However, it has also been reported that vitamin A deficiency impaired the phagocytic activity and intracellular killing capacity of peritoneal macrophages against *Staphylococcus aureus* (334). In a chicken model, peritoneal macrophages from chickens fed a marginal vitamin A diet did not show significantly impaired phagocytosis, but the oxygen dependent-killing capacity was markedly reduced (268).

In reference to cytokines, earlier and stronger interleukin (IL)-12 responses were found in vitamin A deficient mice infected with *Borrelia burgdorferi* (35), whereas retinoic acid treatment inhibited IL-12 production and transcriptional levels from mouse macrophages stimulated with *E. coli* lipopolysaccharide (LPS) (206, 324). Cytokines produced by macrophages from vitamin A deficient rats contributed to increased inflammation (330). The spontaneous release of nitric oxide from peritoneal phagocytes was five times higher in vitamin A deficient rats than control rats, which probably lead to more severe tissue damage.

The RA effect on cytokines produced from the human acute monocyte leukemia cell line (THP-1) was investigated (324). After stimulation of this cell line with LPS, the expression level of IL-10 was 2.2-5.6 times higher in the presence of RA than in the
absence. In contrast, RA down-regulated the expression level of TNF-\(\alpha\) and IL-12 produced from the LPS stimulated THP-1. In addition, IL-10 secreting cells increased, whereas TNF-\(\alpha\) secreting cells decreased in the LPS stimulated THP-1 cells after RA treatment. Similar results were also demonstrated in a mouse model (163). Splenic macrophages isolated from mice treated with RA down-regulated IL-12 production in response to LPS or heated-killed *Listeria monocytogenes* (HKL). Macrophages incubated with keyhole limpet hemocyanin (KLH)-primed CD4\(^+\) T cells purified from mouse lymph nodes inhibited IFN-\(\gamma\) and enhanced IL-4 production by the antigen-primed CD4\(^+\) T cells, resulting in an inhibition of Th1- but enhancement of Th2-mediated immune response in the RA treated mice. As a possible mechanism of suppressed Th1 immune response by RA, NF\(\kappa\)B-mediated IL-12 production from macrophages was inhibited by RA, resulting in an unfavorable milieu for Th1 development (206).

**1.1.3.4. Natural killer (NK) and NKT cells**

NK cells are cytolytic lymphocytes associated with innate immunity and responsible for lysis of various target cells, e.g., virus infected cells and tumor cells without recognizing a specific antigen. The lytic mediators such as perforins and proteases are the major mediators of the cytolytic activity of NK cells. Effects of vitamin A deficiency on numbers of NK cells and their function have been demonstrated (79, 246, 344). Compared to rats fed a control diet (4mg retinol equivalents (RE)/kg), rats fed marginal vitamin A (0.3mg RE/kg) showed lower percentages and numbers of NK cells in peripheral blood, whereas vitamin A supplemented rats (50mg RE/kg) showed significantly increased percentages and numbers of NK cells (79). In addition, a
significantly positive correlation between cytotoxicity and percentage of NK cells in peripheral blood mononuclear cells (PBMC) and spleen was shown. Lytic activity per NK cell was significantly lower in vitamin A deficient rats compared to controls in spleen, but not in PBMC. This study suggests that vitamin A plays an important role in maintaining the number of NK cells and their functional activity (344). Besides the decreased number of NK cells described above, specific cytokine expression, i.e., TNF-α released from NK cells in peripheral blood was investigated in a cross-sectional study of Ghanaian adults (160). In the vitamin A deficient group (< 20µg of RA/dL), the percentage of TNF-α expressing CD3−CD56+ NK cells was significantly reduced, compared to the normal vitamin A group (≥ 20µg of RA/dL) (160).

The lipid antigens presented by CD1d (a major histocompatibility complex [MHC] class I-like molecule) on the surface of antigen-presenting cells activate a specific T lymphocyte subset, natural killer T (NKT) cells. Activated NKT cells immediately produce IFN-γ and IL-4, cytokines representative of Th1 and Th2, respectively. The gene expression levels of CD1d in monocytes were investigated in vivo with and without a physiologic concentration of RA (10 nM), resulting in significantly increased CD1d mRNA in presence of RA, compared to without RA. Thus, it was suggested that RA-dependent CD1d gene expression plays an important role in activating NKT cells (47). In addition, activation of CD1d-restricted NKT cells was shown to be dependent upon lipid-activated transcription factor, i.e., peroxisome proliferator-activated receptor (PPAR)γ in dendritic cells (DCs) (290). Synthetic PPARγ activator, i.e., rosiglitazone (RSG) induced retinol and retinal dehydrogenases, resulting in high concentrations of intracellular RA in DCs. Consequently, the RA-retinoic acid receptor (RAR)α complexes
up-regulated CD1d gene expression in dendritic cells. The elevated CD1d gene expression was positively correlated with NKT cell expansion and the cytokine repertoire (290). In contrast to NK cells, numbers of NKT cells in peripheral blood of vitamin A deficient rats were increased (80). Marginal vitamin A deficiency in older rats significantly increased the number of NKT cells compared with control and supplemented dietary groups. In addition, marginal vitamin A deficiency significantly increased the number of NKT cells relative to NK cells. It is possible that the increased number of NKT cells is a compensatory mechanism which counteracts the reduced cytokine production from NK cells, suggesting that vitamin A status affects reciprocal regulation between NK and NKT cells (80).

1.1.3.5. Dendritic cells

In developing countries, children supplemented with vitamin A tend to show less severity of diarrheal disease, resulting in reduced mortality and morbidity (279). Thus, vitamin A has been considered one of the micronutrients which participate in intestinal immunity. It was demonstrated in vitro that the addition of RA, one of the vitamin A metabolites, imprints gut-homing receptors on B and T lymphocytes. Gut-associated lymphoid tissue (GALT)-dendritic cells (DCs) are responsible for imprinting T and B lymphocytes with lamina propria (LP) homing receptors (161). T cells activated in Peyer’s patches (PPs) and mesenteric lymph nodes (MLNs) express the integrin α4β7 and chemokine receptor (CCR) 9, which interacted with mucosal addressin cell adhesion molecule (MadCAM)-1 and thymus-expressed chemokine ligand (CCL25) in the small intestine tract, respectively. Interestingly, the gut-homing markers, α4β7 and CCR9 are
normally induced by DCs isolated from GALT, but not from peripheral lymph nodes or spleen. It was reported that vitamin A restricted or depleted dietary regimen reduces the number of α4β7 expressing T lymphocytes in the small intestinal tract (157).

In order to convert retinol to RA, retinoid dehydrogenase is required. It was reported that DCs isolated from MLNs showed higher expression levels of retinal dehydrogenase (RALDH)-2 than those from peripheral lymph nodes and spleen, possibly implying that GALT-DCs are preferentially differentiated to produce RA, resulting in imprinting of gut-homing receptors on T lymphocytes. Primed T lymphocytes in PPs preferentially induce OX40, a member of the tumor necrosis factor superfamily. It was suggested that signals from the interaction between OX40 on T lymphocytes and OX40 ligand on dendritic cells may be related to the expression of enzymes involved in RA synthesis (161). In addition, epithelial cells covering the PPs tend to produce another type of retinal dehydrogenase, RALDH-1. Taken together, GALT seems to be an optimal place for production of RA, followed by imprinting of gut-homing receptors on T cells, resulting in induction of gut-immunity (161).

The RA-mediated gut-homing receptor imprinting has also been demonstrated with B lymphocytes (201). Secretory IgA and the number of IgA-secreting B lymphocytes play an important role in preventing infections by microbes involved in intestinal diseases. The RA produced by GALT-DCs imprints the primed B lymphocytes with gut-homing receptors, α4β7 and CCR 9. Primed B lymphocytes produce higher concentrations of IgA after co-culture with PP-DCs than without DCs or with peripheral lymph node (PLN)-DC. Taken together, GALT-DCs and GALT-DC derived RA induce T cell-independent expression of IgA as well as gut-homing receptors. In addition,
besides GALT-DCs and GALT-DC derived RA, Th2 cytokines, i.e., IL-6 and IL-5 are necessary to augment secretory IgA (201).

Further analysis of vitamin A effects on immunity has extended into oral tolerance mechanisms (22, 65). It was reported that oral tolerance, immunologic unresponsiveness, can be induced by the RA produced from GALT-DCs which convert naïve T lymphocytes into regulatory T lymphocytes. The DCs in PPs and MLNs showing a distinctive phenotype, CD103+, but not CD103-, generated more efficiently FoxP3+, transcription factor in regulatory T lymphocytes. In addition, TGF-β is necessary to induce RA-dependent regulatory T lymphocytes by CD103+ DCs, indicating that RA alone is not enough to induce Foxp3+ regulatory T lymphocytes, but TGF-β significantly enhanced FoxP3+ regulatory T lymphocyte induction under subimmunogenic conditions (22, 65). As described above, the RA can induce the gut-homing receptor on FoxP3+ regulatory T lymphocytes, resulting in preferential localization into the small intestinal tract. In addition, RA modulates the microenvironment for subimmunogenic conditions by interfering with co-stimulation signals from an interaction of CD80/86 on DCs and CD28 on T lymphocytes (22).

Immunological homeostasis in the intestinal tract was described as the reciprocal regulation between macrophages and DCs. One macrophage population, CD11b+F4/80+CD11c- in the lamina propria of the small intestine constitutively produced higher levels of the anti-inflammatory cytokine IL-10, and lower levels of pro-inflammatory cytokines (87). Like GALT-DCs, these macrophages also express high levels of retinal dehydrogenase, converting the retinal into RA. Constitutively expressed IL-10 and retinal dehydrogenase in theses macrophages was involved in converting naïve
CD4+ T lymphocytes to Foxp3+ regulatory T lymphocytes, which were further promoted in synergy with exogenous TGF-β (87). Furthermore, the macrophages suppressed the differentiation of Th1 and Th17 cells (87). In contrast, lamina propria (LP)-DCs, i.e., CD11b+ DCs functioned differently unlike the lamina propria macrophages in which LP-DCs stimulated the differentiation of inflammatory Th17 cells in vitro (87). Taken together, reciprocal regulation between CD11b+F4/80+CD11c- macrophages and CD11b+ DCs in the LP may reflect the balance between tolerance and immune response in the intestine.

1.1.3.6. T lymphocytes

1.1.3.6.1. Vitamin A effects on Th1 and Th2-mediated immunity

The impairment of Th2 mediated adaptive immune responses under vitamin A deficiency was demonstrated for antibody and cytokine repertoires induced by infectious agents. Mice infected with Trichinella spiralis showed typical Th2 mediated immune responses in which IgG1 and IgE antibody levels were elevated, abundant IL-4 and IL-5 were produced, and very little IFN-γ and IL-2 were detected (36). However, vitamin A deficient mice showed compromised immune responses in which the frequencies of IgG1 secreting B lymphocytes and bone marrow eosinophils were reduced in response to Trichinella spiralis. Splenocytes and mesenteric lymph node cells from vitamin A deficient mice secreted more IFN-γ than vitamin A sufficient mice did, whereas the release of IL-2, IL-4, and IL-5 from mesenteric lymph nodes cells was significantly reduced in vitamin A deficient mice (36). In addition, immune responses in a mouse model of asthma, a Th2 mediated respiratory disease, were investigated in association
with vitamin A status (263). Vitamin A deficient mice showed reduced IgE and IgG1 levels in serum as well as IL-4 and IL-5 concentrations in bronchoalveolar lavage (BAL) specimens in parallel with significantly decreased pulmonary eosinophilia in BAL (263).

To investigate the reason for decreased antigen specific-IgG1 Ab titers in vitamin A deficiency, macrophages, B lymphocytes, and T lymphocytes from vitamin A deficient mice were examined (37). Macrophages and B lymphocytes in vitamin A deficient mice were functionally normal, whereas T lymphocytes showed improper function, they proliferated in response to antigens, but they failed to stimulate B lymphocytes to produce IgG1 antibody. Gene transcription of IL-4 and IL-5 cytokines involved in isotype switching, is retinoid-dependent. Thus, lower retinoic acid levels in vitamin A deficient mice may decrease the ability of retinoic acid nuclear receptors, e.g., RARs and RXRs to transcribe genes required for antibody response, e.g., IL-4 and IL-5 (37, 77).

In contrast to pathogen-specific antibody, significantly elevated total immunoglobulin was observed in vitamin A deficiency (111, 281). Influenza A specific IgA Ab secreting cells isolated from salivary glands in vitamin A deficient mice were fewer than those from controls, resulting in lower influenza A specific salivary IgA Ab titers. Although virus specific salivary IgA Ab titers were lower in vitamin A deficient mice, total salivary IgA concentrations were significantly higher in vitamin A deficient mice than controls. In addition, vitamin A deficient mice had greatly increased transcriptional levels of polymeric immunoglobulin receptor (pIgR) which plays an important role in IgA transport into glandular secretions such as saliva, bile, tears, etc. The higher concentrations of total salivary IgA are probably attributable to the higher expression levels of pIgR (111, 281). Interestingly, similar results were reported in
broiler chicks vaccinated with Newcastle disease virus (NDV) in which higher total IgA levels in the tracheal homogenates of vitamin A deficient chicks were observed than those in controls (76).

Th2-mediated responses influenced by vitamin A treatment were also demonstrated in a vaccine development study (338). All-trans retinoic acid (ATRA), one of the acid derivatives of vitamin A, was used to investigate whether or not it is an immune modulator, resulting in Th2 immune responses to a DNA vaccine, TR421-hCGβ (eukaryotic expression vector TR421 containing human chorionic gonadotropin β gene), compared with vector TR421 control. For Ab responses, ATRA administered to mice resulted in lower vaccine specific-IgG2a Ab titers, resulting in elevated ratios of IgG1 to IgG2a Abs, indicating a dominance of Th2- over Th1-mediated immune response. In cytokine expression, treatment of mice with ATRA increased transcriptional levels of IL-4 and IL-6, and decreased IFN-γ and TNF-α. The ratio of IL-4 to IFN-γ, an indicator of the balance between Th2 and Th1, was significantly higher in the ATRA treatment group, implying that ATRA treatment favors Th2 responses. In addition, ATRA treatment enhanced transcriptional levels of the Th2 transcription factor GATA-3, whereas it decreased that of the Th1 transcription factor, T-bet (78, 338).

1.1.3.6.2. Vitamin A effects on Foxp3+ regulatory T cells and Th17-mediated immunity

Recently, it was established that retinoids promote the generation of FoxP3+ regulatory T cells and suppresses the differentiation of Th17 cells in peripheral and intestinal lymphoid tissues (203, 322). Dendritic cells have been demonstrated to be key
players to induce FoxP3+ regulatory T cells without co-stimulation, i.e., under subimmunogenic conditions (322). Retinoic acid (RA) and TGF-β generated from CD103+ expressing dendritic cells (DCs) in gut-associated lymphoid tissues (GALT) provide a favorable microenvironment to convert naïve T cells into FoxP3+ regulatory T cells. In addition, RA imprinted gut-homing receptors, α4β7 integrin and CCR9 on FoxP3+ regulatory T cells which migrate to the GALT (22, 322).

On the other hand, ATRA, 9-cis RA or other RAR agonists suppressed the generation of Th17 cells (203). Th17 cell are the newly identified subset of inflammatory T cells, characterized by the expression of IL-17, IL-21, and IL-22, which participate in autoimmunity and inflammation. It was demonstrated that gene transcription of Th17 is mainly controlled by RORγt, orphan nuclear receptor whose expression is decreased by RA. The optimal microenvironment for differentiation of Th17 cells requires not only TGF-β, but also IL-6 which RA might directly counteract upon differentiation of FoxP3+ regulatory T cells (166, 203). Likewise, RA controls the reciprocal differentiation of TGF-β dependent regulatory T cells and Th17 cells.

1.1.3.7. B lymphocytes

1.1.3.7.1. Antibody response to T cell dependent antigens

Vitamin A effects on antibody responses are dependent on the nature of the foreign antigen; T cell dependent and T cell independent antigens. Vitamin A was shown to have a significant effect on antibody responses to T cell dependent antigens. Vitamin A deficient rats perorally immunized with cholera toxin (CT) had decreased CT specific-IgA Ab concentrations in serum and bile, and showed decreased numbers of CT specific-
IgA Ab secreting cells in mesenteric lymph nodes (MLN) (332). Another T cell dependent antigen, tetanus toxoid (TT) has been used to investigate vitamin A effects on antibody responses in vitamin A deficiency (169). Rats fed vitamin A free diets generated very low concentrations of TT specific-IgM and IgG Abs in primary and secondary responses to TT, compared to rats with vitamin A supplemented diets (169). In addition, after being repleted with retinyl palmitate, the vitamin A deficient rats showed quantitatively normal secondary antibody responses to TT, possibly implying that immunologic memory for TT was generated and maintained during vitamin A deficiency (169). To investigate the effect of immunologic memory responses to TT, the ratio of the secondary IgG TT Abs to primary IgG TT Abs was used. As a result, significantly higher ratios were observed in vitamin A supplemented rats compared to the control group (262). Antigens were also evaluated for antibody responses in vitamin A deficiency. T cell dependent antigens, i.e., β-lactoglobulin (β-LG) and sheep red blood cells (SRBC) were used to investigate antibody responses in rats (245, 333). Similar to the effects of vitamin A deficiency on Ab response to antigens of pathogens, vitamin A deficient rats showed significantly reduced serum IgG and IgM Ab production in response to β-LG and SRBC, compared with control rats (245, 333).

1.1.3.7.2. Antibody response to T cell independent antigens

Unlike antibody responses to T cell dependent (TD) antigens, vitamin A deficient rats immunized subcutaneously with Ficoll conjugated to Picrylsulphonic acid (TNP), known as a T cell independent (TI) antigen, did not show any reduction in the levels of serum IgG and IgM Abs, compared to the levels of control rats (333). However, vitamin
A effects on antibody responses to TI antigens were inconsistent. The inconsistency of vitamin A effects on antibody responses to TI antigens differs between two types of TI antigens; Type 1 TI antigen, e.g., lipopolysaccharides (LPS) and type 2 TI antigen, e.g., capsular polysaccharides (96). For the type 2 TI antigens, capsular polysaccharides from *Streptococcus pneumoniae* and *Neisseria meningitidis* were used to monitor plasma IgM Ab levels in vitamin A deficient rats, resulting in significantly lower responses, compared to control rats (245). In contrast, for type 1 TI antigen, the antibody responses were not compromised; plasma IgM Ab responses to LPS from *Pseudomonas aeruginosa* and *Serratia marcescens* in vitamin A deficient rats were similar to those observed in control rats (245).

In summary, vitamin A deficiency compromises antibody responses to antigens classified as TD and type 2 TI, but not to antibody responses to type 1 TI antigens. Nevertheless, it was demonstrated that in vitamin A deficient rats, antibody responses to TD antigen can be enhanced by co-immunization with LPS (10, 245). Compared to vitamin A deficient rats immunized with TT alone, vitamin A deficient rats co-immunized intraperitoneally with TT and *P. aeruginosa* LPS had significantly increased anti-TT IgG titers, comparable to anti-TT IgG titers in vitamin A sufficient rats (10). In addition, the co-immunization effect on antibody responses was demonstrated with *S. pneumoniae* capsular polysaccharide and *P. aeruginosa* LPS in vitamin A deficient rats, resulting in significantly increased *S. pneumoniae* capsular polysaccharide specific plasma IgM Ab levels which were undetectable without LPS co-immunization (245). In the same co-immunization study, tumor necrosis factor (TNF)-α was considered a mediator of antibody responses; treatment with anti-TNF-α serum significantly reduced
anti-TT IgG levels in vitamin A deficient rats after co-immunization with TT and LPS (10, 245).
1.2. Bovine Immunology

1.2.1. Antibodies and B lymphocytes

Cattle have genes for all antibody isotypes, i.e., IgM, IgD, IgG1, IgG2, IgG3, IgE and IgA (343). The Cγ subclass genes have been identified: IgG1, IgG2a and IgG2b. The majority of serum IgG is of the IgG1 subclass which constitutes 50% of total serum IgG. The serum IgG concentrations were shown to vary greatly in each individual because of heritability (215). In Holstein-Charolais crossbred cattle, calves with more Holstein cattle genes had an inclination to produce more bovine respiratory syncytial virus (BRSV) specific IgG Abs than those with more Charolais cattle genes (215). In addition, serum IgG1 concentrations show seasonal variations in neonatal calves fed colostrum (113). Serum IgG1 concentrations were lowest in the winter, but increased during the spring and early summer. The highest serum IgG1 concentrations were observed in September, followed by decreases thereafter (113). Failure of passive transfer of IgG1, defined as less than 8mg of serum IgG1/ml, is seasonal-dependent. Calves born in summer/early fall season, i.e., from June to October showed lower failure rates than those born in winter/spring season, i.e., from November to May (113).

The Fc receptors and antibodies play an important role in specific antibody transfer and protect IgG Ab from degradation. Effector mechanisms associated with Fc receptors and antigen specific antibodies have been studied for neutrophils and macrophages. It was demonstrated that Fcγ receptor associated with IgG in bovine neutrophils mediates superoxide (O$_2^-$) production and intracellular signal transduction including protein kinase C (PKC) and tyrosine phosphorylation, resulting in enhancement of neutrophil functions (143, 178, 208). In addition, it was suggested that neutrophils
enhance the bactericidal activity of IgG2, but not of IgG1 (146). The Fc receptors for bovine IgG1 and IgG2 were detected on macrophages in response to parasitic protozoal and bacterial agents, resulting in antibody-dependent cellular cytotoxicity (ADCC) (146, 299).

Besides immune responses, Fc receptors also play an important role in transfer of maternal antibodies in neonatal calves. Surface membrane Fc receptors for IgG (FcγR) on duodenal and jejunal epithelial cells were shown to facilitate the transfer of maternal IgG across the intestinal epithelium into the circulatory system (313). In addition, the FcγR expressed on intestinal epithelium in calves is structurally very similar to the FcγR expressed on human placenta (313). Neonatal Fc receptor (FcRn) was shown to transcytose IgG through mammary epithelial cells, resulting in accumulation of IgG in colostrum and milk (40). Bovine IgG1 has low affinity for the FcRn and thus was preferentially transferred to the colostrum, whereas Bovine IgG2 binds strongly to the FcRn, followed by its being recycled to the circulation more efficiently. The IgG2 binding to FcRn normally showed a longer half-life (40).

The number of available V, D and J gene segments in cattle was shown to be very restricted compared with humans and rodents (199). In humans and rodents, multiple gene segment rearrangement is the major mechanism to generate the diversity of the antibody repertoire. However, cattle fail to produce significant diversity in the immunoglobulin heavy chain, resulting in a very limited antibody repertoire. Unlike humans and rodents, gene conversion and somatic hypermutation were shown to be major mechanisms to generate diverse V regions in cattle (34, 199). In gene conversion, cattle have relatively large numbers of pseudogenes in the V-region germ line (34,
343). These pseudogenes can be excised and inserted into the functional V gene segment to generate new sequences, or diverse V-regions. Somatic hypermutation was also detected in the ileal Peyer’s patches and spleen of cattle. It was also suggested that as a compensatory mechanism for the limited diversity, very long sequences of complementarity determining region (CDR) 3 are located in heavy chains (343).

Antibody class switching is positively or negatively regulated by cytokines, e.g., IFN-γ, IL-2, IL-4, IL-10, IL-13, TGF-β, etc (98, 99). Resting B lymphocytes stimulated with polyclonal anti-bovine IgM and co-cultured with IFN-γ were used to stimulate immunoglobulin production in vitro (98). As a result, IgM and IgG2 levels were significantly increased, whereas IgG1 and IgA levels did not change significantly, suggesting that IFN-γ plays an important role in controlling IgG2 production (98, 101). In contrast, the activated bovine B cells co-cultured with IL-4 stimulated an IgG1 and IgE antibody response (98). In addition, in response to the cytokine combination of TGF-β, IL-2 and IL-10, the B lymphocyte population isolated from cattle preferentially produced IgA (98, 101). In either IFN-γ or IL-4 stimulation experiments, co-culture with IL-2 augmented the antibody production without an isotype change (98, 99).

The bovine B lymphocytes activated with anti-bovine IgM and cytokines, i.e., IFN-γ and IL-4 described above simulated T lymphocyte independent type 2 (TI-2) antigen mediated antibody responses. The association between antibody isotypes and cytokines was also demonstrated under T lymphocyte dependent (TD) activation using murine fibroblast cell lines transfected with bovine CD40L (100). Compared with TI-2 conditions, lower levels of IgG2 antibody was produced in the IFN-γ stimulated B lymphocytes. However, the IgA antibody, not detectable under TI-2 conditions, was
modestly increased (100). In contrast, in the presence of IL-4, B lymphocyte proliferation and IgM and IgG antibody secretion were enhanced, comparable to that under TI-2 conditions (100). Therefore, it was suggested that the IFN-γ mediated Th1 microenvironment is not efficient to produce antibodies under the TD conditions (100).

1.2.2. CD4+ T lymphocytes

The function of CD4+ MHC class II-restricted T lymphocytes in cattle is consistent with that in rodents and humans; the naïve T lymphocytes are sensitized with antigens processed and presented by antigen-presenting cells, resulting in proliferation and differentiation of these T lymphocytes. Especially, a strong association between the CD4+ T lymphocyte and antigen-specific humoral immune responses has been demonstrated in calves using anti-CD4 monoclonal antibodies (mAbs). Sera from calves depleted of CD4+ T lymphocytes showed significantly reduced hemagglutinating titers against human O red blood cells and lower ovalbumin specific-IgG1 and IgG2 Ab titers (151). Long-term depletion of CD4+ T lymphocytes was created by thymectomy and anti-CD4 mAbs, resulting in significantly reduced IgG1 specific and non-detectable IgG2 antibody responses to ovalbumin compared with thymus-intact control calves (312). Consistent results were generated in response to pathogens. The CD4+ T lymphocyte depleted calves showed suppressed antibody responses to *Anaplasma marginale*; significantly decreased major surface proteins-2 (MSP-2)-specific IgG1 Ab and non-detectable MSP-2-specific IgG2 Ab titers compared with control calves (311). The CD4+ T lymphocyte has been shown to affect local antibody responses as well. After being depleted of CD4+ T lymphocytes using mAbs, calves showed significantly
reduced fecal bovine rotavirus-specific IgM and IgA antibody responses (216). In addition, abrogated antibody responses were described in BRSV infected calves. Two of three calves depleted of the CD4+ T lymphocytes failed to develop an antibody response to BRSV in sera and lung washings (293).

Besides effects on antibody responses, the effects of CD4+ T lymphocytes have been described in terms of peripheral blood mononuclear cell (PBMC) proliferation, control of primary infections, clinical symptoms and histopathology. The PBMCs isolated from the CD4+ T lymphocyte depleted calves showed diminished responses to ovalbumin and mitogens, e.g., purified phytohemagglutinin, concanavalin A and pokeweed (151, 312). In the calves intranasally infected with bovine viral diarrhea virus (BVDV), CD4+ T lymphocytes play an important role in containment of virus replication, resulting in increased duration of viremia and virus titers in blood from CD4+ T lymphocyte depleted calves (148). In addition, similar results were described in CD4+ T lymphocyte depleted calves in response to *A. marginale*; the calves showed enhanced percentage of parasitized erythrocytes, resulting in a significantly reduced proportion of erythrocytes. The CD4+ T lymphocyte depleted calves showed a strong association between transient fever and the highest titer of *A. marginale* in blood (311). In contrast, there was no significant difference in bovine rotavirus excretion between CD4+ T lymphocyte depleted and control calves, reflecting that CD4+ T lymphocytes are not involved in limiting local replication of bovine rotavirus (216). The formation of chancre, local skin reaction mediated by trypanosome-infected tsetse flies has been shown to be CD4+ T lymphocyte-dependent in cattle. Cattle depleted of CD4+ T lymphocytes showed significantly reduced numbers of and skin thickness of the chancre compared
with those of non-depleted groups (207). Compared to CD8+ T lymphocyte depleted calves, less active histological lesions were generated in CD4+ T lymphocyte depleted calves, characterized by lung consolidation associated with proliferative aveloar and large bronchiolar epithelia, comparable to the histopathology of non-depleted calves infected with BRSV (297).

Unlike humans and rodents, bovine T lymphocytes are not readily polarized in naturally occurring infections in which Th0 lymphocytes co-express IFN-γ and IL-4 (30, 31). However, a few infectious agents and in vitro experiments with various cytokine-induced T lymphocyte polarization were associated with distinctive antibody isotypes (30, 99). In vitro studies suggested that Th1- and Th2- mediated immune responses up-regulate IgG2 and IgG1 antibodies, respectively (99). In addition, bovine IFN-γ was shown to preferentially induce IgG2 over IgG1, whereas bovine IL-4 and IL-13 strongly induced IgG1 and IgE (98, 99, 101). In cattle chronically infected with Fasciola hepatica, serum IgG1 antibody levels were consistently enhanced, while much lower levels of serum IgG2 antibodies were detected (58). At the same time, peripheral blood lymphocytes failed to produce IFN-γ in response to F. hepatica. Therefore, it was suggested that chronic infection by F. hepatica induced predominantly Th2 mediated immune responses in cattle. In contrast, cattle immunized with Babesia bigemina rohoptry-associated protein (RAP) 1 and B. bovis genomic DNA showed Th1 mediated immune responses characterized by predominantly induced IFN-γ, low levels of IL-4, and preferential IgG2 over IgG1 antibody production (29).

However, it was suggested that the T lymphocyte subsets might change with extended time periods (242). The PBMCs from cattle were activated in vitro with
soluble *Onchocerca* extract in the onchocerciasis model or bovine tuberculin purified protein derivative in the tuberculosis model. In response to *Onchocerca* extract, IL-4 was predominantly induced over IFN-γ; however, IFN-γ was strongly but transiently induced at certain time points. The cytokine profiles in the tuberculosis model were characterized with strongly sustained IFN-γ through the study periods, and sharply stimulated IL-4 at a certain time point, followed by an undetectable response. It was suggested that IL-4 and IFN-γ play an important role in counter-balancing Th1- and Th2-biased responses in tuberculosis and onchocerciasis, respectively. In bovine immunology, infections may be more efficiently resolved with a balanced immune response.

In most studies of infectious diseases, CD4+ T lymphocytes have been considered helper cells for production of antibody isotypes, maturation of CD8+ T lymphocytes and activation of phagocytes. However, recently, the CD4+ T lymphocytes were investigated from the aspect of cytotoxicity in cattle (50, 126, 278, 323). It was demonstrated that the target cell killing by CD4+ cytotoxic T lymphocytes is mediated through both perforin-granzyme and Fas-pathways inducing apoptosis. However, their major roles in immune responses to infectious agents are still unclear.

### 1.2.3. CD8+ T lymphocytes

CD8+ MHC class I-restricted T lymphocytes in cattle have cytolytic activity and produce IFN-γ like in rodents and humans. In cattle, it was demonstrated that CD8+ T lymphocytes play an important role in controlling virus shedding and histopathology. In BRSV infected calves, the depletion of CD8+ T lymphocyte significantly increased the duration of nasopharyngeal shedding of BRSV. In addition, higher BRSV titers
from lung washings were detected in CD8+ T lymphocyte depleted calves (293). This delayed virus clearance caused by depleting CD8+ T lymphocytes was strongly associated with an increased area of pneumonic consolidation and severe hypertrophy and desquamation of the respiratory epithelium (293, 297). In calves infected with bovine rotavirus, calves depleted of CD8+ T lymphocytes showed increased fecal shedding compared with control calves (216). Likewise, CD8+ T lymphocytes seem to be responsible for limiting primary virus infection. However, it was suggested that CD8+ T lymphocytes are not involved in controlling primary infection of BVDV, based on no significant effect of CD8+ T lymphocyte depletion on BVDV titer in blood and duration of viremia compared with control calves (148).

The CD8+ T lymphocytes are not always associated with controlling primary infection and histopathology. In a study of bovine tuberculosis, it was suggested that the CD8+ T lymphocytes can mediate deleterious immunopathology. Calves depleted of CD8+ T lymphocytes were intratracheally inoculated with *Mycobacterium bovis*, resulting in lower lesion scores in head lymph nodes than in control calves. In addition, IFN-\(\gamma\) produced from whole blood cells was significantly higher in control calves than in CD8+ T lymphocyte-depleted calves, reflecting that decreased IFN-\(\gamma\) production directly or indirectly caused by depletion of CD8+ T lymphocytes is associated with lower pathogenesis (320).

The CD8+ T lymphocytes do not seem to affect antibody responses. Calves depleted of CD8+ T lymphocytes were administered BRSV intranasally and intratracheally, resulting in no significant differences in mean titers of isotype antibodies, i.e., IgG1, IgM and IgA Abs in serum, and IgM and IgA Abs in lung washings (293).
In addition, similar results were generated from calves infected with bovine rotavirus (BRV). The depletion of CD8+ T lymphocytes did not affect BRV-specific IgM and IgA antibody responses in feces and sera compared with control calves (216). In addition, the CD8+ T lymphocytes are not involved in controlling trypanosome infections in cattle. There were no significant differences in local skin reactions (chancre), parasitemia or anemia between CD8+ T lymphocyte depleted and control calves (207, 269).

1.2.4. γδ T lymphocytes

The γδ TCR+ T lymphocytes play a more important role in immune responses of cattle compared with those in rodents and humans. The proportion of γδ TCR+ T lymphocyte is relatively higher in bovine than in human and rodent immune systems (141, 162, 243). The proportion of γδ TCR+ T lymphocytes is age-dependent: accounting 5-17% and 10-40% of PBMCs in adult cattle and young calves, respectively, reflecting that the γδ TCR+ T lymphocyte would be influential in immunity of calves (13, 162, 335). The γδ TCR+ T lymphocytes can be found in various lymphoid tissues; however, large proportions of the γδ TCR+ T lymphocytes have been detected in the lamina propria and gut mucosal surface (151). Especially, the majority of the intraepithelial lymphocyte population was shown to be composed of the γδ TCR+ T lymphocytes which plays an important role in the first line of defense against pathogens (162). For example, in an innate immune response, pattern recognition receptor (PRR) on the γδ TCR+ T lymphocyte, e.g., Dectin-1, CD36, CD11b and NOD2 recognizes pathogen-associated molecule pattern (PAMP) of pathogens, consequently resulting in activation of the γδ TCR+ T lymphocyte (162).
In cattle, there are several phenotypes of $\gamma\delta$ TCR+ T lymphocytes in peripheral blood, but Workshop Cluster 1 (WC1, 215 kDa) expressing $\gamma\delta$ TCR+ T lymphocytes were demonstrated as the major phenotype, whereas $\gamma\delta$ TCR+ T lymphocytes in tissues do not express WC1 antigen (336). It has been demonstrated indirectly that the WC1+ $\gamma\delta$ T cells have a suppressive effect on immune responses (151). Calves depleted of WC1+ $\gamma\delta$ T lymphocytes from peripheral blood using mAbs had significantly enhanced antibody responses compared with non-depleted control calves (149, 151). For example, calves depleted of WC1+ $\gamma\delta$ T lymphocytes showed significantly enhanced IgM and IgA antibody responses to BRSV (293). In addition, proliferation of PBMCs in response to pokeweed mitogen was significantly increased in the depleted calves (149, 151).

The WC1+ $\gamma\delta$ T lymphocytes also play an important role in presenting antigens to CD4+ T lymphocytes and cytolytic activity like NK cells. Most $\gamma\delta$ TCR+ T lymphocytes express MHC class II as well as B7 family members i.e., CD80 and CD86, characteristic antigens normally expressed on activated antigen presenting cells (APCs) (60). The WC1+ $\gamma\delta$ T lymphocytes pulsed with either BRSV or ovalbumin (OVA) antigens enhanced the proliferation of resting memory CD4+ T lymphocytes isolated from calves immunized with BRSV or OVA (60). In addition, clathrin-dependent endocytosis was shown to be the major way of antigen uptake in $\gamma\delta$ TCR+ T lymphocytes (60). In alymphocytic cattle infected with bovine leukemia virus (BLV), WC1+ $\gamma\delta$, but not CD8+ T lymphocytes showed cytotoxic activity against BLV envelopes in which the specific type of major histocompatibility complex (MHC) was not required (187). The non-MHC-restricted NK cell-like cytotoxicity was also demonstrated in WC1+ $\gamma\delta$ T lymphocytes isolated from parasite infected cattle (28, 75). In addition, cytokine
production by activated WC1+ γδ T lymphocytes has been reported: antigen-stimulated WC1+ γδ T lymphocytes expressed high levels of TNF-α, IL-12 and IFN-γ, but not TNF-β, IL-2, IL-4, IL-6, IL-7 IL-10 (28, 105).

In the histological study of skin delayed-type hypersensitivity (DTH) responses, WC1+ γδ T cells were the first lymphocyte localized in early tuberculous lesions and granulomas in calves, indicating that WC1+ γδ T lymphocytes are involved in the early stages of infection and recruiting effector cells to infection sites (233). In addition, it was suggested that WC1+ γδ T lymphocytes express more L-selectin and ligand for E-selectin compared with αβ T lymphocytes, which probably contributes to rapid migration of WC1+ γδ T lymphocytes in early stage of infection (233). In the study of immune responses to bovine tuberculosis, WC1+ γδ T cells were shown to affect T cell polarization in calves infected with Mycobacterium bovis (149). The WC1+ γδ T cell depleted calves using anti-WC1 mAb showed Th2-biased immune responses to M. bovis, characterized by decreased IFN-γ and IgG2 Ab production as well as increased IL-4 (149).

However, it was also demonstrated that WC1+ γδ T lymphocytes in cattle are not major effector cells to control primary infection and pathology (148, 149, 293). For example, virus excretion between WC1+ γδ T lymphocyte depleted and control calves did not differ significantly (216). In response to BRSV, no distinctive pulmonary lesions were observed in WC1+ γδ T lymphocyte depleted calves, whereas CD8+ depleted lymphocyte calves showed greatly enhanced pulmonary lesions (297).
1.2.5. Dendritic cells

Dendritic cells (DCs) play an important role in linking innate and adaptive immunity in response to infectious agents and vaccination. In addition, cytokines and surface molecules expressed by DCs have a significant effect on differentiation of T lymphocytes, i.e., Th1 versus Th2 differentiation. Phenotypically and functionally diverse DCs have been identified, particularly in afferent lymph of cattle. All DCs in the afferent lymph express DEC-205 (CD 205), an endocytic receptor belonging to a family of C-type multilectins (117). The DEC-205 expressing DCs have subpopulations according to the expression of mannose receptor or SIRPα (117). The two major subpopulations of DEC-205+ DCs, i.e., DEC-205+SIRPα+ and DEC-205+SIRPα- have distinctive cytokine secretion, resulting in different functions of T lymphocytes. After, presentation of antigen, the DEC-205+SIRPα+ DCs were shown to produce more IL-1α and IL-10, whereas the DEC-205+SIRPα- DCs preferentially express IL-12 and IFN-γ. The cytokine repertoires from the two subpopulations of DCs play an important role in guiding host immune responses to be inflammatory or anti-inflammatory. Therefore, the IL-12 and IFN-γ produced from DEC-205+SIRPα- DCs induce predominantly Th1-mediated immune responses. In contrast, IL-10 produced from DEC-205+SIRPα+ DCs down-regulate the Th-1 mediated immune response, resulting in an anti-inflammatory response. In addition, it was demonstrated that IL-1α produced from DEC-205+SIRPα+ DCs contributes to stimulation of CD8+ T lymphocytes, whereas DEC-205+SIRPα- DCs were shown to stimulate CD8+ T lymphocytes very poorly because of no IL-1α production.
Besides cytokines, antigens expressed on the surface of DCs have been shown to be involved in stimulating biased immune responses. The CD26 was shown to be an exopeptidase which truncates the N-terminal amino acid residues from macrophage-derived chemokine (MDC), resulting in termination of function of MDC (118). The MDC, also known as CCL22, secreted by DCs and macrophages, plays an important role in attracting CCR4 expressing cells, e.g., Th2 lymphocytes. Thus, it was suggested that CD26+DCs preferentially induce Th1- over Th2-mediated immune responses because CD26 abrogates MDC biological activity (118).

Different surface phenotypes of DCs derived from afferent lymph, spleen, and peripheral blood were investigated (346). Between splenic and peripheral blood DCs, CD13 was shown to be the most distinctive surface phenotype; high expression levels of CD13 were detected on splenic DCs (346). After maturation in vitro, surface antigens were more differentially expressed between splenic and peripheral blood DCs; the expression level of CD11a and CD11c was higher in splenic than in peripheral blood DCs, whereas that of CD11b was higher in peripheral blood than in splenic DCs (346). Compared with splenic and peripheral blood DCs, most differential surface markers of afferent lymph DCs were CD1b and CD45RO; splenic and peripheral blood DCs did not express CD1b and CD45RO (147, 150, 346). Within afferent lymph DCs, besides SIRPα, two other antigens, i.e., CD11a and CD13 were demonstrated as important surface markers in two major subpopulations: SIRPα+CD26-CD11a-CD13- and SIRPα-CD26+CD11a+CD13+ (346). In addition, it was demonstrated that afferent lymph DCs are a more mature phenotype compared with splenic and peripheral blood DCs according to the expression level of CD80 and CD86 (346).
It was suggested that bovine DCs directly stimulate B lymphocytes without T lymphocytes, resulting in antibody class switching (14). In the presence of the DCs, bovine B lymphocytes co-cultured with IL-2 up-regulated IgM and IgG1 antibodies without increased IgG2 and IgA antibodies. The transcriptional levels of cytokines in the DCs were also investigated: mixed cytokine profiles including Th2 cytokines, i.e., IL-6, IL-10, IL-4 and IL-13 were increased compared with the control group. In addition, lower levels of CD40L were expressed on the surface of bovine DCs, suggesting that the antibody class switching may be promoted by interaction of CD40 on B lymphocytes with CD40L on peripheral blood derived DCs.

1.2.6. Macrophages

Macrophages are responsible for antigen specific- and non-specific immunity: non-specific phagocytic activity for cell debris and foreign antigens results in innate immunity and activation of lymphocytes in response to pathogens results in adaptive immunity. Bovine macrophages have been extensively studied for Mycobacterium infection, because the macrophage is considered the major replication site of the bacteria. Bovine macrophages have different immune responses to a variety of infectious agents (267, 326). Genomic DNA from bacteria and parasites was shown to stimulate macrophages, resulting in production of proinflammatory cytokines and nitric oxide (NO). However, the amount of the cytokines and NO were dependent upon the pathogen (267). Bovine macrophages were activated by DNA from Escherichia coli, Babesia bovis, Trypanosoma cruzi and T. brucei, resulting in increased IL-12, TNF-α and NO from the macrophages. The intensity of the macrophage stimulation was ranked in the order of E.
coli ≥ T. cruzi > T. brucei > B. bovis. Interestingly, the different amount of macrophage stimulation was strongly correlated with the frequency of CpG dinucleotides in genomic DNA: E. coli (7.7%) > T. cruzi (4.6%) > T. brucei (3.9%) > B. bovis (3.4%), suggesting that toll-like receptor 9 (recognizes unmethylated CpG dinucleotides) mediated-macrophage activation is dependent upon the proportion of CpG in genomic DNA.

In addition, bovine macrophages were differently stimulated by different bacterial strains (326). Bovine macrophages were infected with three different Mycobacterium avium subspecies paratuberculosis strains isolated from cattle, sheep and humans. Bovine macrophages infected with bovine M. avium subspecies paratuberculosis up-regulated IL-10, and down-regulated TNF-α, indicating an anti-inflammatory response. Compared with bovine M. avium subspecies paratuberculosis, human and sheep M. avium subspecies paratuberculosis down-regulated IL-10, and up-regulated TNF-α, leading to a pro-inflammatory response. According to gene expression levels analyzed by RT-PCR, bovine macrophages infected with M. avium subspecies paratuberculosis had enhanced expression of TGF-β and failed to up-regulate MHC class II molecules (326). Therefore, it seems that bovine M. avium subspecies paratuberculosis is well-adapted to bovine macrophages resulting in up-regulated anti-inflammatory and down-regulated pro-inflammatory cytokines as well as interference with antigen-presentation capacity.

The differential effects on bovine macrophages were also investigated with two different subspecies of Mycobacterium (326). Twenty-seven genes were differentially expressed between M. avium subspecies paratuberculosis- and M. avium subspecies avium-infected bovine macrophages. The two subsets of Mycobacterium spp.-
infected macrophages did not differ significantly in their ability to ingest bacteria. However, bovine macrophages infected with *M. avium* subspecies *paratuberculosis* showed a lower degree of killing, phagosome acidification and apoptosis, compared with those infected with *M. avium* subspecies *avium*. The *M. avium* subspecies *paratuberculosis* and *M. avium* subspecies *avium* are similar genetically and phenotypically; however, *M. avium* subspecies *paratuberculosis* and *M. avium* subspecies *avium* cause chronic and transient infection, respectively in cattle. In summary, the different gene expression profiles between *M. avium* subspecies *paratuberculosis*- and *M. avium* subspecies *avium*-infected bovine macrophages may alter the subsequent immune responses, resulting in different disease patterns.

1.2.7. Natural killer cells

Natural killer (NK) cells are one type of cytotoxic lymphocyte that play an important role in lysing tumor cells, virally infected cells, and MHC disparate cells. Activation and inhibitory receptors on NK cells play an important role in recognizing abnormal cells and regulating NK cell cytotoxicity. Many viruses suppress MHC class I expression, considered one strategy to evade host immunity. In addition, metastatic tumor cells do not express MHC class I. Unlike CD8+ T lymphocytes, MHC class I-antigen complex was shown to present activating as well as inhibitory signals to NK cells through receptors, e.g., the Ly49 receptor family (C-type lectins) in mice and the killer cell immunoglobulin-like receptor (KIR) family in humans (176). Inhibitory receptors have an immunoreceptor tyrosine-based inhibition motif (ITIM) in the cytoplasmic domain where tyrosine phosphorylation recruits the inhibitory protein tyrosine
phosphatase SHP-1, resulting in inhibition of NK cell effector mechanisms (176). In contrast, activation receptors have an immunoreceptor tyrosin-based activation motifs (ITAM) in the cytoplasmic domain which binds to Syk or ZAP70, resulting in activation of NK cells. In addition, the NK cell activation receptors contain a charged amino acid, e.g., lysine or arginine in the transmembrane domains (176).

In cattle, Ly49, four members of KIR (CD158), killer cell lectin-like receptor (KLR, CD94) and NKp46 have been identified as receptors expressed on NK cells (197, 284). The predicted proteins of Ly49 gene isolated from cattle contain ITIM like the inhibitory Ly49 receptors in mice (284). Especially, among the four members of KIR, two bovine KIRs, i.e., KIR2DL1 and KIR3DL1 contain ITIM, suggesting an inhibitory function, whereas the other two members, i.e., KIR2DS1 and KIR3DS1 lack ITIM, but contain an arginine motif in transmembrane domains, suggesting an activating function (197).

The CD94 expressed on NK cells is the MHC receptor which recognizes any disrupted MHC synthesis regardless of MHC genetic diversification (23). In cattle, it was reported that MHC class I-like gene family A1 (MHCLA1), a nonclassical MHC class I, does not seem to interact with α3 domain and is normally expressed on virus-infected cells (177). Although the MHCLA1 was shown by phylogenetic analysis to be related to NK cell stimulatory ligands, the MHCLA1 as a ligand for CD94 has not yet been demonstrated. Effector mechanisms for interaction of CD94 with MHCLA1 can be extrapolated from human and rodent studies; if the MHCLA1 is associated with the CD94 expressed on NK cells, the inhibitory signal from MHC class I-antigen complex is subdued, resulting in activation of NK cells to lyse the target cells.
The NKp46 (CD335) has been shown to be a pan-NK cell surface marker in humans and rodents as well as cattle. The NKp46 in cattle was demonstrated to be an activation receptor (283). Giemsa-stained cytospin preparations of NKp46+ cell populations cultured with IL-2 showed acidophilic granules in one side of the cell. In addition, preincubation of the NKp46+ cell population with anti-NKp46 mAb induced efficient lysis, suggesting that NKp46+ cells contain characteristic effector mechanisms of NK cells (283).

In addition, it was demonstrated in human and rodent studies that CD16 expressed on NK cells is a FcγRIII, involved in antibody-dependent cellular cytotoxicity (ADCC), resulting in high expression of IFN-γ, IL-2αR (CD25) and TNF (8, 39, 176). In cattle, CD16 expressing NK cells contain intracellular perforin and have NKp46-mediated cytotoxicity (27). In addition, the CD16 expressing NK cells cultured with IL-2 and IL-12 showed intracellular and secreted IFN-γ (27). It was reported in human and rodent studies that upon inflammatory stimuli, NK cells are recruited to lymph nodes and produce IFN-γ, resulting in Th1 polarization (191). In response to DC isolated from neonatal calves subcutaneously immunized with Mycobacterium bovis bacillus Calmette-Guerin (BCG), CD3-CD8+ NK cells showed a significant proliferative response and were the major population for IFN-γ production (145). In this study, it was suggested that the CD3-CD8+ NK cells play an important role in developing Th1 immune response to Mycobacterium or vaccination (145).
1.3. **Bovine coronavirus**

1.3.1. **Taxonomy**

In 1968, a previously unrecognized virus was identified by electron microscopy (EM). It was characterized by a pleiomorphic shape ranging from 80–160 nm in diameter; a double layer of petal shaped fringe projections including a surface hemagglutinin, a lipid membrane, an RNA genome and replication in the cytoplasm (5). Because of their characteristic appearance recalling a solar corona, the name coronavirus (CoV) was proposed and accepted by the international committee on taxonomy of viruses (ICTV) (5). The CoV is a member of the family *Coronaviridae* in the order *Nidovirales*, characterized by an envelope and single stranded positive sense RNA genome (122). The order *Nidovirales* has three families; the *Coronaviridae* which includes the two genera, *coronavirus* and *torovirus*, the *Arteriviridae* which includes the genus *arterivirus*, and the *Roniviridae* which includes the genus *okavirus*. The CoVs are classified into three established groups with two subgroups for group 1 (1a and 1b) and 2 (2a and 2b) on the basis of serological cross-reactivity and genome sequence analysis (46, 90, 119, 123, 170, 183, 327). Group 1a CoV includes transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), canine enteric coronavirus (CECoV), feline enteric coronavirus (FECoV) and feline infectious peritonitis virus (FIPV). Group 1b CoV includes porcine epidemic diarrhea virus (PEDV), Bat (*Miniopterus*) CoV, human CoV (HCoV)-229E and HCoV-NL63. Group 2a coronavirus includes bovine coronavirus (BCoV), mouse hepatitis virus (MHV), canine respiratory coronavirus (CRCoV), hemagglutinating encephalomyelitis virus (HEV), wild ruminant CoVs, human CoVs, HCoV-OC43 and HCoV-HKU1. Group 2b coronavirus includes severe acute respiratory
syndrome (SARS) CoV, civet cat (*Paguma larvata*) CoV and raccoon dog (*Nyctereutes procyonoides*) CoV, horseshoe bat (*Rhinolophus sinicus*) CoV. Group 3 coronavirus includes avian CoVs, infectious bronchitis virus (IBV) and turkey coronavirus (TCoV).

In 1972, the BCoV was first detected from a case of neonatal calf diarrhea by electron microscopy (277). The BCoV is a pneumoenteric virus which has two tissue tropisms for replication: the epithelium in the intestinal and respiratory tract. Infection by BCoV results in calf diarrhea (CD), winter dysentery (WD) in adult cattle and respiratory disease in neonatal calves and adult cattle (48, 249, 253, 256). Comparative studies of BCoVs isolated from the intestinal (BECV) and respiratory (BRCV) tracts have focused on biological and antigenic properties as well as nucleotide sequence (51, 135-137, 304). The majority of BRCV were similar to the BECV in terms of hemagglutination (HA), receptor-destroying enzyme activity (RDE) and hemagglutination inhibition (HI). However, antigenic variation was detected between BECV and BRCV using mAbs specific for the hemagglutinin esterase (HE) and spike (S) glycoproteins (135, 136). Using phylogenetic analysis based on the amino acid sequence of the S glycoprotein, BECV and BRCV were classified into two major groups (Groups I and II) with two subgroups (Groups Ia and IIa) (137). Compared with the prototype Mebus strain, BCoV strains in each group showed amino acid substitutions, resulting in predicted changes in secondary structure. Groups I and II were differentiated by an amino acid substitution at amino acid residue 118 in which Group II BCoV strains substituted Lysine for Methionine, whereas Group II did not differ from the prototype BCoV Mebus strain. Group I BCoV strains showed seven amino acid substitutions, amino acid residues 113, 115, 146, 148, 501, 510 and 531, whereas Group Ia BCoV strains had five amino acid
substitutions, amino acid residues 113, 115, 146, 148 and 501. Groups II and IIa BCoV strains showed six and four amino acid substitutions, amino acid residues 113, 115, 118, 501, 510 and 531 in group II, and 113, 115 118 and 501 in group IIa BCoV strains. Group II BCoV strains substituted asparagine for lysine, whereas group IIa BCoV strains, lysine was replaced by tyrosine at amino acid residue 115. Groups II and IIa BCoV strains substituted Serine and Phenylalanine for Proline, respectively at amino acid residue 501. In addition, only a few amino acid differences between enteric and respiratory isolates from the same calves were observed in group I BCoV strains (137).

In addition, the BECV and BRCV were isolated from intestinal and respiratory samples respectively of the same cattle with fatal pneumonia (51). The comparative study of their predictive amino acids revealed that most amino acid changes were detected in the pp1a as well as the S glycoprotein.

It was also suggested that BECV can be classified into three groups based upon the HA and RDE (304). Group I BECVs showed low ratios of HA titer with mouse erythrocytes compared to chicken erythrocytes (M/C HA titer ratio), no differences in HA titers against chicken erythrocytes at 4°C and 37°C, and no RDE activity against chicken erythrocytes. Group II BECVs were characterized by low M/C HA titer ratios, no HA against chicken erythrocytes, and RDE activity with chicken erythrocytes. Group III BECVs have high M/C HA titer ratios, no HA against chicken erythrocytes at 37°C and RDE activity with chicken erythrocytes. However, the BECV classification based upon HA and RDE could not explain geographically or clinically different strains, i.e., CD or WD.
1.3.2. Morphology

Spike (S) glycoproteins, small envelope (E) glycoproteins, membrane (M) glycoproteins, nucleocapside (N) phosphoproteins, and haemagglutinin-esterase (HE) glycoproteins structurally contributes to BCoV, resulting in a pleiomorphic appearance with diameters ranging from 80 to 160nm (57). The S and HE glycoproteins are long and short surface projections, respectively, from the BCoV envelope. The S and HE glycoproteins were described as mushroom-like surface projections with a 13nm diameter cap and a 14nm long stalk and fringe-like surface projections, respectively (244). In addition, by EM, BCoV has been observed without S and/or HE glycoproteins during sample storage or preparation (57). The N phosphoproteins are localized internally and interact with the genomic RNA. The genomic RNA associated with N phosphoproteins is enclosed within the virus envelope. The M and E glycoproteins are located in the virus envelope and are typically derived from portions of host plasma membrane.

1.3.3. Genome

The BCoV genome is composed of a non-segmented, positive sense, single-stranded (ss) RNA (129). The genomic RNA shows typical characteristics of eukaryotic mRNA; a 5’ cap and 3’ polyadenylation. The size of the genomic RNA is approximately 27-30 kb, the largest size among RNA viruses currently known. The molecular weight of BCoV genomic RNA was estimated to be $3.8 \times 10^6$, less than that of infectious bronchitis virus (IBV) or mouse hepatitis virus (MHV). In addition, the genomic BCoV RNA was analyzed after being denatured. Unlike porcine and human CoV, no low molecular weight RNAs such as 4S to 7S RNA species were observed. It was reported that genes
encoding structural and non-structural proteins (NSP) are arranged as follows; 5′-UTR (nucleotide [nt] 1–204), ORF1a (nt 205–13,356), ORF1b (nt 13,335–21,488), 32 kDa NSP (nt 21,498–22,334), HE (nt 22,346–23,620), S (nt 23,635–27,726), 4.9 kDa NSP (nt 27,716–27,805), 4.8 kDa NSP (nt 27,883–28,020), 12.7 kDa NSP (nt 28,100–28,429), E (nt 28,416–28,670), M (nt 28,685–29,377), N (nt 29,387–30,733), and 3′-UTR (nt 30,734–30,894) (Figure 1.1) (340). The HE gene was detected in mainly group 2a CoVs, i.e., BCoV, MHV, HEV and HCoV-OC43 (181).

1.3.4. Viral proteins

There are five major BCoV structural proteins, i.e., S glycoprotein, HE glycoprotein, M glycoprotein, E glycoprotein and N phosphoprotein (Figure 1.1). The S glycoprotein is a large petal-shaped protein that protrudes from the BCoV envelope. The S glycoprotein has a size of 180kDa when it is fully glycosylated (223). In polyacrylamide gel electrophoresis (PAGE), the S glycoprotein shows two cleavage products of 90 and 110 kDa. The amino acids 764-768 of the S glycoprotein were identified as a cleavage site, resulting in S1 and S2 subunits. The two subunits have distinct functional domains near the N-terminus in the S1 and C-terminus in the S2 subunit. The S1 subunit is highly immunogenic and induces strongly neutralizing antibodies (137, 250). In addition, the S1 subunit is responsible for binding to receptors containing N-acetyl-9-O-acetyleneuraminic acid (Neu 5, 9Ac2) during initiation of infection as well as agglutination. It was reported that the S glycoprotein is a more potent haemagglutinin than the HE glycoprotein (264). The S2 subunit is a transmembrane protein composed of hydrophobic α-helices which mediate fusion between the viral
envelope and host cellular membrane (250). In addition, it was demonstrated that during replication of BCoV, expression of the S2 subunit can result in the fusion of neighbouring cells, resulting in the formation of multi-nucleate cells or syncytia (337). Likewise, proteolysis for cleavage of the S glycoprotein is required for virus entry into host cells and cytopathic effects. The variation in tissue tropism of coronaviruses is largely attributed to variations of the S glycoprotein. The amino acid sequences of S glycoproteins between respiratory and intestinal BCoV isolates from cattle with fatal pneumonia were investigated. Eight amino acids differed between the two different isolates; 4 amino acids in each S1 and S2 subunit (51).

The HE glycoprotein is a disulfide linked dimer of approximately 120-140 kDa (168, 222). Pretreatment of the glycoprotein with 2-mercaptoethanol generates two identical monomers of 65 kDa. This HE glycoprotein is unique to group 2a CoVs including BCoV, because many coronaviruses do not have functional HE genes. The S and HE glycoproteins share common features since they recognize the same receptor-determinant, Neu 5, 9Ac2. They contain neutralizing epitopes and induce neutralizing antibodies (235, 250, 264). However, it was demonstrated with chimeric viruses that the HE glycoproteins of BCoV are not necessary to initiate infection in human rectal tumor (HRT)-18 cells (235). Mouse hepatitis viruses (MHV) expressing BCoV S or HE glycoproteins were used to infect the HRT-18 cells. The BCoV S glycoprotein expressing chimeric virus entered and replicated in the HRT-18 cells, whereas the BCoV HE glycoprotein expressing chimeric virus could not infect the HRT-18 cells. The HE glycoprotein also has esterase activity which removes acetyl groups from the Neu 5, 9Ac2, resulting in receptor-destroying enzyme (RDE) activity (222). In addition, it was
suggested that the RDE activity of the HE glycoprotein contributes to virus entry into host cells (321). Interestingly, the HE glycoprotein mediated receptor binding and destroying activity can also be detected in influenza C virus. Therefore, it was assumed that coronavirus acquired the HE gene from the influenza C virus by a non-homologous recombination event (142, 342).

The M glycoprotein is an integral membrane glycoprotein of approximately 24-26 kDa, and has three distinct domains (168). The hydrophilic N-terminal domains are glycosylated and exposed outside of the virus envelope. The hydrophobic central domain is an α-helical structure and passes through the viral envelope three times. The C-terminal domain is located internally and interacts with the N phosphoprotein. It was demonstrated that the M glycoprotein retains the other glycoproteins, i.e., the S and HE glycoproteins at internal membranes and mainly contributes to the BCoV assembly (212, 213). In addition, the M glycoprotein was shown to be a potent inducer of interferon alpha (IFN-α) (19, 20). The BCoV M glycoprotein expressed-pseudoparticles were generated using a recombinant expression system in rabbit kidney epithelial cells, and incubated with peripheral blood mononuclear cells, resulting in induction of IFN-α. In addition, the M glycoprotein was the least immunogenic structural protein in BRCV infected cattle (179).

The E glycoprotein is an integral membrane glycoprotein of approximately 9-12 kDa (119, 189, 266, 307). It was suggested that this small membrane protein plays an important role in BCoV assembly as well as IFN-α generation from peripheral blood mononuclear cells incubated with the M glycoprotein (19, 20).

Unlike the HE, S, E and M glycoproteins, the N proteins are phosphorylated and
located internally (168). The molecular weight of the N phosphoprotein is approximately 50-52 kDa. The N phosphoprotein is associated with viral RNA, resulting in a long, flexible and helical nucleocapsid (250). Isotopic labeling of viral polypeptides demonstrated that the molar ratio of the N phosphoprotein and M glycoprotein is almost one in cultured BCoV, and the N phosphoprotein directly interacts with the M glycoprotein (168). The N phosphoprotein efficiently bound the leader sequence and 434 nucleotides at the 3’ end of the N ORF in BCoV-defective interfering RNA, resulting in formation of the helical nucleocapsid (62). In addition, it was suggested that the N phosphoprotein binds the 291 nucleotides within ORF 1b, and packaging signal containing RNA transcripts, which leads to packaging genomic as well as subgenomic BCoV RNA (61).

Besides structural proteins, the non-structural proteins of BCoV also have been investigated (Figure 1.1) (51). Approximately two-thirds of genomic BCoV RNA is the open reading frame 1 (ORF1) composed of two overlapped ORFs, i.e., ORF1a and ORF1b. The ORF1a encodes a polyprotein (pp)1a, and the ORF1a and ORF1b together encode pp1ab. The ORFs include the slippery sequence UUUAAAC located immediately upstream of a stem-loop structure and near the ORF1a/b junction, resulting in – 1 frameshift (51). The domains in the pp1a and pp1ab have been identified; aspartic or glutamic acid residue domain (Ac), papain-like proteases 1 (PL1), conserved domain of unknown function (X), PL2, transmembrane domain 1 (TM1), TM2, poliovirus 3C-like protease (3CL\textsuperscript{pro}) and TM3 in the pp1a, and RNA dependent RNA polymerase (RdRp), zinc finger domain (ZD), RNA helicase (HEL) and conserved sequence domain in nidoviruses (ND) in the pp1ab. The replication and transcription of CoVs are mediated
by the viral replicase including RdRp, HEL and protease. The PL and 3CLpro have proteolytic activity for polyproteins, resulting in release of the RdRp and helicase. The RNA helicase plays an important role in unwinding double-stranded (ds) RNA, and moves in a 5’ to 3’ direction. In addition, it was suggested that the RNA helicase mediates RNA 5’-triphosphatase activity which may be involved in capping the 5’CoV RNAs (156). Upon entry and uncoating, the RdRp is translated, followed by synthesis of the full-length antigenomic RNA. On the basis of the antigenomic RNA, the RdRp synthesizes the genomic RNA or transcribes subgenomic mRNAs, directly used to translate structural proteins (165).

It was also reported that four other nonstructural proteins (NSPs), not necessary to replicate were encoded in the BCoV genome: 32kDa, 4.9kDa, 4.8kDa and 12.7kDa NSP (1, 25, 69, 132). The genes encoding 4.9kDa, 4.8kDa and 12.7kDa NSP are located between the S and E gene (1). The consensus sequence, CYAAAC is detected before the ORFs encoding the NSPs, suggesting that the consensus sequence is involved in the position for transcription start. In addition, the gene encoding the 32kDa NSP is located immediately upstream from the HE gene, and the 32kDa protein was demonstrated to be a phosphorylated nonstructural protein in BCoV infected cells (68, 69).

1.3.5. Morphogenesis

The BCoV binds to the Neu 5, 9Ac2 containing receptors via the S and HE glycoproteins (264). However, it was reported that the HE glycoproteins are not necessary to initiate infection in HRT-18 cells (235). It was suggested that rather than receptor binding activity, the RDE activity of the HE glycoprotein contributes to the virus
entry into host cells (321). Proteolytic cleavage of the S glycoprotein to generate S2 subunits is critically important, because the S2 subunit-mediated cell fusion between virus envelope and plasma membrane of target cells initiates virus entry into the host (229, 230, 288, 337). Unfortunately, little is known about the mechanism of the BCoV uncoating and the genomic BCoV RNA release. The uncoated BCoV replicates in the cytoplasm of target cells. Once the genomic RNA is released into the cytoplasm, host ribosomes synthesize the RdRp by using the genomic RNA as mRNA. The RdRp replicates the complement full length negative sense RNA from the genomic RNA. The negative sense RNA plays a role as a template for seven subgenomic mRNAs as well as for the full length genomic RNA (165). A kinetic study demonstrated that the synthesis of genomic RNA and subgenomic mRNA are regulated differently (165). In the early infection phase, at 4 to 8 hours post infection, the subgenomic mRNA was shown to be maximally synthesized i.e., greater than 90% of the total BCoV RNA, resulting in the peak of BCoV protein synthesis. However, at the same time point, only 7% of the total RNA was the genomic RNA. In the later infection phase, at 70 to 72 hours post infection, the proportion of genomic RNA increased markedly and accounted for 88% of the total RNA, concurrent with the production of viral particles (165). Each subgenomic mRNA contains a single or several open reading frame (ORFs); however, only the 5’ORF in each subgenomic mRNA is translated. The NSPs and N phosphoproteins are synthesized by free ribosomes in the cytoplasm of target cells, whereas the other structural proteins, HE, S, E and M are synthesized by ribosomes at the rough endoplasmic reticulum (RER). After translation, the M and E membrane proteins are localized at the RER and Golgi apparatus which are normally considered the actual budding sites (182). The newly
synthesized genomic RNA complexes with N phosphoproteins to generate helical structures of nucleocapsid. The C-terminal domain of the M glycoprotein interacts with the N phosphoprotein. Thus, the nucleocapsids are arrayed on the RER and Golgi apparatus and budding into vesicles occurs. The vesicles containing BCoV are transported to the plasma membrane and released into the lumen.

1.3.6. Pathogenesis

The BCoV has been shown to be a causative agent of neonatal calf diarrhea (NCD), winter dysentery (WD) in adults and respiratory tract disease including shipping fever in calves and adult cattle (57). The BCoV replicates in the epithelium of the intestinal and respiratory tract and is transmitted in cattle through the fecal, oronasal and respiratory routes. Neonatal calf diarrhea caused by the BCoV is common in calves less than 3 weeks of age. Major clinical signs are profuse watery or hemorrhagic diarrhea, resulting in high morbidity and mortality. Diarrhea normally develops approximately 2-3 days after experimental infection and lasts for 3-6 days. The BCoV can be detected in feces during the diarrhea period or for a couple of days after the diarrhea is resolved (251, 257). During acute infection, calves are often listless, anorexic, pyretic and dehydrated. Epithelial cells in both the small intestine and colon are the major replication site of BCoV which causes villous atrophy followed by migration of immature epithelium on villi. At the same time, the number of goblet cells decrease and mucin production is reduced. Consequently, the change in intestinal architecture leads to the loss of the absorptive capacity in the gut. Secretory function in the immature epithelium causes increased fluid secretion in the gut lumen. In addition, the immature epithelium cannot
produce digestive enzymes such as maltase, sucrase, lactase, dipeptidase, etc. Thus, the undigested nutrients accumulated in the gut lumen give rise to increased microbial activity and an osmotic imbalance-mediated diarrhea. After severe BCoV infection of the intestinal tract, diarrhea may cause dehydration, acidosis and hypoglycemia, resulting in high mortality. However, BCoV infection was shown to be self-limiting in well-nourished calves. The intestinal crypt epithelium can regenerate replacement villous epithelium that eventually regains normal function.

The BCoV also causes a respiratory disease syndrome in calves and adult cattle. The respiratory infection may be mild or even subclinical, although pneumonia is also reported (51, 179, 196, 285, 286). Clinical signs are usually detected in calves between 2 and 16 weeks of age. The primary replication sites are the epithelium of the nasal cavity and trachea, resulting in mild upper respiratory symptoms, e.g., sneezing from rhinitis and coughing from tracheitis. In addition, the lower respiratory tract can be infected with BCoV, which leads to lung lesions. However, BCoV has also been shown to be involved in more severe lower respiratory disease and it can predispose calves to secondary bacterial infections. For example, the BCoV was recently recognized as one of the causative agents of the bovine respiratory disease complex (BRDC) (51, 179, 285, 286). The BCoV was isolated from nasal samples and lung tissues of cattle with shipping fever pneumonia. In addition, respiratory tract infections with BCoV, followed by secondary infections with Pasteurella spp. were observed in natural outbreaks of shipping fever (286). Under field conditions, BCoV can be isolated simultaneously from both the intestinal and respiratory tracts of infected calves, indicating that major infection routes are fecal-oral as well as aerosol-nasal transmission. It was suggested that the upper
respiratory tract plays an important role in initial replication and migration into the gut. As BCoV replicates in the upper respiratory tract, mucous production by goblet cells increases, and BCoV nasal shedding occurs. However, substantial amounts of BCoV coated with mucous which may act as a protective shield, may enable survival of BCoV under the low pH in the stomach and its transport to and replication in the intestinal tract (137, 296).

During the winter season, i.e., from November to March, acute diarrhea outbreaks in adult cattle have been reported (93, 210, 253, 256). This disease is referred to as winter dysentery (WD), and is characterized by hemorrhagic diarrhea, respiratory signs, listlessness and reduced milk production (85, 158, 300). The precise etiology of WD is unknown. However, it was reported that WD outbreaks are closely associated with BCoV infection (93, 253, 271). The crypt epithelium in the large intestine of WD-affected cattle showed degeneration, necrosis, and desquamation as well as BCoV antigens (210). In addition, cows without serum BCoV Ab had severe anemia due to the massive hemorrhage in the intestinal tract (210).

In addition, WD was experimentally reproduced in seronegative adult dairy cows (301, 306). In one study, seronegative lactating adult dairy cows were kept with a transmitter calf (source of BCoV transmission to cows) inoculated oronasally with BCoV-containing feces collected from cows affected by WD (301). The direct nose contact between cows and the transmitter calf or its feces gave rise to profuse watery diarrhea with small amounts of blood and sweet stinking smell in the affected cows, characteristic for WD. Moreover, markedly decreased milk production was detected; the range of milk production in the affected cows was 19-56% of the milk production before
the BCoV infection (301). In another study, seronegative adult dairy cows were also inoculated with BCoV strains (DBA strain of WD, and DB2 and 216XF strain of NCD) through a surgically-placed duodenal catheter (306). In this experiment, WD was reproduced in two dairy cows inoculated with NCD BCoV strains and in four dairy cows inoculated with WD BCoV strain, characterized by black and semi-liquid BCoV-associated diarrheal feces and BCoV fecal shedding. Unlike the experiment using the transmitter calf and lactating cows, no bloody diarrhea was observed in the inoculated non-lactating cows (306).

The BCoV infection is not always associated with WD, because subclinical BCoV shedding was detected from healthy adult cattle (59). Therefore, it was suggested that environmental risk-factors seem to exacerbate the BCoV infection and clinical signs: winter season, last gestation period, housing cattle in tie stall or stanchion barns, same equipment used to handle manure and feed, and high density herd (59, 158, 271, 329).

1.3.7. Zoonotic potential

Evidence for the zoonotic potential of BCoV has emerged. It was reported that humans can be exposed to BCoV when they cared for diarrheic calves inoculated orally with the BCoV, resulting in high neutralizing antibody titers against enteropathogenic BCoV in human sera (287). The HECV-4408 strain isolated from the stools of a child with acute diarrhea showed more than 99% homology with enteric BCoV LY138 strain in terms of nucleotide and predicted amino acid of S and HE genes (341). Moreover, gnotobiotic calves orally inoculated with the HECV-4408 strain induced cross-protective immunity against the BCoV DB2 strain (130). In addition, according to analysis of the S
gene sequence, BCoV and human coronavirus (HCoV)-OC43 share a common ancestor, and it was suggested that HCoV-OC43 may have originated from BCoV (319).

1.3.8. Diagnosis

The optimal diagnostic techniques for BCoV detection include virus isolation using cell cultures, antibody and antigen ELISA, virus neutralizing antibody assays and reverse transcriptase-polymerase chain reaction (RT-PCR). The optimal conditions for BCoV propagation using various cell cultures was described in detail (21). The Madin-Darby bovine kidney (MDBK) and human rectal tumor (HRT)-18 cells treated with trypsin were shown to be useful to enhance replication of the enteric BCoV strain.

Some diagnostic tests were developed on the basis of BCoV specific properties. For example, the haemagglutinating activity, haemagglutination inhibition and RDE activity test have been used to investigate BCoV specificity based on the HE gene (132, 135, 321).

For large epidemiological studies, enzyme-linked immunosorbent assays (ELISAs) are the most widely used for BCoV diagnosis, because of rapid evaluation and cost-effectiveness (136, 140, 271, 272, 296). The agreement between ELISA and protein A-colloidal gold immunoelectron microscopy (PAG-IEM) was 85% in terms of BCoV detection in the feces and nasal secretions of infected calves (139). A comparison between antigen-capture ELISAs using polyclonal and mAbs was evaluated. It was demonstrated that ELISA using mAbs for BCoV capture more clearly differentiate BCoV positive samples from BCoV negative samples. In addition, the ELISA using mAbs showed excellent agreement with traditional diagnostic methods, i.e., electron
microscopy and immunoelectron microscopy (kappa value = 0.96) (272, 273).

Hybridization assays have also been used for diagnosis of BCoV. The $^{32}$P-labeled BCoV-specific recombinant plasmid probes to the N and M genes were used to detect genomic BCoV RNA (316-318).

The RT-PCR has been frequently used for BCoV detection due to exceptionally high sensitivity and specificity as well as reproducibility. The nested PCR and one-step RT-PCR with BCoV-specific primers targeting the N gene were shown to be highly sensitive in detecting BCoV in nasal and fecal samples BCoV (49). Especially, low titers of the BCoV from subclinically infected calves were detected only by the nested PCR (49). The one-step RT-PCR with BCoV-specific primers targeting the S1 subunit of S gene was also developed to detect respiratory and enteric BCoVs (137). In addition, primers targeting the M gene were used to detect the BCoV in feedlot calves affected by bovine respiratory disease (83). Besides the BCoV-specific primers, pancorona-specific primers targeting the RdRp gene and group 2a CoV-specific primers targeting the N gene have been used to detect BCoV as well as the related ruminant CoVs, e.g., CoVs isolated from giraffe, sambar deer, waterbuck, sable antelope and white-tailed deer (4, 132).

Real-time RT-PCR has been used to simultaneously amplify and quantify the BCoV target gene based upon two types of fluorescence generation; hybridization of fluorescent reporter probes with complementary DNA, e.g., TaqMan-based real-time RT-PCR and intercalation of fluorescent dyes with double-stranded DNA, e.g., SYBR Green based real-time RT-PCR (83, 84, 97).
1.3.9. Epidemiology

The BCoV causes intestinal and respiratory tract disease in cattle. Its prevalence in feedlot or dairy cattle has been reported from different countries (38, 133, 134, 221, 309). Calves in the first 3 weeks of life are subject to enteritis caused by BCoV, resulting in severe diarrhea. Calves recovered from BCoV infection were normally protected from diarrhea after challenge with either a heterologous or homologous strain of BCoV (49). However, persistence of BCoV in neonatal calves was reported; the BCoV antigens were detected in crypt or Peyer’s patches for 3 weeks after infection (164). In addition, BCoV can remain viable in the environment. Thus, farms that previously experienced a BCoV-mediated diarrhea outbreak can have other yearly outbreaks.

The BCoV mediated enteric disease in calves has been investigated worldwide. In Ontario, Canada, BCoV in fecal samples from neonatal calves with severe diarrhea was detected using ELISA, resulting in 17.3% positive (38). In Ohio, BCoV in fecal samples was detected using the BCoV antigen ELISA and RT-PCR, resulting in 53% and 96% positive calves (average 7 months old), respectively (133). In Switzerland, fecal samples from dairy calves suffering from acute diarrhea (1-21 days old) were tested for BCoV using BCoV antigen ELISA, resulting in 7.8% prevalence (309). In South Korea, 15.6% of fecal samples from calves with diarrhea were positive for BCoV using RT-PCR and nested PCR. In addition, according to the phylogenetic analysis based on the amino acid sequences of the HE gene, the circulating Korean BCoV strains showed distinct patterns compared with the BCoV strains isolated from the USA and Canada (221). In addition, it was demonstrated that BCoV fecal shedding was age-dependent in cattle with diarrhea; the percentage of BCoV fecal shedding was 37.1% in 0-30-day-old calves,
25.6% in 4-12-month-old cattle and 18.2% in 2-7-year-old cows (134).

Besides the enteric disease, the respiratory tract disease caused by BCoV has also been investigated in calves. In Tennessee, it was suggested that BCoV plays an important role in respiratory disease outbreaks in cattle (4-8-month-old), based on BCoV isolation from nasal samples and lung tissues of cattle with shipping fever pneumonia (286). In addition, respiratory tract infections with the BCoV, followed by secondary infections with Pasteurella spp. were observed in natural outbreaks of shipping fever (286). In Ohio, BCoV in nasal samples was detected using BCoV antigen ELISA and RT-PCR, resulting in 48% and 84% positive calves (average 7 months old), respectively (133). In Ontario, Canada, 2% of feedlot calves that died of respiratory disease within 60 days after arrival showed evidence of BCoV infection based on virus isolation and BCoV antigen ELISA (110). In Italy, there were three outbreaks of bovine respiratory disease in calves (2-3-month-old) as a result of single BCoV infection without any other respiratory pathogens, e.g., bovine respiratory syncytial virus (BRSV), bovine herpes virus (BHV)-1 and bovine viral diarrhea virus (BVDV). Based on RT-PCR, 62.5% of calves with respiratory disease showed BCoV nasal shedding (83). In Finland, infectious agents involved in bovine respiratory disease were investigated. Among herds with respiratory distress (abnormal sounds on auscultation of the respiratory tract, fever > 39.5°C, elevated respiratory rate (>40/min), cough or nasal discharge), 89% of the total herds showed BCoV positive calves based on BCoV antibody ELISA (131).

The BCoV has been shown to be one of the etiological agents of winter dysentery (WD). The WD-affected cattle showed hemorrhagic diarrhea, respiratory signs, listlessness and reduced milk production (85, 158, 300). However, outbreaks of WD also
have been reported in the warmer season in Italy and South Korea. In a dairy herd in southern Italy, using virus isolation and RT-PCR, BCoV was detected in rectal, nasal and ocular swabs collected from adult cattle with clinical signs, e.g., severe profuse watery and bloody diarrhea, pyretic, anorexic, mild cough and nasal discharge as well as markedly decreased milk production in September (average 30 °C temperature) (85). In South Korea, BCoV fecal shedding was detected in cattle affected by winter dysentery (WD). The prevalence of BCoV detected from fecal samples varied according to the diagnostic technique: 36.5%, 35.1%, 33% and 79.4% of total fecal samples were positive by electron microscopy (EM), BCoV antigen ELISA, one-step RT-PCR and nested PCR (159). Interestingly, compared to previously reported WD-BCoV strains (159), some BCoV strains isolated in this study lacked RDE activity, suggesting that the South Korean BCoV strains have a distinct evolutionary pathway (220). It was also reported that WD is more frequently observed in large herds than in smaller herds, probably resulting from more chance to contact potential BCoV carriers (300). The BCoV carriers are clinically normal cows which show persisting BCoV excretion. Therefore, the carriers can be sources of infection for neonatal calves (71, 72).

In dairy cows, BCoV infection led to decreased milk production, resulting in significant economic losses (158). In addition, BCoV antibody levels in bulk milk were measured to estimate BCoV infection in dairy cattle (300). Eighty-nine % of all bulk milk samples were BCoV Ab positive using BCoV Ab ELISA, which corresponded with the high incidence of winter dysentery (227, 300).
1.3.10. Prevention and Control

Avoiding stressful environmental conditions as well as ensuring colostrum consumption are the most important means to prevent and control BCoV infection in cattle. An increased incidence of BCoV and its clinical signs has been associated with various environmental-risks (transport and crowding) (179, 285, 296), high herd density (158, 329), cattle housed in tie stall or stanchion barns and same equipment used to handle manure and feed (271), etc. To minimize environmental-risks for BCoV infections in cattle, pastures should be rotated to minimize contamination of calving grounds for new-born calves and all cattle with signs of intestinal and respiratory disease should be segregated immediately to minimize BCoV transmission (33).

Because of severe watery diarrhea, maintaining hydration and electrolyte balance is a key factor in treatment of calves suffering from BCoV enteritis. Fluids should be administered either orally or intravenously. Oral administration of absorbent agents, e.g., charcoal, clay, and clay minerals can be used to reduce the severity of diarrhea. However, it was suggested that infection can be enhanced by the adsorbent bound virus complex (56).

Unlike in humans and rodents, bovine immunoglobulins cannot be transferred through the placenta and calves are born agammaglobulinemic. To obtain passive maternal antibodies, colostrum consumption is critically important for neonatal calves. In particular, IgG1 is selectively concentrated in bovine colostrum from serum prior to parturition (17). The IgG1 in colostrum is absorbed across the intestinal epithelium and enters into the circulatory system via the lymphatics. Moreover, passively acquired serum IgG1 BCoV Ab titers in calves closely paralleled the virus neutralizing antibody
responses (95, 251). In addition, neonatal calves may have reduced or inadequate immune responses to vaccines because of the immaturity of their immune system. Therefore, colostrum containing high levels of BCoV specific Abs will protect the neonatal calves from BCoV disease (73, 171, 260). There are commercial inactivated vaccines for parenteral use in pregnant cows to induce lactogenic immunity against BCoV such as Guardian (Schering Animal Health), Scour Bos4 and 9 (Novartis Animal Health), and ScourGuard 3 and 4® (K)C (Pfizer Animal Health), marketed in the U.S.

Vaccinated calves also showed significant protection from BCoV diseases (204, 232, 328). In an extra-label, experimental BCoV vaccine trial in feedlot cattle, intranasal administration of a commercial, modified-live vaccine against BCoV reduced the risk of calves developing respiratory disease on entry to the feedlot (232). It was reported that a serial cell culture adapted BCoV had potential as a modified-live vaccine to protect calves from diarrhea, but no independent vaccine trials were done to substantiate this claim (328). In addition, in utero administration of a BCoV vaccine was shown to efficiently induce humoral immune responses in neonatal calves (204). At 9 to 49 days before parturition, the administration of attenuated BCoV into amniotic fluid elicited high titers of serum and intestinal IgA, IgM and IgG BCoV Ab in neonatal calves. Upon challenge with virulent BCoV, no diarrhea or lesions in intestinal epithelium were observed in the neonatal calves. However, practical application of the in utero administration should be very cautious, because it can frequently give rise to abortion and premature birth (204).

Vaccine studies to prevent BCoV infection and disease in adult cattle have been conducted (59, 291, 292). Cattle were vaccinated intramuscularly with BCoV-infected
cell lysates containing large amounts of hemagglutinin esterase (HE) antigen with an oil adjuvant. High titers of serum neutralizing antibodies were induced, and the vaccinated cattle did not develop clinical disease after challenge with virulent BCoV (292). In addition, high titers of HI antibodies persisted for 9 months after vaccination, and there was a significant and high correlation between the HI antibody and serum neutralizing antibody titers (291). Besides an inactivated BCoV vaccine, modified-live BCoV vaccines were also used in healthy cattle shedding BCoV subclinically to investigate BCoV shedding before and after parturition (59). In non-vaccinated cattle, the incidence of BCoV shedding was significantly higher after parturition than before parturition, whereas the incidence in vaccinated cattle did not show a significantly increased incidence after parturition (59).
1.4. Bovine rotavirus

1.4.1. Taxonomy

Reo-like virus was first described as an etiological agent of neonatal calf diarrhea (NCD), serologically unrelated to classical reoviruses (198). In 1974, the name ‘reo-like virus’ was replaced by ‘rotavirus (RV)’, because of its ‘wheel-like’ appearance (Rota-in Latin means wheel) (106). The international committee on taxonomy of viruses (ICTV) placed RV in the family Reoviridae. The RVs are characterized as non-enveloped and triple-layered viruses containing a genome with 11 segments of dsRNA (192). Rotaviruses are classified serologically into serogroups that share cross-reacting antigens detectable by serological tests within a defined serogroup. The RV detection by ELISA using mAbs against RV inner capsid viral protein (VP6) is a major method for epidemiological monitoring of RV infections. Antigenic analysis (ELISA), analysis of viral ds RNA by polyacrylamide gel electrophoresis or RT-PCR are methods used to differentiate RVs into 7 groups, A to G, and to subdivide group A RVs further into 2 main subgroups, I and II (26, 107). The VP6 subgroup specificity of the group A RV strains was determined by mAbs for specific epitopes on VP6, e.g., mAb 255/60 for subgroup I and mAb 361/9 for subgroup II (127, 186). For example, human strain SA11 and Wa are classified into subgroup I and II, respectively (127). In cattle, subgroup I VP6 specificity has been shown to be dominant, whereas subgroup II VP6 specificity has not been confirmed yet in the bovine species (53, 107, 186).

Group A RV is an important pathogen of humans, cattle, foals, piglets, lambs, goats, dogs, cats and other animals. Group B RV can infect calves, lambs, piglets, goats and humans. Group C RV can infect swine and humans. Group D, F and G RVs are
poultry pathogens, and group E RV infects swine (88). According to electropherotyping techniques, the migration pattern of the 11 dsRNA segments is distinctive among the RV groups. The 11 segments of dsRNA can be re-classified into 4 groups based on their sizes; from segments 1 to 4 into the first class, segments 5 and 6 into the second class, from segments 7 to 9 into the third class and segments 10 and 11 into the fourth class. Therefore, group A RV has a 4-2-3-2 pattern. Group B RV has a 4-2-2-3 pattern (from segments 1 to 4 into the first class, segments 5 and 6 into the second class, segments 7 and 8 into the third class, and from segments 9 to 11 into the fourth class), whereas group C RV has a 4-3-2-2 migration profile (from segments 1 to 4 into the first class, from segments 5 to 7 into the second class, segments 8 and 9 into the third class, and segments 10 and 11 into the fourth class) (180, 275, 295, 325).

Viruses within group A RV were classified further into serotypes based on two outer capsid proteins, VP4 and VP7. The VP7 is denoted as G type as it is a glycoprotein, and VP4, as P type as it is a protease sensitive protein (325). Thus, RV classification is binomial in which distinct serotypes of VP4 and VP7 are recognized by neutralization activity using neutralizing mAb to each of these two proteins (66, 185). The neutralizing mAb for G and P serotypes were produced from prototype viruses or mono-reassortants. Since nucleotide sequencing techniques have been developed, P and G genotyping has been widely used for classification (16, 44, 314). The G and P genotypes are determined by RT-PCR with primers targeting VP4 and VP7 gene and sequence-based analysis. Among the group A RV, 15 G types and 26 P types have been reported for RVs affecting humans and domestic animals (64). Of the 15 G types, G1, G5, G6, G8, G10 and G15 serotypes have been identified for bovine RVs (BRVs); however, the predominant BRV
G types are G6, G8 and G10 serotypes (64, 219, 239). In contrast to G type classification, genotypes of VP4 (as designated as [P] genotypes) using RT-PCR or sequence analysis have been applied for P type classification, because of a lack of readily available typing mAbs to differentiate VP4 types. Of the 26 P types, P[1], P[5], P[11] and P[21] genotypes have been identified for BRVs: P[1], P[5] and P[11] are the predominant bovine rotavirus P types (64). The correlation between G serotype and genotype has been established, but the correlation was less clear between P serotype and genotype (194). Therefore, serotype and genotype have been denoted together in which the P serotype is denoted, followed by P genotype being denoted within square brackets such as P6[1], P7[5] and P8[11] for BRVs (194). Besides VP6, VP7 and VP4, the sequences of the rotavirus enterotoxin NSP4 nucleotide and its amino acids were analyzed to examine the species of origin, relatedness and diversity of NSP4 (54). This analyses revealed four genotypes (A to D) in which the NSP4 genotype A included RV strains isolated from bovine, lapine, equine and human species, the NSP4 genotype B contained porcine, human and equine strains, the NSP4 genotype C involved simian, feline and human strains, and the NSP4 genotype D included only murine strains (54).

Recently, a modified classification of RV was suggested using all 11 genomic RNA segments (194). The recommended nomenclature was Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, representing the genotypes of the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6, encoding gene segments, respectively, where the x indicates the number of the genotype. For example, the bovine strain BRV 033 is G6-P[1]-I2-R2-C2-M2-A3-N2-T6-E2-H3. However, genotyping for only a few strains of BRV based on all 11 genomic RNA segments have been done. To date, two genotypes of VP6 (I2 and
4), one genotype of VP1 (R2), one genotype of VP2 (C2), one genotype of VP3 (M2),
five genotypes of NSP1 (A1, 3, 11, 13, and 14), two genotypes of NSP2 (N1 and 2), three
genotypes of NSP3 (T1, 6 and 7), two genotypes of NSP4 (E1 and 2) and two genotypes of
NSP5/6 (H1 and 3) have been determined (194).

Some RVs were very similar to group A RV in terms of morphology, size,
number of dsRNA genome segments, and tropism for villous enterocytes, but were
antigenically distinct. The RVs were collectively referred to as nongroup A RVs (255).
Saif et al. identified the first nongroup A RV (group C RV) from swine with diarrhea
(252). Nongroup A RVs have also been identified in cattle, especially group B and C
RVs (225, 255, 275, 303). As noted earlier, the migration patterns of the 11 segments of
dsRNA in group B and C RV are 4-2-2-3 and 4-3-2-2, respectively. Amino acid
sequences of the VP6 genes among group A, B and C BRVs were analyzed (303). In
general, group B BRV showed a marked divergence of the deduced amino acid sequences
of the VP6 gene where the sequence identity between group B BRV Nemuro strain and
group A BRV RF strain was 10.3 % and between group B BRV Nemuro strain and group
C BRV shintoku strain was 14.6%. In addition, relatively higher sequence identity,
41.1% was observed between group A BRV RF strain and group C BRV shintoku strain
(303).

It was suggested that dual infection with different BRV groups may enhance fecal
shedding of a heterologous nongroup A BRV which may not induce virus shedding upon
single infection (42). Gnotobiotic calves infected with a porcine-like group C WD534tc
strain isolated from an adult cow diarrhea did not shed the group C RV shedding;
however, calves co-infected with WD534tc and a group A BRV strain shed both group A
and C BRV. In addition, co-infected calves had more pronounced villous atrophy through all regions of the small intestine, compared with calves infected singly with either WD534tc or group A BRV strain. This study suggested that synergic effects of dual infection may be a mechanism for adaptation of heterologous RVs to new host species (42).

1.4.2. Morphology

The RVs contain a double stranded (ds) RNA genome surrounded by triple-layered icosahedral capsid proteins. The virus is non-enveloped and has a wheel-like shape of approximately 50 ~ 76 nm diameter (102, 128). In addition, short spike-like proteins (VP4) protrude from the surface of the VP7 outer capsid. By EM, there are three types of virus particles: the triple-layered particle (TLP); the double-layered particle (DLP) and the inner core particle (102, 128). The TLP, the mature infectious RV particle is approximately 75 nm in diameter and composed of three layers of proteins, outer capsid VP7 and spike VP4, middle capsid VP6, and inner capsid VP2, which surround the genomic dsRNA. The outer shell of the TLP is formed by 260 trimers of VP7, arranged in a skewed symmetry with $T=13l$ (l for levo) icosahedron and 60 VP4 spikes. Once the RV enters into target cells, the VP7 outer capsid proteins are removed in the cytoplasm to produce the DLP. The DLP is approximately 70 nm in diameter and composed of 260 trimers of VP6, arranged with a $T=13l$ icosahedral lattice, the most abundant protein in RV. The 11 genomic segments are packaged in an inner core (approximately 50 nm in diameter) comprised of 120 units of dimer VP2, arranged with $T=2$ icosahedron. Besides VP2, the inner core includes VP1 and VP3. The VP1 and
VP3 proteins form complexes which interact with the VP2 inner layer (128).

1.4.3. Genome

The RV genome is composed of 11 segments of dsRNA within the core capsid. For group A RVs, the sizes of the segments range from 667 nucleotides (nt.) (segment 11) to 3,302 nt. (segment 1) base pairs, resulting in approximately 18,522 nt. in the total genome (102). In addition, the other sizes of RNA segments are 2,687 nt. in segment 2, 2,591 nt. in segment 3, 2,362 nt. in segment 4, 1,581 nt. in segment 5, 1,356 nt. in segment 6, 1,104 nt. in segment 7, 1,059 nt. in segment 8, 1,062 nt. in segment 9 and 751 nt. in segment 10. Each RV genome segment contains a single open reading frame (ORF). Unlike coronavirus (CoV), the RV genome is associated with only the 5’ capping structure, but lacks a 3’-polyadenylation tail.

1.4.4. Viral proteins

The RV is composed of 6 structural proteins, VP1, VP2, VP3, VP4, VP6, and VP7, and 6 non-structural proteins, NSP1, NSP2, NSP3, NSP4, NSP5, and NSP6 (102). The structural proteins are located in the core (VP1, VP2 and VP3), inner capsid (VP6) and outer capsid layer (VP4 and VP7). The VP1, encoded by genome segment 1, is an RNA-dependent RNA polymerase which plays an important role in producing the mRNA transcripts for the synthesis of viral proteins and copies of the RV genomic RNA segments for newly produced virus particles (102). The VP2, encoded by genome segment 2, is the third most abundant protein in RV. The VP2 is an essential protein for proper function of the RNA polymerase complex. It has high affinity for the dsRNA,
suggesting that it plays an important role in packaging of the genomic RNA into the virion (175). The VP3, encoded by genome segment 3, is a guanylyltransferase, a capping enzyme which forms the 5’ cap in the post-transcriptional modification of mRNA, resulting in stabilization of viral mRNA by protecting it from nucleases (102). The VP4, encoded by genome segment 4, is a spike-like protein protruding from the outer capsid VP7 layer (102). This protein is a viral hemagglutinin which helps the RV to attach to the receptors on host cells, resulting in virus entry. The P serotype is determined by this VP4 protein. Cleavage of the VP4 protein by trypsin-like enzymes into VP5 and VP8 enhances attachment and entry. In addition, this VP4 protein induces neutralizing antibody. The VP6, encoded by genome segment 6, is the most abundant protein and a highly immunogenic antigen (102). The VP6 is found in trimeric form in the inner capsid layer. It contains antigenic determinants for serogroup (7 RV serogroups, A to G) and for subgroup (subgroups I and II in group A RV). Unlike VP4 and VP7, neutralizing antibodies are not induced by VP6. However, it was demonstrated in vivo that a VP6 specific mAb can inhibit RV infection (103). The VP6 specific mAb attachment to RV induced a conformational change of the VP6 trimer, resulting in prevention of viral transcription. Thus, VP6 is also essential for transcription. The VP7, encoded by genome segment 8, is the second most abundant protein which forms the outer capsid layer (102). The G serotype is determined by this VP7 protein. Like VP4, this VP7 protein is highly immunogenic and induces neutralizing antibody. It was demonstrated that the VP7 protein has Ca\textsuperscript{2+} binding sites which play an important role in dissociating the VP7 outer capsid layer in the cytoplasm, resulting in conversion to DLP, or viral transcriptase (91, 247).
The functions of non-structural proteins are partially understood. It was recently demonstrated that the NSP1, encoded by genome segment 5, binds interferon regulatory factor-3 (IRF3) in the infected cell cytoplasm (125). The IRF3 is the transcription factor which induces type I interferon (IFN) in response to virus infection. However, NSP1-IRF3 complexes activate proteasome degradation, resulting in inhibition of IRF3 mediated type I IFN expression. The N-terminal zinc-binding domain in NSP1 plays an important role in the proteasome-dependent IRF3 degradation (125). In addition, it was suggested that NSP1 has RNA-binding activity and serves to recruit viral mRNA into the viroplasm (152). The NSP2, encoded by genome segment 8, was shown to be one of the proteins composing viral replicase (9). Like the NSP1, NSP2 also has an RNA-binding activity and binds to 11 ds RNA genomic segments in RV-infected cells. The NSP2 was detected in the viroplasm along with NSP5, VP1 and VP2. The siRNA-mediated NSP2 knockdown showed a lack of viroplasm formation, suggesting that the NSP2 is a very important protein to form viroplasm (228). The NSP3, encoded by genome segment 7, binds the 3’-end consensus sequence UGACC of the RV mRNAs, and can transport viral mRNAs to the viroplasms. This sequence-specific RNA binding activity of NSP3 can protect viral mRNA from degradation and encapsidate the RNA genomic segments (234). In addition, it was reported that NSP3 plays an important role in the shutdown of cellular protein synthesis (218). The NSP4, encoded by genome segment 10, is involved in the morphogenesis of virus particles as well as viral pathogenesis by acting as an enterotoxin. The double-layered particles (DLPs) in the viroplasms are associated with NSP4 located in the endoplasmic reticulum (ER), which facilitates DLP budding into the ER lumen. Finally, the outer capsid proteins, VP7 and VP4 are assembled into the DLPs, resulting in
triple-layered particles (TLPs) (12, 228). In addition, the transmembrane or secreted soluble form of NSP4 functions as a viral enterotoxin to increase intracellular \( \text{Ca}^{2+} \) concentrations and induce diarrhea (32). The NSP5, encoded by genome segment 11, is a phosphorylated protein (228). Like NSP2, viroplasm formation is also dependent on NSP5. The core proteins, i.e., VP1 and VP2, accumulate in the viroplasm because the NSP2 and NSP5 have affinity for VP1 and VP2, respectively (228). The NSP5 and NSP6 are both encoded by segment 11 containing alternative initiation codons. The NSP6 was shown to interact with NSP5 (121). It was demonstrated that NSP6 is localized to viroplasms in infected cells (120). In contrast to the other NSPs, NSP6 has a high turnover rate. Thus, it was completely degraded within 2 hours after synthesis (237). Little is known about the biological functions of NSP6.

### 1.4.5. Morphogenesis

The RV has a preferential replication site (tissue tropism), the cytoplasm of enterocytes in the intestinal villi (106). The RV receptor for entry still remains controversial and is not completely understood. Generally, there are two types of RV based on whether or not the virus binds sialic acid (SA) residues on the cell surface; SA-dependent and -independent. Once the RV enters the intestinal tract, the outer capsid protein VP4 is cleaved by intestinal proteases, e.g., chymotrypsin, into two subunits, VP5 and VP8, resulting in enhanced virus infectivity. In the case of SA-dependent RVs, interaction between VP8 and the SA residue mediates initial binding, followed by VP5 containing hydrophobic domains interacting with cell membranes (receptor mediated-endocytosis). However, in the case of SA-independent RVs, only VP5 directly mediates
host entry (direct penetration). In addition, the position of SA residues in gangliosides can be a determinant for RV strains. The SA-dependent RVs (neuraminidase-sensitive strains), such as simian SA11 and bovine NCDV, bind to external SA residues in gangliosides, whereas SA-independent RVs (neuraminidase-insensitive strains), such as bovine UK, recognize gangliosides with internal SA (52, 86, 155). Cellular integrins also can be RV co-receptors (67). The RV outer capsid proteins, VP4 and VP7, have ligand peptides of $\alpha_2\beta_1$, $\alpha_4\beta_1$ and $\alpha_x\beta_2$ integrins. In addition, mAbs to these integrins partially inhibit RV infection. After entry into host cells, low Ca$^{2+}$ concentrations in the cytoplasm induce the dissociation of VP4 and VP7, resulting in generation of DLPs, the viral transcriptases (91, 247). The DLPs play an important role in RV transcription, resulting in full-length mRNA synthesis from dsRNA genomic templates. After uncoating, RNA-dependent RNA polymerases (VP1) in the DLPs are activated. The RNA-dependent RNA polymerase and guanylyl transferase (VP3) produce positive strand RNAs which play a role as mRNA, followed by being translated in the cytoplasm. Core (VP1, VP2 and VP3) and inner capsid (VP6) proteins along with NSPs are assembled in the cytoplasm. The interaction between NSP4 located in the ER and VP6 proteins facilitate the budding of assembled DLPs into the ER lumen (12, 228, 231). During this budding into the ER, the DLPs transiently obtain a lipid membrane. Finally, VP7 proteins are assembled onto the DLPs in the ER cisternae, resulting in removal of the lipid membrane. It is unclear how and where the VP4 proteins are assembled into the virus particles. However, it was suggested that VP4 proteins are synthesized on free cytosolic ribosomes, move to the plasma membrane of the infected host cells and assemble later into viral particles in the plasma membrane, resulting in infectious TLPs.
The progeny RVs are released from the cells by lysis (205).

**1.4.6. Pathogenesis**

The BRV infection was normally characterized by its localization to the small intestine of neonatal calves, resulting in disruption of efficient absorptive surfaces to produce a malabsorptive diarrhea (88). The RV induced-diarrhea is caused by multiple factors involving direct effects of virus and indirect effects of the host response (144, 188, 238, 248). In the small intestine, villous enterocytes are non-replicating mature cells covering the villi that have critical digestive and absorptive functions. The crypt epithelial cells, the progenitors of the villus enterocytes, actively secrete Cl⁻ ions into the intestinal lumen, but lack the absorptive function. In enterocytes infected with RV, pathological changes vary from slight lesions, e.g., enterocyte vacuolization and loss, to substantial lesions, e.g., pronounced villus atrophy and crypt hyperplasia. Diarrhea induced by RV infection has been attributable to maldigestion and malabsorption. Due to the destruction of the brush border membranes, undigested disaccharides, carbohydrates, fats and proteins accumulate in the intestinal lumen. The undigested materials result in an osmotic imbalance and insufficient water absorption, leading to osmotic diarrhea.

The virus NSP4 is an enterotoxin that induces diarrhea in neonatal mice, but its role in other species is unclear (32). In mice, the NSP4, or cleaved NSP4 peptides can induce diarrheal symptoms without significant changes in intestinal architecture. Following virus entry, formation of viroplasms are frequently detected (228). The NSP4 is released from viroplasms and secreted from cell via unknown mechanisms. The NSP4 binds to integrin α1β1 and α2β1 on cells, followed by stimulation of a signaling pathway
through phospholipase C (PLC) and inositol-triphosphate (IP$_3$) (188, 238, 248). Consequently, Ca$^{2+}$ is released from the ER. The increase in Ca$^{2+}$ induces disruption of the microvillus cytoskeleton. On the other hand, intracellular NSP4 released from viroplasms disrupts tight junctions as well as the microvillus cytoskeleton. As a result of disruption of tight junctions, water and electrolytes are released between epithelial cells (238, 248, 265). In addition, it was demonstrated for the mouse model that activation of the enteric nervous system (ENS) is responsible for approximately 67% of the fluid and electrolyte secretion in RV induced-diarrhea (238). In crypt cells, basolaterally or apically released NSP4 can directly stimulate the ENS, resulting in a high concentration of intracellular Ca$^{2+}$, followed by induction of Cl$^-$ secretion (238). In case of malnourished hosts, the symptoms from RV infection are more severe; the intestinal recovery is much slower, and immune responses to RV are abnormal (347).

Diarrhea is the major clinical sign in calves infected with RV (11, 144, 184). Oral-fecal transmission has been considered the major infection route in calves. Moreover, RV is frequently detected in virus-contaminated milk, water and feed (64). Once calves are infected with RV, virus shedding commences at post-inoculation day 2 or 3 and continues for approximately one week (251). In neonatal calves, the mortality rate caused by RV infection varies from 5 to 20%; however, it is increased in calves fed insufficient amounts of colostrum and under stressful conditions (88). Unless calves contract infections by secondary pathogens, the diarrheal feces normally do not contain blood. Prolonged RV infection in very young animals or sequential secondary bacterial infections lead to death due to severe dehydration (88). In the recovery stage, enterocytes are regenerated and the intestinal architecture returns to normal. In addition, calves
recovered from RV induced-diarrhea usually return to normal body weight by 3 to 4 weeks after infection. Likewise, RV infection seems to be self-limiting (88, 144).

1.4.7. Zoonotic potential

In nature, many RVs may have restricted host ranges. However, uncommon RV types with outer capsid proteins more commonly found in cattle have been detected from human infections (88). It was suggested that genetic reassortment between human and bovine RV strains during co-infection of the same host cell is the major driving force for zoonotic transmission (88). The role of the bovine species as a source of human infection was suggested from close relatedness between human DS1-like RV and BRV strains in terms of amino acid sequences (63, 193, 236). Especially, phylogenetic dendrograms based on the amino acid sequences of VP1, VP2, VP3, VP6, NSP2 and NSP4 clustered the human DS1-like RV and BRV strains closely, indicating high amino acid sequence identity. These studies also suggested that there was a common origin between human DS1-like RV and BRV strains. Parental human RV and BRV strains may exchange genomic RNA segments in the same host cell, resulting in viral chimeras and new assembled virus particles. The complete genome sequence of a newly detected human RV strain B1711 (G6P[6], isolated from a 13-month-old child) was analyzed to determine possible reassortment (195). The analysis of VP3 and VP7-encoding gene segments clustered the human RV strain B1711 with BRV strains, suggesting that the new human RV strain was a reassortant strain with VP3 and VP7 gene segments originated from a BRV strain (195). The BRV G10 serotype-derived reassortant strains have also been involved in infection of infants (74). The RV strain I321 (G10P[11])
isolated from neonates in India caused asymptomatic infections (74). The amino acid sequences of VP4 and VP7 in the I321 strain were highly homologous to those in the BRV B223 strain, suggesting that both surface proteins may be derived from BRVs (74).

In addition, BRV isolated from calves with diarrhea showed close relatedness (99.9%) to a human RV G8 strain, HMG035 in terms of the nucleotide sequence of the VP7 gene (2).

It was suggested by Dunn et al. that RV NSP1 gene may determine host range restriction (92). The nucleotide sequence of the NSP1 gene of BRV G3 strain J63 showed high homology with the NSP1 gene of human RV G8 strain MP409 isolated from a diarrheic child, suggesting that gene reassortment had occurred (315). Dual infection by human and BRVs has been observed in an infant with severe diarrhea under natural conditions (209). Two type of RVs were isolated from the infant; G1P[8] and G1P[5]. Although BRVs may not replicate as efficiently in the human intestine, a BRV-like G1P[5] was detected in the infant. The recovery of BRV genes from co-infection may be attributed to reassortment, resulting in successful adaptation to a new host (209).

Close contact between humans and domestic livestock provides a means for zoonotic transmission (64, 88). In addition, it was suggested that zoonotic transmission could be mediated by contamination of water, food and air (88, 89).

### 1.4.8. Diagnosis

Various diagnostic methods have been applied to detect BRV, e.g., EM, polyacrylamide gel electrophoresis (PAGE)-based electropherotyping, ELISA, restriction fragment length polymorphism (RFLP) and RT-PCR (43, 219, 303). Especially, PAGE based-electropherotyping is a useful and widely used technique for virus detection and...
for molecular epidemiology studies (88, 294, 325). Isolation or detection of virus in intestinal contents or feces is accomplished by using cell cultures or EM procedures, respectively. A monkey kidney epithelial cell line, MA104 is widely popularly used to isolate BRV, and EM was the major procedure for direct virus detection (259). In addition, a cell culture immunofluorescence test (CCIF) has been applied to detect infectious RV using Abs to BRV conjugated to fluorescein isothiocyanate (FITC) (186). Enzyme immunoosorbent linked assay (ELISA) has been developed to increase sensitivity and specificity. In large epidemiologic studies, ELISA has been used as the main methodology, because of cost-effectiveness (55, 107, 112, 153, 154). The RT-PCR has been developed to detect RV more rapidly, sensitively and reliably (43). Primers targeting the outer capsid genes, VP4 and VP7 are commonly used to detect RV and genotype RV, e.g., P and G-typing of RVs, respectively. The sequence analysis of VP4 and VP7 made it possible to differentiate various BRV isolates. Although diagnostic methods have been improved using cutting-edge technologies, RV can be detected easily by conventional EM which in turn still remains as a gold standard for RV detection.

1.4.9. Epidemiology

In animals, RV is detected ubiquitously in a wide host range including avian, bovine, ovine, canine, feline, equine and porcine species (88). In cattle, group A, B and C RVs have been reported; however, group A BRV is considered the major RV serogroup accounting for most bovine infections (115, 254). The prevalence of RV in calves has been reported from different countries (55, 107, 109, 112, 153, 154, 185, 200, 240). In five U.S. states, surveillance of group A BRVs was conducted in calves using
ELISA (185). Seventy-nine percent of the total fecal samples (n=308) from diarrheic beef and dairy calves from South Dakota, Ohio, Michigan, Nebraska and Washington were positive for group A RV. Of the positive fecal samples, 54% were positive for G6 serotype, 14% were positive for G10 serotype, 4% were positive for both G6 and G10 serotypes, and 28% were negative for both G6 and G10. In the five states, G6 was the most prevalent strain, followed by G10. Compared with other states, the fecal samples from Ohio calves showed more positives for BRV G10 serotype (22% of positive fecal samples vs. 18% in Nebraska, 17% in South Dakota and 8.3% in Michigan), whereas no positives for BRV G10 serotype were observed in Washington (185). The fecal samples from diarrheic calves from Ohio, Nebraska, Washington and Canada were analyzed by dot and Northern blot hybridization assay with nucleic acid probes representing P and G type specificities (224). The G typing analysis indicated that G6 (36.3%) type was most prevalent, followed by G10 (12.7%) and G8 (2.9%). In P typing analysis, P[5] type (20.4%, UK-like) was most prevalent, followed by P[11] type (9.3%, B223-like) and P[1] type (2.2%, NCDV-like) (224). In Ohio, 16.4% of fecal samples from neonatal calves were positive using ELISA with hyperimmune anti-serum against group A BRV Lincoln strain and cell culture immunofluorescence test (CCIF) with FITC-conjugated pig anti-group A BRV serum (186). The field samples were also characterized by RT-PCR and restriction fragment length polymorphism (RFLP) (43). The full length VP7 genes and partial length VP4 genes were amplified with RT-PCR, followed by treatment with four enzymes, EcoRV, NlaIV, BamHI and HpaII for digesting the amplified gene products. Of 86 BRV fecal samples from diarrheic calves in Ohio, South Dakota, Nebraska, California and Wyoming, analysis of G typing revealed that the G6 (60.5%) type was
most prevalent, followed by G10 (19.8%) and G8 (7%). In P typing analysis, P[5] type (64%) was most prevalent, followed by P[11] type (28%) and P[1] type (1.2%). In addition, the G6P[5] type was most prevalent in the field samples (43). In Italy, RV was detected in fecal samples from dairy and beef calves by nested RT-PCR assays, resulting in 96.1% positives. Of the RV positive samples from calves, G6 (78.5%) was the most prevalent strain, followed by G10 (9.9%) and G8 (4.7%) (200). In Ireland, 91% of fecal samples from calves were positive for RV by latex agglutination and ELISA (240). Nested RT-PCR was used to determine G genotypes, resulting in G6 as the predominant strain, but G10 and G6/G10 mixed types were also detected. In Canada, ELISA was used to detect RV in fecal samples from dairy calves, resulting in 26.4% of fecal samples being positive (154). The G serotyping using ELISA showed that G6 and G10 serotypes were prevalent. However, the proportion of G serotypes between diarrheic and non-diarrheic calves differed; the proportion of the G6 serotype was higher in diarrheic than non-diarrheic calves. In Australia, 48.7% of fecal samples from beef and dairy calves under going diarrhea outbreaks were positive for RV using ELISA (153). The VP7 sequence analysis confirmed that G6 and G10 strains were the major serotypes of RV in calf diarrhea outbreaks. In Argentina, fecal samples from calves in dairy and beef herds involved in diarrhea outbreaks were collected and analyzed for group A RV by ELISA, resulting in 62.5% of the total being positive for RV (112). Genotyping by heminested multiplex RT-PCR for P and G genotypes indicated that 60% of RV positive calves were P[5]G6, 4.4% were P[11]G10, and 2.4% were P[5]G10. In Venezuela, 11.7% of total diarrheic dairy calves were positive for RV infection using ELISA (55). Using G and P serotype-specific mAbs, serotypes G6 and G10 were the major circulating RVs in two
different dairy farms. In Tunisia, 30% of dairy calves tested positive for RV by ELISA. Genotyping by RT-PCR showed that G8 viral strains were most prevalent, although G6 or mixed strains (G6+G8) were also detected (107). In Japan, 36.8% of fecal samples from diarrheic beef calves were positive for RV shedding using ELISA. Using ELISA with G serotype-specific mAbs and RT-PCR with P genotype-specific primers, 60.6% of isolated RVs from fecal samples were identified as G8 serotype which was frequently combined with P[1] or [11] (109).

Group B BRV was associated with sporadic cases of diarrhea in cattle (45). The RT-PCR with primers targeting a partial-length group B BRV VP7 gene were used to detect group B BRV using 90 fecal samples from diarrheic calves from Ohio, California, Wyoming, South Dakota, Nebraska, and 81 fecal samples from diarrheic adult cows (winter dysentery cases) from Ohio, New York and California. For all five states, the prevalence of group B BRV fecal shedding was 5.6% of the fecal samples from diarrheic calves and 18.5% of the fecal samples from diarrheic adult cows. For individual states, the prevalence of group B BRV in diarrheic calves was 5.7%, 6.5% and 16.7% of fecal samples from Ohio, South Dakota and California, respectively. However, no group B BRV from diarrheic calves was observed in Wyoming and Nebraska. The prevalence of group B BRV in diarrheic adult cows were 40%, 20% and 16.9% of fecal samples from New York, California, Ohio, respectively (45).

In addition, the seroprevalence of group C BRV was investigated in the U.S. and Japan (302). Using ELISA with hyperimmune antisera to group C BRV Shintoku strain, 47% and 56% of bovine sera collected from cattle in the U.S. and Japan, respectively, had group C BRV specific Abs. According to serological results, group C BRV
infections are common in cattle in the U.S. and Japan (302).

In the U.S., 3.7% of fecal samples from diarrheic adult cows were positive for group C BRV using RT-PCR with primers targeting a partial-length group C BRV Shintoku VP6 gene (42). The group C BRV isolated from the positive sample in the U.S. was designated as WD534tc/C. The WD534tc/C strain was more closely related to group C porcine Cowden strain than group C BRV Shintoku strain, characterized by aggregation by anti-Cowden serum, high sequence homology (over 98%) of VP6 gene with the Cowden strain, identical virus neutralization titer of hyperimmune antiserum to Cowden strain. To date, only two group C BRV strains, i.e., Shintoku and porcine-like WD534tc strains have been reported in cattle (42, 305). One possible reason could be the weak pathogenicity of group C BRV, resulting in inapparent clinical signs in affected cattle. In addition, the majority of cattle infected with group C BRV may shed low titers in feces over a short time period (226).

The etiological factors contributing to RV infection and disease include: insufficient intake of colostrum, young age, micronutrient deficiency, insufficient lactogenic antibody from the dam, unhygienic environments, temperature variations, high herd density and secondary bacterial or virus infections. Interestingly, BRV infection was detected more frequently in female than male calves. Significantly higher titers of RV Abs were detected in females than males. In addition, female calves tended to shed more RV compared with males (138). It was reported that herd types, geographic region and year have an effect on the distribution of RV G types (82). In Swedish cattle herds, the G6 type was the major serotype in beef herds, whereas G10 type was the predominant type in dairy herds (82). The herd type-specific BRV G types were also detected in the
U.S. (185). The G6 type was more prevalent in beef (67%) than in dairy (47.5%) herds, and the G10 type was more frequently detected in dairy (17.5%) than in beef (5.5%) herds (185). Similarly in Argentina, distribution of G and P types was dependent upon herd type; P[5]G6 was the major strain in beef herds, and P[11]G6 or P[11]G10 were the prevalent types in dairy herds (112). In terms of geographic specificity, the G10 serotype was the predominant strain in southern, western and central regions of India, whereas a higher prevalence of G6 or G8 serotypes was reported from other countries (109, 200, 240, 315). Periodic changes in the serotypes were investigated in Japan where the predominant serotype was G10 in 1995, G8 in 1996 and G6 in 1997 and 1998 (108).

1.4.10. Prevention and Control

The morbidity and mortality of calves from RV infections can be minimized by proper management and hygiene on farms (144). Because secondary bacterial infections usually give rise to more severe disease, antibiotic administration would be helpful for their prevention (144). Rehydration and electrolyte therapy are essential for calves with diarrhea to restore the fluid balance (144). The feeding of colostrum containing RV Abs is a key management strategy to protect neonates from RV infection. In order to produce maternal antibodies against RV, the dams should be immunized with RV vaccines a few weeks before parturition. Intramuscular and intra-mammary vaccination of the dam significantly stimulated enhanced serum and colostrum antibody titers to BRV (70, 104, 167, 254, 258, 261). Especially, stimulation of antibody accumulation in colostrum before parturition is critical for passive immunity in calves, because unlike in humans and rodents, bovine immunoglobulins cannot be transferred through the placenta and calves
are born agammaglobulinemic. Colostrum collected from dams that received intramuscular and intramammary inoculations of adjuvanted modified-live Ohio Agricultural Research and Development Center (OARDC) RV vaccine had significantly higher IgG1, IgG2, IgA and IgM and neutralizing BRV Ab titers, compared to colostrum from uninoculated controls (258, 261). In addition, recombinant virus-like particles (VLP) were used to stimulate lactogenic Abs (104). Colostrum from dams vaccinated intramuscularly and intramamarily with recombinant VLP, prepared by the coexpression in baculovirus of RF strain VP2 and SA11 strain VP4, VP6 and VP7, had high neutralizing and IgA Ab titers (70, 104, 258). It was demonstrated that colostrum plays an important role in passively protecting calves from BRV-associated diarrhea and shedding (104, 258). Bovine rotavirus Ab-seronegative calves were fed colostrum pooled from their dams who were vaccinated with the adjuvanted modified-live OARDC RV vaccine, followed by challenge with virulent NCDV BRV strain. All calves had enhanced IgG1 BRV Ab titers and were protected against diarrhea and RV fecal shedding. However, diarrhea and RV fecal shedding occurred in colostrum deprived-calves and in calves fed colostrum pooled from unvaccinated dams. In addition, the colostrum fed calves had delayed onset and shortened duration of diarrhea and RV fecal shedding (258). In the VLP study, serum IgG, IgA and fecal IgA BRV Ab titers were significantly enhanced in calves fed colostrum from dams vaccinated with recombinant RV VLP vaccine (104, 254). After challenge, all calves fed the VLP colostrum were protected from BRV-associated diarrhea and showed less BRV fecal shedding.

Significantly enhanced antibody production in colostrum was induced most effectively by a combination of intramuscular primary vaccination and intramammary
booster (104, 167, 258, 261). The colostrum from these vaccinated dams was significantly associated with enhanced protection against RV-associated diarrhea and RV fecal shedding in calves.

The oral administration of a live attenuated BRV vaccine is important to stimulate active immunity to prevent BRV-associated diarrhea and fecal shedding in calves (254). It was suggested that the passively transferred BRV Abs from the colostrum may cause the failure of live oral BRV vaccines in calves (81, 254). For example, dairy calves inoculated orally with a commercial modified live BRV vaccine did not have significantly reduced BRV-associated diarrhea after challenge, compared with control calves given a placebo (81). The possible reason for the low vaccine efficacy in newborn calves, could be passively transferred antibodies localized in the intestinal tract, which may neutralize the live oral vaccine virus and inhibit active immunity in calves (254).

In addition, probiotics, dietary supplements containing potentially beneficial bacteria or yeast, were suggested to confer health benefit on animals and humans (190, 298, 339). Little is known about probiotic effects on BRV-associated diseases and immune responses to BRV in calves. However, probiotic effects were demonstrated with gnotobiotic pigs colonized with *Lactobacillus* bacteria, a probiotic microorganism (339). The gnotobiotic pigs orally inoculated with *L. acidophilus* strain NCFM had significantly enhanced virus neutralizing and serum IgM, IgA and IgG Ab titers in response to attenuated Wa human RV vaccine, compared with gnotobiotic pigs without *L. acidophilus* colonization. In addition, IFN-γ producing CD8+ T cells in ileum and spleen, and IgA and IgG Ab-secreting cells in ileum were significantly enhanced in the gnotobiotic pigs colonized with *L. acidophilus*. In cattle management, probiotics have
been used to prevent or treat calf diarrhea (298). Calves orally administered a multispecies probiotic containing six different bacteria (*L. acidophilus* W55, *L. salivarius* W57, *L. paracasei* W56, *L. plantarum* W59, *Lactococcus lactis* W58 and *Enterococcus faecium* W54) mixed with milk replacers had significantly lowered incidence and duration of diarrhea, compared with control groups fed milk replacers without the multispecies probiotic. In addition, the administration of the multispecies probiotic significantly reduced the percentage of calves that required antibiotic treatment of gastrointestinal, respiratory, or other disease (298). Besides lactic acid bacteria, e.g., of *Lactobacillus* species, yeast also has been widely used in calves (190). Calves fed grains containing cultures of *Saccharomyces cerevisiae* (2% of grain ingredient composition) had improved fecal scores (median fecal scores, 1.2 in yeast-feeding vs. 1.3 in control calves, *p* < 0.08; 1=firm, 2 = soft or moderate, 3=runny or mild diarrhea and 4=watery and profuse diarrhea) and lower incidence and duration of diarrhea. Like the multispecies probiotic described above, the feeding of yeast cultures in grain significantly reduced the proportion of calves treated with antibiotics, compared with control calves fed grains without cultures of *S. cerevisiae* (190).
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Figure 1.1: Diagram of BCoV genome organization. The BCoV genome includes 11 open reading frames (ORFs) flanked by 5’- and 3’-untranslated regions (UTRs). The approximate positions of predicted functional domains are shown in ORF1a and ORF1b: aspartic or glutamic acid residue domain (Ac), papain-like proteases 1 (PL1), conserved domain of unknown function (X), PL2, transmembrane domain 1 (TM1), TM2, poliovirus 3C-like protease (3CL\text{pro}) and TM3 in the ORF1a, and RNA dependent RNA polymerase (RdRp), zinc finger domain (ZD), RNA helicase (HEL) and conserved sequence domain in nidoviruses (ND) in the ORF1b [Adapted from Chouljenko et al. (2001) J. Gen. Virol. 82:2927-33]. The ORF1a and ORF1b are overlapped by 22nt at the ORF1a/b junction including ‘slippery’ sequence. Other proteins are located downstream of ORF1: 32kDa, 32kDa non-structural protein (NSP); HE, hemagglutinin-esterase protein; S, spike protein; 4.9kDa, 4.9kDa NSP; 4.8kDa, 4.8kDa NSP; 12.7kDa, 12.7kDa NSP; E, small membrane protein; M, membrane protein; and N, nucleocapsid protein.
CHAPTER 2

VITAMIN A EFFECTS ON ANTIBODY RESPONSES IN FEEDLOT CALVES INTRAMUSCULARLY VACCINATED WITH BOVINE CORONAVIRUS

2.1 SUMMARY

Dietary vitamin A restriction in feedlot calves has been used to increase intramuscular fat deposition, or marbling, resulting in higher quality beef production in terms of palatability. However, it is unclear whether vitamin A restriction which increases the beef carcass value, exerts detrimental effects on the immune response to pathogens or vaccines in cattle. To investigate the effects of vitamin A on antibody (Ab) response, Angus steers (n=40) were randomly assigned to two groups that received either low (LVA group; 1100 IU/kg of dietary dry matter (DM), n=20) or high (HVA group; 3300 IU/kg of dietary DM, n=20) dietary vitamin A. Because approximately 90-112 days are required to decrease vitamin A in serum from the liver stores, all calves were vaccinated intramuscularly with an inactivated bovine coronavirus (BCoV) vaccine at post-arrival day (PAD) 112 and boosted at PAD 126 to determine the effect of vitamin A status on vaccine-induced BCoV Ab responses at PAD 140. Twenty calves (50% of total calves) shed BCoV either in feces or nasally at least once shortly after arrival (at
The prevalence of BCoV shedding increased from 21% at PAD 0 to 41% at PAD 4, but with no BCoV shedding detected thereafter (at PAD 35, 112 and 140). The BCoV fecal shedding was negatively associated with pre-existing IgA BCoV Ab titers at PAD 4. In addition, BCoV nasal shedding was negatively associated with pre-existing IgA and IgM Ab titers at PAD 4. Serum IgG1- and IgA-BCoV Ab seroconversion at PAD 35 was positively associated with average BCoV RNA copy numbers detected in fecal samples at PAD 0 and 4. Serum IgG1 BCoV Ab titers and the ratios of IgG1 to IgG2 Abs in the HVA group were significantly higher at PAD 140 than at PAD 112, whereas those in the LVA group did not differ significantly. At PAD 140, serum IgG1 BCoV Ab titers and the ratios of IgG1 to IgG2 Abs were significantly higher in the HVA than in the LVA group. This study suggests that the low vitamin A diet suppresses the Th2 associated Ab (IgG1) responses.

2.2 INTRODUCTION

Vitamin A and its bioactive metabolites are essential for epithelial cell integrity, cell proliferation and differentiation, apoptosis and general development, as well as immune functions (5, 78, 81, 82). Vitamin A deficiency is one of the major risk factors for enteritis and pneumonia caused by infectious agents (7, 74). Clinical trials showed that vitamin A supplementation has a significant effect on reducing morbidity and/or mortality in children with acute measles (6, 21), diarrhea (37, 74), respiratory infections (50, 57, 74), malaria (52, 70), human immunodeficiency virus infection (20, 69), etc. In addition, vitamin A effects on immunity to infectious agents was demonstrated in community- and hospital-based studies (39, 40, 60, 68). The important role of vitamin A
in immune response to and resistance against infectious agents has been studied mainly clinically for human disease or experimentally in mouse models with an emphasis on systemic responses. The association between host vitamin A status and many animal infectious diseases is poorly understood.

It is unclear whether dietary vitamin A restriction, which has been used to increase intramuscular fat (marbling) resulting in higher quality beef production in terms of meat palatability, influences occurrence or severity of infectious disease in feedlot calves (1, 29, 71). Moreover, it is unknown whether vitamin A restriction, which increases the beef carcass value, exerts detrimental effects on immune responses to infectious pathogens. In calves, vitamin A effects on the immune system have been reported in terms of antibody (Ab) response (29, 35), mononuclear leukocyte population (51), ileum villus height and follicle size in Peyer’s patches (67), superoxide (O$_2^-$) production, intracellular signaling in neutrophils (36), and nitric oxide (NO) production from blood mononuclear leukocytes (58). However, the majority of the studies listed above reported immune responses induced by mitogens or non-replicating antigens, which neglects responses to pathogens under field conditions, and overlooks the critical point of disease-associated vitamin A effects (61).

Bovine coronavirus is a non-segmented, positive-sense, single-stranded (ss) RNA virus in the Coronavirus genus, the Coronaviridae family and the Nidovirales order. The BCoV has two major tissue tropisms: the epithelium of the respiratory and intestinal tracts. Infection results in diarrhea in calves, winter dysentery (WD) in adult cattle and nasolacrimal discharge associated with cough and pneumonia in calves and adult cattle (44, 47, 76). Bovine CoV and BCoV-associated clinical signs were frequently detected
in feedlot and dairy cattle where fecal-oronasal infection was the major transmission route under field conditions (25, 31). The incidence of BCoV infections and clinical signs were associated with two major risk factors: environmental risks, i.e., stressful conditions during transport and crowding (44, 75, 79); a specific outbreak period from October to January (49, 63, 64, 73); high density herds (38, 84); cattle housed in tie stall or stanchion barns; the same equipment used to handle manure and feed (72); and immunological risks, low serum IgG BCoV Ab titers in calves at arrival to feedlots (18, 79); or in dairy cattle (73). Other immunological risks included low serum IgA BCoV Ab titers in dairy calves associated with diarrhea duration (31); associated with BCoV nasal shedding and low nasal or lachrymal IgA BCoV Ab titers in dairy calves (33). However, little is known about nutritional effects on BCoV infections and immune responses to BCoV.

The objective of this study was to understand the impact of vitamin A status in cattle on their immune responses to BCoV vaccination. We investigated Ab responses in naturally infected feedlot calves that were subsequently vaccinated intramuscularly with BCoV. These calves were fed either low (LVA group; 1100 IU/kg of dietary dry matter (DM)) or high (HVA group; 3300 IU/kg of dietary DM) dietary vitamin A. Real-Time RT-PCR was used to detect BCoV shedding in the fecal and nasal specimens. Serum and fecal samples were assayed by ELISA for IgG1, IgG2, IgM, IgA and fecal IgA isotype Abs to BCoV. Our findings indicate that the LVA dietary regimen compromised IgG1 BCoV Ab titers and the ratio of IgG1 to IgG2 Ab in response to inactivated BCoV vaccine, suggesting suppressed Th2 associated Ab (IgG1) responses.
2.3 MATERIALS AND METHODS

The study design is briefly diagrammed with the number of calves, vitamin A status, study time points, samplings and events in Figure 2.1.

**Feedlot calves and vitamin A dietary regimen.** This study was commenced on 10/05/2006 when forty Angus calves (average age 199 days, range 163-220 days) arrived at The Ohio State University feedlot from two OARDC branch experimental stations located in Belle Valley and Coshocton, Ohio. The dates noted in this study were calculated from their arrival at the feedlot (Post-Arrival Day: PAD). Calves were vaccinated and boosted for infectious bovine rhinotracheitis, parainfluenza-3, *Haemophilus somnus*, *Pasteurella* and *Clostridium* (Quadraplex, Somnugen 2P, and Dybelon, respectively; Bioceutic, St. Joseph, MO) and dewormed with Ivomec pour-on (Merck, Rahway, NJ) six weeks prior to arrival at the feedlot. Calves (N\text{total} = 40) were randomly assigned to two groups at PAD 0: high vitamin A (HVA, N\text{HVA} = 20) and low vitamin A (LVA, N\text{LVA} = 20) groups. The high and low dietary vitamin A were defined as greater than or less than the 2200 international units (IU)/kg of dietary dry matter (DM), respectively as recommended by the National Research Council. The LVA group received 1100 IU/kg of dietary DM to simulate low levels in vitamin A restricted feedlot calves and the HVA group received 3300 IU/kg of dietary DM. Supplemental vitamin A was fed at PAD 0, then daily throughout the 140 day study period (PAD 140). Each calf was isolated in a pen and fed approximately 7.5kg of feed/day individually during the experiment. The feed was composed of (DM basis %) 5% corn silage, 80% whole shell corn and 15% supplements. The supplements for the HVA group contained 0.06% vitamin A, whereas those for the LVA group contained no vitamin A. In the LAV group,
vitamin A in serum was assumed to decrease at approximately 90-112 days after the low vitamin A diet started, resulting in an increased difference of serum vitamin A concentrations between the HVA and LVA group (29, 30). The hypothetical vitamin A status in serum is indicated by the grey and white arrows representing prior to and post serum vitamin A decreases, respectively (Figure 2.1). Two calves in each group were euthanized on 01/22/07, and one calf in the LVA group died on 01/06/07.

**Antibiotic treatments.** An unexpected respiratory disease outbreak occurred on 01/01/2007 (at PAD 88), and all calves were treated with antibiotics. Banamine was administered if calf rectal temperatures were $\geq 103.5$ °F. Draxxin was administered as the first antibiotic for calves with rectal temperatures $\geq 103.5$ °F. Nuflor or Excenel was administered as the second choice antibiotic if there was no response to the first antibiotic, and as the last choice antibiotic, Micotil was administered if there were no responses to the first and second antibiotics.

**Vaccination and booster.** The commercial vaccine, ScourGuard 3®(K)C (Pfizer, New York, NY) was used for vaccination at PAD 112 and booster at PAD 126. ScourGuard 3®(K)C is a veterinary vaccine containing inactivated BCoV Mebus strain, Rotavirus G6 and G10, *Escherichia coli* (*E. coli*) K99 bacterin and *Clostridium perfringens* Type C toxoid. This vaccine is adjuvanted with Quil-A Saponin to enhance the immune response. Two ml of the vaccine was administered intramuscularly into the brachiocephalicus as recommended by the manufacturer at PAD 112 to prime and at PAD 126 to boost immunity.

**Sampling.** Nasal swab specimens, fecal and blood samples were collected from calves at PAD 0, 4, 35, 112 and 140. Nasal samples were diluted 1:5 in Minimum
Essential Medium (MEM) containing 1% antibiotic-antimycotic, followed by centrifugation at 1000 x g for 11 minutes at 4 ºC. Fecal samples were diluted 1:10 in MEM containing 1% antibiotic-antimycotic, and centrifuged at 850 x g for 20 minutes at 4ºC. Supernatants from nasal and fecal samples were stored at -70ºC. Ten to 15ml of blood was collected via jugular venipuncture, followed by centrifugation at 2000 x g for 20 minutes. Serum was collected, heat-inactivated at 56 ºC for 30 minutes and stored at -20ºC. The processed nasal and fecal samples were used for BCoV detection and quantification by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Antibody-enzyme-linked immunosorbent assay (Ab-ELISA) was performed using serum and fecal samples to evaluate systemic and local Ab responses to BCoV, respectively.

**RNA extraction.** Total RNA was extracted from fecal and nasal samples with TRIzol LS reagent (Gibco, Life Tech, Grand Island, NY). Two hundred microliters of fecal or nasal samples were mixed with 600µl TRIzol LS and incubated for 5 minutes at room temperature. The samples were treated with 160µl of chloroform, vortexed and incubated for 10 minutes at room temperature. The samples were then centrifuged at 18,300 x g for 15 minutes at 4 ºC, 400µl of supernatant was transferred to a new tube, and mixed with 400µl of 100% isopropyl alcohol. After 10 minutes of incubation at room temperature, the samples were centrifuged at 18,300 x g for 10 minutes at 4ºC. The pellet was reconstituted with 800µl of 75% EtOH and centrifuged at 11,600 x g for 5 minutes at 4ºC, and the supernatant was discarded. The pellet was dried with low spin for 10 minutes, reconstituted with 40µl of 0.1% diethylpyrocarbonated (DEPC) treated H₂O, and incubated at 55ºC for 10 minutes. The total RNA was stored at -70ºC until used.
**Antibody ELISA.** An indirect Ab capture ELISA was used to measure BCoV specific-serum IgG1, IgG2, IgA, IgM, and fecal IgA (fIgA). The 96-well ELISA plates (MaxiSorp high protein-binding capacity ELISA plates; Nunc, San Diego, CA) were coated with 100µl of monoclonal Abs against bovine IgG1 (1:500, Serotec, Raleigh, NC), IgG2 (1:500, Serotec, Raleigh, NC), IgA (1:500, Serotec, Raleigh, NC), and IgM (1:1000, Sigma, St. Louis, MO) in coating buffer (15mM Na₂CO₃, 35mM NaHCO₃, pH 9.6) overnight at 4°C. Plates were washed 4 times between incubations with phosphate-buffered saline (PBS, pH 7.4) and 0.05% Tween 20 (PBS-Tw). The plates were blocked with PBS/1% bovine serum albumin (EMD chemicals, Gibbstown, NJ) to prevent non-specific binding. After washing, a 4-fold dilution of the serum samples was added to the plates; dilutions from 1:400 to 1:1,638,400 were used for IgG1 and IgG2, and from 1:4 to 1:16,384 for IgA, IgM and fIgA detection. Serum samples out of the titer range were retested using dilutions greater than 1,638,400 for IgG1 and IgG2, and 16384 for IgA, IgM and fIgA detection. Sera from gnotobiotic calves infected with the BCoV Mebus strain or naïve calves were used as positive and negative controls, respectively. The negative controls were added in the same dilutions as the serum samples described above. After the plates were incubated for 1 hour at room temperature and washed, 100µl of 10⁶ plaque forming units per milliliter (pfu/ml) BCoV Mebus strain and mock infected human rectal tumor (HRT)-18 cells were added to each well. After incubation for 1 hour at room temperature, 100µl of guinea pig anti-BCoV Mebus strain hyperimmune sera diluted 1:5000 were added to each well. After 1 hour incubation at room temperature, 100µl of 1:2500 diluted goat anti-guinea pig IgG (H+L) horseradish-peroxidase (HRP) conjugated (KPL, Gaithersburg, MD) was added to the plates. After 1 hour incubation at
room temperature, 100µl of HRP substrate [ABTS® Peroxidase Substrate System, equal ratio of 2,2’-azino-di-(3-ethylbenzthiazoline-6-sulfonate) to H2O2, KPL, Gaithersburg, MD] was added. After 20 minutes of incubation at room temperature, the optical density was measured at 405 nm with a microplate spectrophotometer (SpectraMax 340PC384, Sunnyvale, CA). The Abs detected from serum and fecal samples were quantified by endpoint titration, defined as the reciprocal of the highest dilution showing an optical density value above the cut-off (average of negative controls + 2.077* standard deviation of negative controls) (28).

**Real-time RT-PCR.** A one-step reverse transcription (RT)-PCR assay was performed to detect and quantify BCoV from fecal and nasal samples. The oligonucleotide primers, forward primer, 5’-GYKGTTWTATKTTAARCC-3’, and reverse primer, 5’-CATTRGCDGAAACAGCTTG-3’, were designed to anneal to the ORF1b sequence, resulting in 99-bp amplicons. TaqMan Probe, FAM 5’-ACTAGTAGTTGATGCAACTACTGCTTTTGC-3’ BHQ1, was also designed to anneal to ORF1b between the forward and reverse primer. Reaction components in RT-PCR were composed of 5 µl of total RNA plus 20µl of RT-PCR reaction buffer including 5µl of 5 x reaction buffer (Qiagen, Valencia, CA), 1µl of RT-PCR enzyme mixture (Qiagen, Valencia, CA), 400µM of deoxynucleoside triphosphates (dNTP) (Promega, Madison, WI), 800µM of MgCl2 (Promega, Madison, WI), 4µM of each primer (Integrated DNA technologies, Coralville, IA), 0.2µM of TaqMan Probe (Integrated DNA technologies, Coralville, IA), 20 units RNasin®RNase inhibitor (Promega, Madison, WI), 1.25 units GoTaq®Flexi DNA polymerase (Promega, Madison, WI), and 7.75µl of RNase-free water (Qiagen, Valencia, CA). Total RNA extracts from 2 x 10^7
pfu/ml BCoV Mebus strain was diluted with 0.1% diethylpyrocarbonated (DEPC) buffer to generate ten-fold serially diluted standards ranging from 10 to $10^7$ pfu/ml. Therefore, BCoV detected from fecal and nasal samples were quantified as pfu/ml. In addition, total RNA extracts from minimum essential medium (MEM) containing 1% antibiotic-antimycotic were used as negative control. SmartCycler system (Cepheid, Sunnyvale, CA) was used to generate the kinetic curve of PCR amplification with specific conditions of reverse transcription and PCR; one cycle of reverse transcription with 50.0°C for 1800 seconds and 95.0°C for 900 seconds; 45 cycles of PCR with 95.0°C for 15 seconds in denaturation, 50.0°C for 30 seconds in annealing, and 72.0°C for 20 seconds in extension. The detection limit in our quantitative RT-real time PCR was $10^2$ pfu/ml.

**Serum retinol concentration.** Ten milliliters of jugular blood samples were collected from calves. The blood samples were immediately wrapped in aluminum foil to avoid retinol light damage, and kept on ice. Serum was prepared by centrifugation at 2,200 x g for 10 minutes at 4°C, and stored at -20°C until analysis of serum retinol concentration using high performance liquid chromatography (HPLC) (30). The serum samples were extracted with hexane and dried under N₂ gas at 37°C, reconstituted with ethanol, and injected into a HPLC apparatus equipped with a reverse-phase column (Supelcosil LC-18, 25 cm x 4.6 mm, Supelco Inc., Bellefonte, PA). The solvent used was initially 75% water and 25% methanol (vol/vol) and then was changed linearly to 100% methanol over 2 min. The flow rate was 1.8 mL/min. All procedures were performed in the dark to avoid retinol light damage.

**Statistical analysis.** Immunoglobulin (Ig) titers quantified by Ab-ELISA and Real-time RT-PCR data were transformed to base 10 logarithms for statistical analysis.
Analysis of variance (ANOVA) was used following analysis of homoskedasticity of dependent variables by means of Cochran C and Hartley $F_{max}$. Although normality assumption was violated, ANOVA was used on the basis of balanced sample size in comparison because of its robustness. The Mann-Whitney test was used to determine statistical significance when the normality assumption was violated and the comparison groups had different sample sizes. Repeated measures ANOVA was used to determine differences among PADs. Duncan’s multiple range test was used for multiple comparisons. To determine associations between binomial dependent variables and continuous independent variables, logistic regression analysis was used with two significance tests; likelihood ratio test (LRT) for significance of the full model, and Wald test for significance of a single coefficient. In logistic regression analysis, the non-detectable level was considered half of the lowest detection limitation: 50 pfu/ml. In the 2 x 2 contingency tables, Fisher’s exact test was used to determine the association between two categorical data. In addition, the strength of relationship between two categorical data was analyzed by relative ratio (RR) calculated by dividing prevalence in exposed group by prevalence in unexposed group. Data analyzed with ANOVA were plotted with means and whiskers denoting 95% confidence intervals. Data analyzed with Mann-Whitney test were plotted with mediums and whiskers representing non-outlier range calculated by upper quartile + 1.5 x interquartile range (IQR) and lower quartile – 1.5 x IQR. All statistical analyses were performed with the Statistica 8 software package (StatSoft, Inc., Tulsa, Ok). All tests were considered significant at a probability of $p < 0.05$. 

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2.4 RESULTS

Frequencies of BCoV shedding in calves during vitamin A treatment and BCoV vaccination are summarized in Tables 2.1 and 2.2. Before the decreased vitamin A levels in serum occurred (at PAD 0, 4 and 35), we investigated the isotype Ab responses induced at PAD 35 by natural BCoV infection at PAD 0 and 4. After vitamin A decreased in serum (by PAD 112), we investigated BCoV Abs induced at PAD 112 and 140 by an inactivated BCoV vaccine given at PAD 112 and 126.

**BCoV shedding.** BCoV shedding was analyzed using number of BCoV shedding calves (S), prevalence of BCoV shedding (P), and BCoV RNA copy number (C). BCoV fecal and nasal shedding was defined as real-time RT-PCR positives from fecal and nasal samples, respectively. The BCoV shedding was detected at early time points, PAD 0 and 4. At PAD 0, 8 calves (S\textsubscript{HVA} = 3 and S\textsubscript{LVA} = 5) shed BCoV fecally; no nasal shedding was detected at PAD 0. At PAD 4, 16 calves (S\textsubscript{HVA} = 7 and S\textsubscript{LVA} = 9) shed BCoV, of which 8 calves (S\textsubscript{HVA} = 4 and S\textsubscript{LVA} = 4) shed BCoV nasally. Twenty calves (50% of total calves, S\textsubscript{HVA} = 9 and S\textsubscript{LVA} = 11) shed either BCoV fecally or nasally at least one time at either PAD 0 or 4. The prevalence of BCoV shedding increased from 21% (P\textsubscript{HVA} = 15% and P\textsubscript{LVA} = 28%) at PAD 0 to 41% (P\textsubscript{HVA} = 37% and P\textsubscript{LVA} = 45%) at PAD 4. However, BCoV shedding was not detected at PAD 35, 112 and 140. The average BCoV RNA copy number detected from nasal samples was lower than from fecal samples: 4.41 x 10\textsuperscript{3} pfu/ml (C\textsubscript{HVA} = 6.26 x 10\textsuperscript{3} pfu/ml and C\textsubscript{LVA} = 2.56 x 10\textsuperscript{3} pfu/ml) from nasal samples at PAD 4, compared to 7.39 x 10\textsuperscript{6} pfu/ml (C\textsubscript{HVA} = 11 x 10\textsuperscript{6} pfu/ml and C\textsubscript{LVA} = 5.23 x 10\textsuperscript{6} pfu/ml) and 13.87 x 10\textsuperscript{6} pfu/ml (C\textsubscript{HVA} = 15.23 x 10\textsuperscript{6} pfu/ml and C\textsubscript{LVA} = 12.82 x 10\textsuperscript{6} pfu/ml) from fecal samples at PAD 0 and 4, respectively.
**Antibody isotype responses.** The BCoV specific serum Ab isotype geometric mean titers (GMTs), IgG1, IgG2, IgA, IgM and fecal IgA (fIgA), are shown according to PADs in figure 2.2 and 2.3. Isotype Abs were plotted with the HVA and LVA groups at PAD 0, 4, 35, 112 and 140 (Figure 2.2), and with BCoV shedding and non-shedding groups at PAD 0, 4 and 35 (Figure 2.3). Before the decreased vitamin A levels in serum occurred (at PAD 0, 4 and 35), overall Ab isotype titers were calculated regardless of the vitamin A dietary regimens. However, after vitamin A decreased in serum (at PAD 112 and 140), Ab isotype titers were specified according to vitamin A dietary regimen.

(i) **Serum IgG1 BCoV antibody.** Combined serum IgG1 BCoV Ab GMTs representing both the HVA and LVA groups at PAD 0, 4 and 35 were 1903, 800 and 1345, respectively; however, no statistically significant differences for the Ab titers were observed among the three PADs. After BCoV vaccination, serum IgG1 BCoV Ab titers in the HVA group were significantly higher at PAD 140 than at PAD 112 (GMTs, 185 at PAD 112 and 1008 at PAD 140) \( (p < 0.005) \) (Figure 2.2.A). There were no statistically significant differences in serum IgG1 BCoV Ab titers between PAD 112 and 140 in the LVA group. The serum IgG1 Ab titers (GMT, 1008) in the HVA group were significantly higher than those (GMT, 226) in the LVA group at PAD 140 \( (p < 0.05) \), but not at PAD 112 (Figure 2.2.A).

In addition, serum IgG1 BCoV Ab titers were significantly higher at PAD 0 in BCoV non-shedding calves than in BCoV shedding calves \( (p < 0.05) \), indicating that calves with high titer IgG1 BCoV Abs tended not to shed BCoV upon arrival (Figure 2.3.A). After arrival at the feedlot, serum IgG1 BCoV Ab titers in BCoV non-shedding calves decreased significantly at PAD 35 compared with PAD 0 \( (p < 0.05) \). Serum IgG1
BCoV Ab titers in BCoV shedding calves increased at PAD 35 compared with PAD 0; however, the increase was not statistically significant (Figure 2.3.A).

(ii) **Serum IgG2 BCoV antibody.** Overall serum IgG2 BCoV Ab GMTs at PAD 0, 4 and 35 were 4685, 1715 and 586, respectively. Serum IgG2 BCoV Ab titers decreased between PAD 0 and 35. Serum IgG2 BCoV Ab titers at PAD 35 were significantly lower than those at PAD 0 ($p < 0.05$) (Figure 2.2.B). After BCoV vaccination, the serum IgG2 BCoV Ab titers were lower at PAD 140 than at PAD 112 in the HVA calves (GMTs, 588 at PAD 112 and 159 at PAD 140) and in the LVA calves (GMTs, 981 at PAD 112 and 177 at PAD 140) group; however, the differences were not significantly lower (Figure 2.2.B). These data suggest that no booster response occurred to the inactivated BCoV vaccine in either group. Serum IgG2 BCoV Ab titers between the HVA and LVA groups were not significantly different at PAD 112 and 140 (Figure 2.2.B).

Unlike serum IgG1 or IgA BCoV Ab responses, serum IgG2 BCoV Ab titers were significantly lower at PAD 35 than at PAD 0 in calves shedding BCoV ($p < 0.05$) (Figure 2.3.B), suggesting lack of an anamnestic or booster IgG2 response after BCoV infection. Serum IgG2 BCoV Ab titers in BCoV non-shedding calves also decreased between PAD 0 and 35; however, the decreased Ab titers were not statistically significant (Figure 2.3.B). There were no significant differences in serum IgG2 BCoV Ab titers between BCoV shedding and non-shedding calves at PAD 0, 4 and 35 (Figure 2.3.B).

(iii) **Serum IgA BCoV antibody.** Overall serum IgA BCoV Ab GMTs at PAD 0, 4 and 35 were 231, 3327 and 1136, respectively. Serum IgA BCoV Ab titers at PAD 4 and 35 were significantly higher than those at PAD 0 ($p < 0.01$ at PAD 4 and $p < 0.05$ at
PAD 35). After BCoV vaccination, there were no significant differences in serum IgA BCoV Ab titers between PAD 112 and 140 in either group as well as between the HVA and LVA group at both PADs (Figure 2.2.C).

Serum IgA BCoV Ab titers were significantly higher in BCoV non-shedding calves than in BCoV shedding calves \((p < 0.001)\), indicating that like for serum IgG1 BCoV Abs, calves with high serum IgA BCoV Ab titers tended not to shed BCoV upon arrival (Figure 2.3.C). After arrival at the feedlot, serum IgA BCoV Ab titers in BCoV non-shedding calves were significantly lower at PAD 35 than at PAD 4 \((p < 0.05)\) (Figure 2.3.C). In contrast, serum IgA BCoV Ab titers in BCoV shedding calves were significantly higher at PAD 35 than at PAD 0 \((p < 0.001)\) (Figure 2.3.C), suggesting a booster IgA Ab response after infection with BCoV.

(iv) Serum IgM BCoV antibody. Overall serum IgM BCoV Ab GMTs at PAD 0, 4, and 35 were 461, 1176 and 12.1, respectively. Serum IgM BCoV Ab titers increased transiently between PAD 0 and 4 then decreased between PAD 4 and 35 in all calves (Figure 2.2.D). The serum IgM BCoV Ab titers at PAD 35 were significantly lower than those at PAD 0 and 4 \((p < 0.001)\) (Figure 2.2.D). After BCoV vaccination, no significant differences in serum IgM BCoV Ab titers between PAD 112 and 140 occurred in either group or between the HVA and LVA group at both PADs (Figure 2.2.D).

Serum IgM BCoV Ab titers in all calves were significantly lower at PAD 35 than at PAD 4 \((p < 0.001)\) (Figure 2.3.D), but there were no significant differences in serum IgM BCoV Ab titers between BCoV shedding and non-shedding calves at PAD 0, 4, and 35 (Figure 2.3.D), indicative of lack of boosting of primary (IgM) immune responses to BCoV in either group.
(v) **Fecal IgA BCoV antibody.** Overall fecal IgA (fIgA) BCoV Ab GMTs at PAD 0, 4 and 35 were 13.3, 9.73 and 3.61, respectively. There were no significant differences in the fIgA BCoV Ab titers among these three PADs (Figure 2.2.E). After BCoV vaccination, no significant differences occurred in fIgA BCoV Ab titers between PAD 112 and 140 in either group or between the HVA and LVA groups at both PADs (Figure 2.2.E).

In addition, there were no significant differences in fIgA BCoV Ab titers between calves shedding and not shedding BCoV at PAD 0, 4 and 35 as well as among the three PADs in BCoV shedding and non-shedding calves (Figure 2.3.E).

**Calves in the HVA and LVA groups showed statistically similar background levels of BCoV shedding and pre-existing serum BCoV antibodies at PAD 0.** Homogeneity between the HVA and LVA groups at PAD 0 was analyzed in terms of BCoV RNA copy number shed and serology (Figure 2.4). The calves were randomly assigned to the HVA ($N_{HAV} = 20$) and LVA ($N_{LVA} = 20$) groups at PAD 0 (Figure 2.1). The BCoV Ab isotype titers of IgG1, IgG2, IgA, IgM and fIgA were plotted according to the HVA and LVA groups (Figure 2.4). No Ab titers differed statistically between the HAV and LAV groups, indicating that all calves in the HVA and LVA groups had similar BCoV serological backgrounds at PAD 0. In addition, the BCoV detected from the eight BCoV shedding calves (Table 2.1) was quantified and plotted, and did not differ statistically between the HVA and LVA groups (Fig. 2.4). In summary, calves in the two vitamin A dietary groups (HVA and LVA) were uniform and did not differ in terms of BCoV shedding or pre-existing serum BCoV Abs at PAD 0 (prior to the 90-112 days required for vitamin A decrease in serum). Therefore, both groups were similar without
initial bias and required no sub-categorical data analyses.

The probability of BCoV shedding was negatively associated with pre-existing serum IgA and IgM BCoV Ab titers at PAD 4. The BCoV fecal shedding was negatively associated with pre-existing serum IgA BCoV Ab titers at PAD 4 (based on the derived formula, probability of BCoV fecal shedding (%) = 100*[e^{(1.84–0.63*Log10IgA)} / (1+e^{(1.84–0.63*Log10IgA)})]) (Figure 2.5.A). According to the logistic regression equation, the coefficient of serum IgA BCoV Ab titers, –0.63, explains the odds ratio of BCoV fecal shedding relative to the change in pre-existing serum IgA BCoV Ab titers. For example, for a change from 3 to 2 in Log10 IgA BCoV Ab titers, the odds ratio of BCoV fecal shedding is e^{-0.63*(2-3)}, or 1.88, indicating that calves with 2 serum IgA BCoV Ab titers (log10 scale) are approximately 1.88 times more likely to show BCoV fecal shedding than those with 3 serum IgA BCoV Ab titers (log10 scale). In addition, calves with titers greater than 2.92 (log10 scale) serum BCoV IgA Ab have less than 50% probability of BCoV fecal shedding.

The BCoV nasal shedding was also negatively associated with pre-existing serum IgA (solid line) and IgM (dotted line) BCoV Ab titers at PAD 4, (based on the derived formula, probability of BCoV nasal shedding (%) = 100*[e^{(2.37–1.21*Log10IgA)}/(1+e^{(2.37–1.21*Log10IgA)})] and 100*[e^{(0.25–0.61*Log10IgM)}/(1+e^{(0.25–0.61*Log10IgM)})], respectively) (Figure 2.5.B). For the change of pre-existing Ab titers, e.g., from 3 to 2, the odds ratio of BCoV nasal shedding is e^{-1.21*(2-3)}, or 3.35 serum IgA BCoV Ab titers, and e^{-0.61*(2-3)}, or 1.84 serum IgM BCoV Ab titers. According to the estimated odds ratio, calves with 2 serum IgA BCoV Ab titers (as a Log10 scale) tend to be 3.35 times more likely to shed BCoV nasally than those with 3 serum IgA BCoV Ab titers (as a Log10 scale). In the
case of IgM BCoV Ab titers, they were 1.84 times more likely to shed BCoV nasally. For calves with greater than 1.96 and 0.41 serum IgA and IgM BCoV Ab titers (as a Log10 scale), respectively, the probability of BCoV nasal shedding is less than 50%. This relationship could not be demonstrated for the data at PAD 0, because the number of positive calves’ samples (fecal or nasal) by real-time RT-PCR was not sufficient for the logistic regression model. In summary, the probability of BCoV fecal and nasal shedding decreases as pre-existing serum IgA or IgM titers become higher.

**Serum IgG1- and IgA-BCoV antibody seroconversion were positively associated with BCoV shedding.** Seroconversion was defined as an increase in BCoV specific-Ab titers of at least four-fold between PAD 0 and 35. The number of calves shedding BCoV at either PAD 0 or 4 was significantly associated with serum IgG1- \((p < 0.001)\) (Figure 2.6.A) and IgA-BCoV Ab seroconversion \((p < 0.05)\) (Figure 2.6.B), indicating that BCoV infection was significantly associated with increased serum IgG1 and IgA BCoV Ab titers. The strength of the relationship between BCoV shedding and seroconversion was explained with a relative ratio (RR) calculated from dividing the prevalence of seroconversion in the BCoV shedding group by the prevalence of seroconversion in the non-BCoV shedding group. The RR was 6.2 (1.60-23.82 95% confidence intervals) for serum IgG1 BCoV Ab titers and 1.9 (1.07-3.36 95% confidence intervals) for serum IgA BCoV titers, indicating that calves shedding BCoV are 6.2 and 1.9 times more likely to seroconvert to IgG1 and IgA, respectively than those not shedding BCoV. Likewise, single time point BCoV fecal or nasal shedding between PAD 0 and 4 was statistically related with serum IgG1- and IgA-BCoV Ab seroconversion at PAD 35. A logistic regression model was used to determine the
seroconversion pattern of serum IgA (solid line) and IgG1 (dotted line) according to BCoV RNA levels (Figure 2.6.C). Serum IgA- and IgG1-BCoV Ab seroconversion at PAD 35 was positively associated with average BCoV RNA copy numbers detected in fecal samples at PAD 0 and 4 (based on the derived formulas, probability of IgA-BCoV Ab seroconversion (%) = 100*[e^{(-5.52+1.25*average of BCoV copies) / (1+e^{(-5.52+1.25*average of BCoV copies)})}], and probability of IgG1-BCoV Ab seroconversion (%) = 100*[e^{(-7.32+1.28*average of BCoV copies)}/(1+e^{(-7.32+1.28*average of BCoV copies)})]). For BCoV copy number, calves with 10^6 pfu/ml BCoV fecal shedding were 3.49 and 3.60 times more likely to show IgA- and IgG1-BCoV Ab seroconversion, respectively than those with 10^5 pfu/ml BCoV fecal shedding. In summary, according to the 2 x 2 contingency table and logistic regression model, there was a significant positive association between BCoV shedding and IgA- or IgG1-BCoV Ab seroconversion.

The vitamin A dietary regimens had a significant effect on serum retinol concentration. Serum retinol concentrations were investigated with 10 calves in each vitamin A group at PAD 112 and 140. At PAD 112, calves in both the LVA and HVA group had similar serum retinol concentrations (HVA = 34.8 and LVA = 32.0 µg/dl, p > 0.05). However, the serum retinol concentrations were significantly differentiated at PAD 140 where serum retinol concentrations in the LVA group were lower significantly than those in the HVA group (HVA = 33.7 and LVA = 25.1 µg/dl, p < 0.01). In the HVA group, the serum retinol concentrations at PAD 112 did not differ significantly with those at PAD 114 (PAD 112 = 34.8 and PAD 140 = 33.7 µg/dl, p > 0.05), whereas serum retinol concentrations in the LVA group were significantly lower at PAD 140 than at PAD 112 (PAD 112 = 32.0 and PAD 140 = 25.1 µg/dl, p < 0.01). Taken together, calves
fed 3300 IU/kg of dietary DM maintained serum retinol concentration between PAD 112 and 140; however, calves fed 1100 IU/kg of dietary DM failed to show homeostasis of serum retinol concentration, suggesting the depletion of hepatic vitamin A.

**A low vitamin A dietary regimen compromised the serum IgG1 BCoV Ab response to inactivated BCoV vaccine.** To determine the IgG subclass responses to inactivated BCoV vaccine, serum IgG1 BCoV Ab titers and the ratios of IgG1 to IgG2 Abs at PAD 112 (before vaccination) and 140 (after vaccination) were analyzed according to vitamin A treatments (Figure 2.7). Serum IgG1 BCoV Ab titers in the HVA group were significantly higher at PAD 140 than at PAD 112 ($p < 0.005$), whereas those in the LVA group did not differ significantly (Figure 2.7.A). At PAD 140, serum IgG1 BCoV Ab titers were significantly higher in the HVA than in the LVA group ($p < 0.05$). Serum IgG2 BCoV Ab titers in the HVA and LVA groups tended to be lower at PAD 140 than at PAD 112; however, differences were not statistically significant. There were no significant differences in serum IgG2 BCoV Ab titers between the HVA and LVA groups at either PAD (Figure 2.7.B).

In addition, the ratios of IgG1 to IgG2 Abs were used to assess dominance of Ab immune responses (IgG1, Th2) over cell-mediated immune responses (IgG2, Th1) in cattle. The ratios of IgG1 to IgG2 Abs in the HVA group were significantly higher at PAD 140 (post-BCoV vaccination) than at PAD 112 ($p < 0.005$), whereas those in the LVA group did not change significantly (Figure 2.7.C). At PAD 140, the ratios of IgG1 to IgG2 Abs were significantly higher in the HVA than in the LVA group ($p < 0.05$). According to the IgG1 BCoV Ab titers (Figure 2.7.A) and the ratios of IgG1 to IgG2 Abs (Figure 2.7.C), serum IgG1 BCoV Abs were predominantly induced in response to
inactivated BCoV vaccine in the HVA group; however, they were compromised in the LVA group. Thus, vitamin A may be an essential micronutrient to induce IgG1 Ab responses (Th2) to a BCoV inactivated vaccine.

**Calves previously recovered from natural BCoV infection had a compromised serum IgG1 BCoV Ab response to vaccination in the LVA group.** Calves naturally infected with BCoV in the HVA and the LVA group were compared to assess the impact of BCoV infection and BCoV vaccination on Ab responses before the decreased vitamin A (at PAD 0 and 35) and after the decreased vitamin A (at PAD 112 and 140) in serum. As noted in the descriptive analysis section, twenty calves (9 calves in the HVA and 11 calves in LVA group) showed either BCoV fecal or nasal shedding at least one time between PAD 0 and 4. For these twenty calves, the ratios of IgG1 to IgG2 Abs were calculated in the HVA and the LVA group (Figure 2.8). After natural BCoV infection, the ratios of IgG1 to IgG2 Abs tended to be higher at PAD 35 than at PAD 0 in the HVA ($p = 0.0881$) and in the LVA ($p < 0.05$) group (Figure 2.8.A). Likewise, the twenty calves naturally infected with BCoV had predominantly serum IgG1 BCoV Ab at PAD 35. After vaccination, the ratios of IgG1 to IgG2 Abs in the HVA group were significantly higher at PAD 140 than at PAD112 ($p < 0.05$) (Figure 2.8.B). In contrast, in the LVA group, no significant differences in the ratios of IgG1 to IgG2 Abs were observed between PAD 112 and 140. In addition, at PAD 140, the ratios of IgG1 and IgG2 Abs tended to be higher in the HVA than in the LVA group ($p = 0.06$). In summary, IgG1 BCoV Ab responses to BCoV vaccine were compromised in the low vitamin A fed-calves which previously showed elevated IgG1 BCoV Ab responses to natural BCoV infection, suggesting a differential effect of low vitamin A on secondary IgG1 Ab
2.5 DISCUSSION

We studied the effects of two vitamin A diets on the immune responses to enteric bovine coronavirus and rotavirus vaccines administered by the parenteral route in feedlot calves. We also determined natural infection and pre-existing Abs to BCoV after arrival to the feedlot, before serum vitamin A decrease and vaccination. We investigated BCoV shedding using real-time RT-PCR and Ab responses to BCoV using isotype-specific Ab ELISA in calves treated with different vitamin A diets. The numbers of BCoV infections doubled from PAD 0 to 4. In addition, BCoV fecal shedding occurred during the first 4 days, whereas BCoV nasal shedding was only detected at PAD 4. The BCoV shedding pattern observed in this study may reflect fecal-oral transmission of BCoV in which BCoV fecal shedding precedes nasal shedding. A similar transmission pattern has been reported previously (25, 31). It has also been suggested that BCoV replication in the upper respiratory tract may result in subsequent BCoV enteritis in which the virus is swallowed and infects the enterocytes of the small intestine, suggesting that calves may have BCoV respiratory tract infections prior to or simultaneously with BCoV enteric tract infections (31, 33, 65, 66, 79). However, it was not the case in our study since fecal shedding preceded nasal shedding. Monitoring BCoV shedding before and after arrival at feedlot may be required to elucidate the mechanisms of initial replication and the transmission route of BCoV in feedlot calves.

In our study, pre-existing serum BCoV Ab titers were negatively correlated with BCoV shedding, suggesting that pre-existing serum IgA BCoV Abs may play an
important role in preventing fecal and nasal shedding, and pre-existing serum IgM BCoV Abs in preventing nasal shedding. Particularly, serum IgA Ab responses to coronavirus (CoV) have been reported and demonstrated as a useful indicator for protection in several studies. A negative association between serum IgA BCoV Ab titers and diarrhea duration was shown in dairy calves naturally infected with BCoV (31). Serum IgA Ab was induced in swine inoculated orally with transmissible gastroenteritis virus (TGEV) and neutralizing Abs were also detected from the IgA Ab fraction by gel filtration chromatography (41). The number of IgA Ab secreting cells (ASCs) in blood significantly correlated with protection in piglets orally inoculated with virulent porcine epidemic diarrhea virus (PEDV) (23). Chickens previously exposed to infectious bursal disease virus (IBDV) were inoculated intranasally with embryo-adapted infectious bronchitis virus (IBV, M41 strain) and produced negligible levels of serum IgA and IgG Abs which may account for increased susceptibility to IBV infection (80). In addition, one out of six patients with severe acute respiratory syndrome (SARS)-CoV pneumonia who died, showed serum IgA Ab titers below detection levels using SARS-CoV nucleocapsid protein based ELISA (86).

Therefore, the systemic BCoV Abs detected may have originated from virus specific-B cells stimulated in the gut, which migrated to the systemic lymphoid tissue during the lymphocyte homing process. Thus, early virus-specific Abs and ASCs in the circulation may be used as an early indicator of intestinal infection (22, 45, 55, 87). Transiently circulating mucosal lymphocytes initially activated by Vibrio cholerae O1 were detected in patients with acute cholera (two days after onset of illness), resulting in higher titers of V. cholerae immunogen specific IgA Ab in serum than in feces (55). In
conventional pigs orally inoculated with PEDV, PEDV specific IgA ASCs were detected in blood at post-inoculation day (PID) 14, earlier than in mucosal associated lymphoid tissues at PID 21 (22). Turkey poults orally infected with Turkey coronavirus (TCV) produced TCV-specific IgA Ab in serum after 1 week post-infection (PI), whereas the IgA in feces was detectable after 4 week PI (45). In gnotobiotic pigs orally inoculated with virulent Wa strain human rotavirus (HRV), transient trafficking of HRV specific IgA ASCs in peripheral blood were detected at challenge, which led subsequently to high protection rate against virus shedding and diarrhea at post-challenge days (87). Likewise, although BCoV replicates in the intestinal epithelium and induces intestinal immunity, serum immune responses could be considered as an indicator of protection at early time points after infection. In addition, we cannot exclude the possibility of a transient viremic phase in BCoV infection to explain the elevated serum IgA BCoV Ab which may be produced by systemically activated B cells. Transient BCoV viremia was detected in calves orally inoculated with a winter dysentery (WD) BCoV strain at post-inoculation day (PID) 3 (53).

In our study, the association between pre-existing Ab in serum and BCoV shedding revealed that calves with GMTs of serum IgA Ab of 832 had a 50% probability of BCoV fecal shedding; GMTs of serum IgA BCoV of 92 and of serum IgM BCoV Ab of 3 had a 50% probability of BCoV nasal shedding. Thus, according to our findings, it is possible that vaccination of calves against BCoV prior to their arrival at feedlots to produce serum IgA BCoV Ab GMTs greater than 832 or 92 might provide protection of 50% or higher against BCoV fecal shedding or nasal shedding, respectively. It is remarkable that only the very low titer of IgM BCoV Ab was associated with the reduced

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BCoV nasal shedding. Compared with the serum IgA BCoV Ab titers, the serum IgM BCoV Ab titers were approximately 30-fold more efficiently associated with protection against BCoV nasal shedding. Secretory IgM, one of the polymeric Abs along with secretory IgA, has been identified to be more effective in neutralization of virus infection (8, 85). At a lower concentration, the HIV-1 specific IgM expressed in vitro inhibited more efficiently HIV-1 infection of peripheral blood mononuclear cells (PBMCs) (up to 28-fold) than the HIV-1 specific IgG did (85). In addition, the experiment of neutralizing Abs against HIV transcytosis demonstrated that IgM isolated from HIV-positive patients showed more efficacy of the neutralization (up to 80% inhibiting HIV transcytosis) than IgA did (up to 60% inhibiting HIV transcytosis) (8). It was demonstrated that the polymeric structure of Ab enhances the efficacy of virus neutralization due to its higher avidity and steric hindrance for virus receptor binding activity (17, 59). Therefore, the IgM, pentameric form of Ab, is expected to more effectively inhibit virus infection. Moreover, it was demonstrated that calves inoculated orally and intranasally with BCoV developed serum IgM BCoV Ab response to hemagglutinin-esterase (HE) antigen, which elicited virus infectivity-neutralizing Abs (32). It was also demonstrated that serum IgM BCoV Ab response in cattle with shipping fever pneumonia was shown to be closely related to hemagglutinin-inhibiting Ab activity (43).

Storage and release of vitamin A are dependent upon serum retinol concentration; hepatic vitamin A is released into peripheral blood to maintain the serum retinol concentration at approximately 30ug/dl in cattle (34). However, once the hepatic vitamin A concentration reaches a certain minimum threshold, the serum retinol concentration starts to decrease, resulting in failure of serum retinol homeostasis (77). In our study, the
vitamin A concentration used in the HVA group, i.e., 3300 IU/kg of dietary DM maintained the serum retinol concentration between PAD 112 and 140; however, the vitamin A concentration used in the LVA group, i.e., 1100 IU/kg of dietary DM failed to show homeostasis of serum retinol concentration, suggesting that the minimum threshold concentration of the hepatic vitamin A required to decrease the serum retinol concentration may be reached between PAD 112 and 140.

A low vitamin A dietary regimen has been frequently used to increase the amount of intramuscularly deposited fat, or marbling, one of the criteria for higher quality beef production in terms of meat palatability (1, 29, 71). To our knowledge, whether the low vitamin A dietary regimen used in feedlot calves increases the incidence of infections or diseases or reduces vaccine efficacy or immune responses has not been reported. However, it was demonstrated that dairy calves fed a low vitamin A experimental diet developed diarrhea and fever (77). Male Holstein calves fed 2300 IU/kg of dietary DM (3800 IU/kg of dietary DM recommended by the NRC for dairy calves) showed decreased serum vitamin A concentrations during 28 days. Characteristic vitamin A deficiency signs were not observed in the calves; however, a significantly higher incidence of high fecal scores (more watery feces) and high rectal temperatures were observed. The incidence of febrile calves was approximately three times higher in calves fed 2300 IU/kg of dietary DM than in those fed vitamin A concentrations greater than NRC recommendation (77). Thus, optimized low vitamin A concentrations designed for higher quality dairy and/or beef production, but without detrimental effects on animal health, are desirable. In our study, our serological assays revealed the possibility of reduced BCoV vaccine effectiveness associated with the low vitamin A dietary regimen.
It has been suggested that IgG is the major BCoV-specific virus neutralizing and hemagglutination-inhibiting Ab in bovine serum (43). According to previous studies from our laboratory, IgG1 Ab titers to BCoV closely paralleled the virus neutralizing Ab responses in serum (25, 62). In addition, spike (S) and hemagglutinin-esterase (HE) glycoproteins contain the virus antigenic neutralizing epitopes (44). In our study, IgG1 BCoV Ab responses to inactivated BCoV vaccine were compromised in calves under the low vitamin A dietary regimen at PAD 140 (Figure 2.7.A and C). Thus, calves fed with low concentrations of vitamin A could be more susceptible to BCoV in the feedlot or vaccines such as those to BCoV may fail to enhance Ab responses.

The relationship between vitamin A and IgG Ab response has been reported for in vitro and in vivo studies. Retinoic acid, an active metabolite of vitamin A, at concentrations ranging from $10^{-12}$ to $10^{-14}$ M augmented IgG synthesis from formalinized *Staphylococcus aureus* stimulated peripheral blood mononuclear cells (PBMC) (4). In addition, retinol binding protein (RBP) deficient mice had total IgG levels in serum less than approximately 50% of those of wild type mice (56). We demonstrated that a low vitamin A diet (1100 IU/kg) can have a significant impact on Ab responses to a BCoV vaccine in calves. The inactivated BCoV vaccine induced a relatively high titer of IgG1 BCoV Ab in the HVA group, but it did not increase IgG1 BCoV Ab titers in the LVA group. Thus, the prolonged low vitamin A dietary regimen might reduce the immune responses to other vaccines, resulting in compromised Ab responses.

Previous studies have defined vitamin A deficiency in cattle over a wide range of serum retinol concentrations e.g., less than 7-8 µg/dl or less than 20 µg/dl, were suggested and associated with clinical signs of vitamin A deficiency in growing calves (9,
24, 77). In our study, vitamin A deficiency was not induced in the LVA group. However, the serum retinol concentration induced by 1100 IU/kg of dietary DM during 140 days, i.e., 25.1 µg/dl may be low enough to compromise vaccine-induced IgG1 (Th2) Ab responses in calves.

The influence of vitamin A as a micronutrient for enhancing Ab responses is dependent upon the nature of the antigen or pathogen. The Ab response to five different bacterial antigens, e.g., polysaccharides from *Streptococcus pneumoniae* and *Neisseria meningitidis*, lipopolysaccharides from *Pseudomonas aeruginosa* and *Serratia marcescens*, and tetanus toxoid, was investigated in vitamin A-deficient rats (54). Serum IgM Ab concentrations in vitamin A-deficient rats immunized with polysaccharides from *Streptococcus pneumoniae* and *Neisseria meningitidis*, and tetanus toxoid were very low or negligible compared with control rats. In contrast, almost normal IgM Ab concentrations were observed in vitamin A-deficient rats immunized with lipopolysaccharides from *Pseudomonas aeruginosa* and *Serratia marcescens* (54). The results indicate that compromised Ab responses in vitamin A deficiency are dependent upon the type of antigen: polysaccharides from *Streptococcus pneumoniae* and *Neisseria meningitidis* classified as T cell independent (TI) type 2 antigens; lipopolysaccharides from *Pseudomonas aeruginosa* and *Serratia marcescens* as TI type 1 antigens, and tetanus toxoid as a T cell dependent (TD) antigen. Thus, Ab responses were compromised to TI type 2 and TD, but normal to TI type 1 antigens in immunized vitamin A deficiency rats (54). Other animal coronaviruses such as transmissible gastroenteritis virus (TGEV) have been demonstrated to be TD antigens which elicit CD4+ T lymphocytes and B lymphocytes to synthesize neutralizing Abs (2, 3). Thus,
decreased vitamin A compromised serum IgG1 Ab responses in calves given an inactivated BCoV vaccine.

The dominant IgG1 Ab response in calves naturally infected with BCoV or vaccinated with inactivated BCoV in the HVA group is consistent with previous reports (14-16, 46). The T lymphocytes from vitamin A deficient mice over-produced interferon (IFN)-γ which diminished IgG1 secreting B cells and decreased IgG1 production. However, retinoic acid (RA), the active metabolite of vitamin A, decreased T cell secretion of IFN-γ in supplemented mice and fully restored IgG1 production (14-16). In addition, RA positively regulated tetanus toxoid (TT) specific-serum IgG1 and IgG2b, but negatively regulated IgG2a in primary and secondary responses to TT, resulting in a significantly elevated ratios of IgG1 to IgG2a Abs, an indicator of Th2/Th1 lymphocyte imbalance (46). The ratios of IgG1 to IgG2 Abs were used in our study to assess dominance of Ab immune responses (IgG1, Th2) over cell-mediated immune responses (IgG2, Th1) in cattle (27, 42, 83). The IgG subclass expression patterns in cattle are also related to Th1 or Th2 lymphocyte responses: IL-4 and IL-13 induced IgG1 preferentially over IgG2, whereas IFN-γ and IL-12 strongly induced IgG2 over IgG1, respectively (11, 19, 26). According to the relatively higher IgG1 over IgG2 BCoV Ab titers in our study, the results suggest that vitamin A treatment also modulates adaptive immune responses to Th2-like (IgG1) responses in cattle. However, interpretation of bovine cell-mediated-immune responses is complex, because in cattle polarization of T lymphocytes differs from that of human and mice. Most infectious agents stimulate both Th1 and Th2 lymphocytes, expressing both IFN-γ and IL-4 and also unpolarized CD4+ Th0 lymphocytes, which enhance both IgG1 and IgG2 production and also co-expressed IFN-
Additional studies are needed to answer remaining questions on the effect of vitamin A status on immune response and animal health. (i) Do different vaccination routes produce different Ab responses with high and low vitamin A dietary regimens? In our study, calves naturally infected with BCoV showed enhanced serum IgA BCoV Ab titers, whereas intramuscularly vaccinated calves did not show significant serum IgA BCoV Ab responses, reflecting that oronasally or live BCoV exposed animals may develop different Ab responses than ones parenterally inoculated with inactivated BCoV vaccine. Thus, investigation of Ab responses according to vitamin A status, different vaccination routes and vaccine types is critical to determine the adjuvant effect of vitamin A. (ii) Do mucosal immune responses differ between the high and low vitamin A dietary regimen? It is difficult to assess mucosal Ab response with our data because of the paucity of significant fecal IgA Ab responses to BCoV. In this regard, B lymphocyte migration to gut-associated lymphoid tissue (GALT) has been studied with emphasis on integrin α4β7, GALT associated dendritic cells (DCs), mucosal addressin cell adhesion molecule-1 (MAdCAM-1), CCR9, and retinoic acid (RA) (10, 48). (iii) Do memory cells induced by primary infection under normal vitamin A concentrations function properly after secondary stimulation with vaccines under low vitamin A concentrations? Or, do memory cells induced by primary infection under low vitamin A concentrations function properly in secondary stimulation with vaccines under normal vitamin A concentrations? Investigations to correlate vitamin A status and function of memory cells could have a major impact on vaccine development.

To our knowledge, this is the first study to analyze the impact of a micronutrient,
i.e., vitamin A on Ab response to BCoV in feedlot calves. The vitamin A status in feedlot calves had a significant effect on Ab response to an inactivated BCoV vaccine. Serum IgG1 BCoV Abs were induced predominantly under the high vitamin A dietary regimen, whereas they were compromised under the low vitamin A dietary regimen, suggesting that the low vitamin A diet suppresses Th2 associated Ab (IgG1) stimulation.


28. **Frey, A., J. Di Canzio, and D. Zurakowski.** 1998. A statistically defined...


56. Quadro, L., M. V. Gamble, S. Vogel, A. A. Lima, R. Piantedosi, S. R. Moore,


Figure 2.1: Study design diagram. Arrows indicate the status of vitamin A stores in liver: grey and white arrows indicate prior to and after vitamin A decrease or depletion, respectively. Black horizontal bar represents study time frame crossed by vertical bars according to sampling and events, i.e., fecal, nasal, and blood sampling at PAD 0, 4, 35, 112 and 140, calves’ arrival at feedlot at PAD 0, decreased vitamin A concentration in serum at PAD 90, respiratory disease outbreak at PAD 88, vaccination at PAD 112, and booster at PAD 126. The dates in parenthesis refer to calendar dates.
<table>
<thead>
<tr>
<th>Frequency</th>
<th>PAD 0</th>
<th>PAD 4</th>
<th>PAD 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of calves</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>No. of infected calves(^a)</td>
<td>8</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Fecal shedding</td>
<td>8</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Nasal shedding</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Dual shedding</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Prevalence of infection (%)(^b)</td>
<td>21.1</td>
<td>41.0</td>
<td>0</td>
</tr>
<tr>
<td>Fecal shedding</td>
<td>21.1</td>
<td>41.0</td>
<td>0</td>
</tr>
<tr>
<td>Nasal shedding</td>
<td>0</td>
<td>20.5</td>
<td>0</td>
</tr>
<tr>
<td>Dual shedding</td>
<td>0</td>
<td>20.5</td>
<td>0</td>
</tr>
</tbody>
</table>

| BCoV RNA copies\(^c\) (pfu/ml) | Fecal Average (x10^6pfu/ml) | 7.39 | 13.9 | 0   |
| Nasal Average (x10^3pfu/ml)   | ND\(^d\)                  | 4.41 | 0    | 0   |

\(^a\) Calf was considered infected if it showed at least one positive sample (fecal or nasal) by real-time RT-PCR.

\(^b\) Two samples in the LVA group at PAD 0 and one sample in the HVA group at PAD 4 were missing so the prevalence of infection was calculated with n=38 and 39 at PAD 0 and 4, respectively.

\(^c\) Significant differences were observed in BCoV fecal shedding between PAD 0 and 4 (\(p < 0.05\)), and between BCoV fecal and nasal shedding at PAD 4 (\(p < 0.001\)).

\(^d\) Not detected.

**TABLE 2.1:** Frequency of BCoV shedding and BCoV RNA copy numbers in calves before vitamin A decreased in serum.
TABLE 2.2: Frequency of BCoV shedding and BCoV RNA copy numbers in calves after vitamin A decreased in serum.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PAD 112</th>
<th>PAD 140</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Frequency</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>High Vitamin A</strong></td>
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<td></td>
</tr>
<tr>
<td>No. of calves</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>No. of infected calves</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fecal shedding</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nasal shedding</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dual shedding</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Prevalence of infection (%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Low Vitamin A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of calves</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>No. of infected calves</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fecal shedding</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nasal shedding</td>
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<td>0</td>
</tr>
<tr>
<td>Dual shedding</td>
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<td>0</td>
</tr>
<tr>
<td>Prevalence of infection (%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>BCoV RNA copies (pfu/ml)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>High Vitamin A</strong></td>
<td></td>
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</tr>
<tr>
<td>Fecal Average (x10^6 pfu/ml)</td>
<td>ND^d</td>
<td>ND</td>
</tr>
<tr>
<td>Nasal Average (x10^3 pfu/ml)</td>
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<td>ND</td>
</tr>
<tr>
<td><strong>Low Vitamin A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fecal Average (x10^6 pfu/ml)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Nasal Average (x10^3 pfu/ml)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

^a Two calves in the HVA and three calves in the LVA group were euthanized or died by PAD 112.

^b Calf was considered infected if it showed at least one positive sample (fecal or nasal) by real-time RT-PCR.

^d Not detected.
**Figure 2.2:** Antibody isotype geometric mean titers (GMTs) to BCoV in serum and fecal samples of feedlot calves treated with vitamin A: IgG1 (A), IgG2 (B), IgA (C), IgM (D) and fecal IgA (fIgA) (E) are plotted for each PAD. Geometric mean titers (log10) in each vitamin A group are differentiated with symbols: ● in the HVA and ○ in the LVA group. Prior to and after vitamin A depletion from liver stores (at PAD 90-112) is indicated by A+ and A-, respectively. Vaccination at PAD 112 and booster at PAD 126 is marked with V and B, respectively. Vertical bars denote 95% confidence intervals.
Figure 2.3: Serum and fecal BCoV specific antibody isotype geometric mean titers (GMTs) in BCoV shedding (PAD 0 and 4) and non-shedding calves: IgG1 (A), IgG2 (B), IgA (C), IgM (D) and fecal IgA (fIgA) (E) are plotted for each PAD. Geometric mean titers (log_{10}) in each BCoV shedding group are differentiated with symbols: ● BCoV shedding and ○ non-shedding calves. Vertical bars denote 95% confidence intervals. Significant differences between BCoV shedding and non-shedding calves at PAD 0 is indicated with asterisk; *, $p < 0.05$ and **, $p < 0.001$. 
Figure 2.4: Comparison of antibody isotype geometric mean titers (GMTs) and BCoV RNA levels between the HVA and LVA group at PAD 0. Base 10 logarithm transformed-antibody GMTs and -BCoV RNA copy numbers are plotted according to vitamin A treatments. Serum IgG1 (A), IgG2 (B), IgA (C) and IgM (D) BCoV Ab GMTs are depicted as ● in the HVA and ○ in the LVA groups. Whiskers represent 95% confidence intervals. Fecal IgA (fIgA) (E) Ab GMTs and BCoV RNA levels (F) are depicted as ■ in the HVA and □ in the LVA groups. Whiskers represent non-outlier range. One-way ANOVA was used to compare serum antibody titers (A–D) between the HVA and LVA groups. The Mann-Whitney test was used to calculate the differences between the HVA and LVA group for fecal IgA titers (E) and BCoV RNA levels (F).
Figure 2.5: Negative association between probability of BCoV shedding detected by real-time RT-PCR and antibody geometric mean titers (GMTs) at PAD 4. A logistic regression model was used to represent the effect of pre-existing antibody on the pattern of BCoV fecal (A) and nasal (B) shedding. Solid and dotted lines represent serum IgA and IgM, respectively.
Figure 2.6: Association between probability of seroconversion and BCoV fecal shedding in calves. The 2 x 2 contingency tables composed of two categorical data; BCoV shedding, and serum IgG1- (A) and IgA- (B) BCoV Ab seroconversion. Fisher’s exact test was used to calculate the association between frequency of calves showing BCoV shedding and IgG1- (A) or IgA- (B) BCoV Ab seroconversion. Logistic regression model was used to evaluate the effect of quantified BCoV RNA levels on the IgA- (solid line) and IgG1- (dotted line) BCoV Ab seroconversion (C).
**Figure 2.7:** IgG subclass antibody geometric mean titers (GMTs) responses to inactivated BCoV vaccine according to vitamin A status. The IgG subclass BCoV Abs: serum IgG1 (A) and IgG2 (B), and the ratios of IgG1 to IgG2 BCoV Abs (C) are plotted according to PAD 112 (before vaccination) and 140 (after vaccination). Differences in IgG subclass BCoV Ab GMTs and their ratio between PAD 112 and 140 were evaluated with repeated measures ANOVA, followed by multiple comparisons using Duncan’s multiple range test. Serum IgG1, IgG2 BCoV Ab GMTs and the ratios of IgG1 to IgG2 Ab GMTs are depicted as ● in the HVA and ○ in the LVA groups. Whiskers represent 95% confidence intervals. Significant differences between the HVA and LVA groups at PAD 112 and 140 are indicated with asterisk; *, p < 0.05.
Figure 2.7

**IgG1 BCoV antibody titers**

**Post-Arrival Day (PAD)**

**Ratios of IgG1 to IgG2 antibody GMTs**
Figure 2.8: The ratios of IgG1 to IgG2 antibody geometric mean titers (GMTs) responses to natural BCoV infection and to inactivated BCoV vaccination. Data from twenty calves previously shedding BCoV were used to plot the ratio of IgG1 to IgG2 at PAD 0 and 35 (natural infection, A), and PAD 112 and 140 (vaccination, B). Repeated measures ANOVA was used to compare titers and ratios between PAD 0 to 35 and between PAD 112 to 140, followed by multiple comparisons using Duncan’s multiple range test. Vitamin A status is symbolized as ● HVA group and ○ LVA group. Whiskers represent 95% confidence intervals.
CHAPTER 3

VITAMIN A EFFECTS ON BOVINE ROTAVIRUS ANTIBODY RESPONSES
IN FEEDLOT CALVES VACCINATED INTRAMUSCULARLY
WITH AN INACTIVATED BOVINE ROTAVIRUS VACCINE

3.1 SUMMARY

In the previous study, the prolonged low vitamin A dietary regimen reduced the antibody (Ab) response, especially serum IgG1 BCoV Ab, to an inactivated BCoV vaccine. However, it is unclear whether the low vitamin A dietary regimen has detrimental effects on immune responses to all vaccines or to only certain vaccines. Feedlot calves are susceptible to calf diarrhea (CD), caused by multiple etiological agents including bovine coronavirus (BCoV) and bovine rotavirus (BRV). In addition, co-infection by BCoV and BRV has been frequently observed in calves with severe gastroenteritis. Therefore, because calves are subject to multiple infectious diseases, it is important to understand whether vitamin A effects on Ab response to vaccines are pathogen-specific. Thus, Ab response to an inactivated BRV vaccine was investigated in feedlot calves fed either high (HVA group; 3300 IU/kg of dietary dry matter (DM), n=20) or low (LVA group; 1100 IU/kg of dietary DM, n=20) dietary vitamin A. All calves were vaccinated intramuscularly with the inactivated BCoV/BRV vaccine (Scourguard®)
3KC, Pfizer) at PAD 112 and boosted at PAD 126 to determine the effect of vitamin A status on vaccine-induced BRV Ab responses. After BRV vaccination and booster, all serum isotype Ab geometric mean titers (GMTs) were significantly higher at PAD 140 than at PAD 112 regardless of vitamin A treatment groups; however, no statistically significant differences were observed between the HVA and LVA groups at PAD 112 and 140. Thus, vitamin A dietary regimen did not affect Ab titers in response to an inactivated BRV vaccine. In the LVA group, Ab response to inactivated BRV and BCoV differed since serum IgG1 Ab response to the inactivated BCoV vaccine was compromised, whereas those to the inactivated BRV vaccine were significantly increased at PAD 140 compared with PAD 112. This study suggests that vitamin A effects on Ab responses may be vaccine antigen-dependent. Further studies should be performed with various infectious agents or additional vaccines to confirm pathogen-specific vitamin A effects on Ab response.

3.2 INTRODUCTION

Vitamin A is an essential micronutrient for epithelial cell integrity, cell proliferation, cell differentiation, apoptosis, general development, and immune function (7, 50, 52, 53). Vitamin A supplementation has been considered a cost-effective intervention to decrease the incidence of diarrhea and fever in calves (20, 47, 49). Calf diarrhea (CD) is a common cause of morbidity and mortality in the first 3 weeks of life, resulting in economic losses, e.g., $95,500,000/year, the estimated average annual loss of calves from 1970 to 1976 (4, 15, 16, 18, 28, 31, 41). Multiple infectious agents have been involved in CD, e.g, *Escherichia coli*, *Salmonella* spp., *Clostridium perfringens*,
Cryptosporidium, Coccidia, coronavirus and rotavirus (4, 25-27, 38, 41, 45, 51). In addition, the CD is influenced by several factors including herd management, hygienic status, housing facilities, host nutritional status, vaccination and month of birth (8, 33). Bovine rotavirus (BRV), especially group A BRV, has been considered the major etiological agent of calf diarrhea worldwide (4, 15, 23, 27, 31, 32).

Rotavirus (RV) belongs to the family Reoviridae, characterized by non-enveloped triple-layered viral particles and 11 double-stranded (ds) RNA segments. Villous atrophy of the small intestine is the major histopathologic lesion caused by BRV infection, resulting in disruption of absorption and consequently producing intensive diarrhea. Specific interaction of RV infection or immune response to RV and vitamin A deficiency was demonstrated only in a mouse model (1, 2). Vitamin A deficient mice orally inoculated with rotavirus showed complete destruction of the villous tips, exposing the underlying lamina propria, indicating that vitamin A deficiency accentuated rotavirus related pathology (2). In addition, impaired serum Ab and cell-mediated immune responses to rotavirus were observed in vitamin A deficient mice (1).

A vitamin A restricted dietary regimen for feedlot calves has frequently been used to increase intramuscular fat, or marbling, resulting in higher beef carcass value in terms of meat palatability. Because negative effects of vitamin A deficiency on immune response to RV were demonstrated in the mouse model, we hypothesized that feedlot calves fed low vitamin A concentrations might have compromised Ab responses to a BRV vaccine. To date, many researchers have investigated immune response to BRV in experimentally or naturally infected calves and adult cattle (3, 40-42). However, to our knowledge, the association between immune response to BRV vaccines and host
nutritional status, i.e., vitamin A in feedlot calves has not been studied. Thus, in our study, Ab response to an inactivated BRV vaccine in calves fed with high (HVA) and low vitamin A (LVA) dietary regimens was examined. In addition, we also examined if there was a significant effect of vitamin A dietary level on morbidity and mortality from diarrheal disease in general; however, it is unclear whether vitamin A has an effect on the immune responses to all vaccines against diarrheal pathogens or to vaccines against specific pathogens. Because CD has a multiple causal etiology, it is still an issue to be addressed. Therefore, we compared the Ab responses to inactivated BRV vaccine with our previously analyzed Ab responses to the inactivated BCoV vaccine, which possibly could elucidate whether or not vitamin A effects on Ab response to vaccines is antigen-specific.

Our findings indicate that there were no statistically significant differences between the LVA and the HVA groups in terms of Ab responses to inactivated BRV vaccine, whereas the LVA dietary regimen compromised the IgG1 BCoV Ab titers and the ratios of IgG1 to IgG2 Abs in response to the inactivated BCoV vaccine. Thus, vitamin A effects on Ab responses seems to be antigen-dependent, although the two different inactivated viruses are given simultaneously to the calves.

3.3 MATERIALS AND METHODS

The study design is briefly diagrammed with the number of calves, vitamin A status, study time points, samplings and events in Figure 2.1.

**Feedlot calves and vitamin A dietary regimen.** This study commenced on 10/05/2006 when forty Angus calves (199, 163 and 220 days old in average, minimum
and maximum age, respectively) arrived at The Ohio State University feedlot from two OARDC branch experimental stations located in Belle Valley and Coshocton, Ohio. The dates involved in this study were calculated from their arrival at feedlot (Post-Arrival Day: PAD). Calves were vaccinated and boosted for infectious bovine rhinotracheitis, parainfluenza-3, *Haemophilus somnus*, *Pasteurella* and *Clostridium* (Quadraplex, Somnugen 2P, and Dybelon, respectively; Bioceutic, St. Joseph, MO) and dewormed with Ivomec pouron (Merck, Rahway, NJ) six weeks prior to arrival at the feedlot. Calves (N total = 40) were randomly assigned to two groups at PAD 0: high vitamin A (HVA, N HVA = 20) and low vitamin A (LVA, N LVA = 20) groups. High and low dietary vitamin A were defined as greater than or less than the 2200 international units (IU)/kg of dietary dry matter (DM), respectively, as recommended by the National Research Council. The LVA group received 1100 IU/kg of dietary DM to simulate low levels in the vitamin A restricted feedlot calves and the HVA group received 3300 IU/kg of dietary DM. Supplemental vitamin A was fed at PAD 0, then daily throughout the 140 day study period (PAD 140). Each calf was isolated in a pen with 3.9 m² of floor space and fed approximately 7.5 kg of feed/day individually during the experiment. The feed was composed of (DM basis %) 5% corn silage, 80% whole shell corn and 15% supplements. Supplements for the HVA group contained 0.06% vitamin A, whereas those for the LVA group contained no vitamin A. In the LAV group, vitamin A in serum was assumed to decrease at approximately 90-112 days after the low vitamin A diet started, resulting in an increased difference of serum vitamin A concentrations between the HVA and LVA groups (24). The hypothetical vitamin A status in serum is indicated by the grey and white arrows representing prior to and post serum vitamin A decreases, respectively.
(Figure 2.1). Two calves in each group were euthanized on 01/22/07 and one calf in the LVA group died on 01/06/07.

**Antibiotic treatments.** An unexpected respiratory disease outbreak occurred on 01/01/2007 (PAD 88). At PAD 88, all calves were treated with antibiotics for a respiratory disease. Banamine was administered if rectal temperatures of calves was equal to or greater than 103.5 °F. Draxxin was administered as the first antibiotic for a calf with temperatures ≥ 103.5 °F. Nuflor or Excenel was administered as the second choice antibiotic if no response occurred to the first antibiotic, Draxxin. As the last choice antibiotic, Micotil was administered if no response to the second antibiotics, Nuflor and Excenel occurred.

**Vaccination and booster.** A commercial vaccine (ScourGuard 3©(K)C, Pfizer, New York, NY), composed of inactivated BCoV Mebus strain, Rotavirus G6 and G10, *Escherichia coli* (*E. coli*) K99 bacterin and Clostridium perfringens Type C toxoid, was used for vaccination at PAD 112 and booster at PAD 126. This vaccine is adjuvanted with Quil-A Saponin to enhance the immune response. Calves were immunized with 2 ml of the vaccine intramuscularly into neck at PAD 112 and boosted at PAD 126.

**Sampling.** Calves were sampled at PAD 112 and 140 to obtain nasal swab specimens, feces and blood. Nasal samples were diluted 1:5 in Minimum Essential Medium (MEM) containing 1% antibiotic-antimycotic, followed by centrifugation at 1000 x g for 11 minutes at 4 °C. Fecal samples were diluted 1:10 in MEM containing 1% antibiotic-antimycotic and centrifuged at 850 x g for 20 minutes at 4°C. Supernatants from nasal and fecal samples were stored at -70°C. Ten to 15ml of blood was collected via jugular venipuncture, followed by centrifugation at 2000 x g for 20 minutes. Serum
was collected, heat-inactivated at 56 ºC for 30 minutes and stored at -20ºC. Antibody-enzyme-linked immunosorbent assay (Ab-ELISA) was performed with serum and fecal samples to evaluate systemic and local antibody responses, respectively.

**Antibody ELISA.** An indirect Ab capture ELISA was used to measure BRV specific-serum IgG1, IgG2, IgA, IgM, and fecal IgA (fIgA). The 96-well ELISA plates (MaxiSorp high protein-binding capacity ELISA plates; Nunc, San Diego, CA) were coated with 100µl of monoclonal Abs against bovine IgG1 (1:500, Serotec, Raleigh, NC), IgG2 (1:500, Serotec, Raleigh, NC), IgA (1:500, Serotec, Raleigh, NC), and IgM (1:1000, Sigma, St. Louis, MO) in coating buffer (15mM Na₂CO₃, 35mM NaHCO₃, pH 9.6) overnight at 4°C. Plates were washed 4 times between incubations with phosphate-buffered saline (PBS, pH 7.4) and 0.05% Tween 20 (PBS-Tw). The plates were blocked with PBS/1% bovine serum albumin (EMD chemicals, Gibbstown, NJ) to prevent non-specific binding. After washing, a 4-fold dilution of the serum samples was added to the plates; dilutions from 1:400 to 1:1,638,400 was used for IgG1 and IgG2, and from 1:4 to 1:16,384 for IgA, IgM and fIgA detection. Serum samples out of the titer range were retested using dilutions greater than 1,638,400 for IgG1 and IgG2, and 16,384 for IgA, IgM and fIgA detection. Sera from gnotobiotic calves infected with the BRV IND strain or naïve calves were used as positive and negative controls, respectively. The negative controls were added in the same dilutions as the serum samples described above. After the plates were incubated for 1 hour at room temperature and washed, 100µl of optimally diluted BRV IND strain and mock infected African green monkey kidney cells were added to each well. After incubation for 1 hour at room temperature, 100µl of guinea pig anti-BRV IND strain hyperimmune sera diluted 1:5000 were added to each well. After 1
hour incubation at room temperature, 100µl of 1:2500 diluted goat anti-guinea pig IgG (H+L) horseradish-peroxidase (HRP) conjugated (KPL, Gaithersburg, MD) was added to the plates. After 1 hour incubation at room temperature, 100µl of HRP substrate [ABTS® Peroxidase Substrate System, equal ratio of 2,2’-azino-di-(3-ethylbenzthiazoline-6-sulfonate) to H2O2, KPL, Gaithersburg, MD] was added. After 20 minutes of incubation at room temperature, the optical density was measured at 405 nm with a microplate spectrophotometer (SpectraMax 340PC®, Sunnyvale, CA). The Abs detected from serum and fecal samples were quantified by endpoint titration, defined as the reciprocal of the highest dilution showing optical density values above the cut-off (average of negative controls + 2.077* standard deviation of negative controls) (22).

Statistical analysis. The immunoglobulin (Ig) titers quantified by Ab-ELISA were transformed to base 10 logarithms for statistical analysis. Repeated measures analysis of variance (ANOVA) was used to determine the statistical differences among PADs after analysis of homoskedasticity of dependent variables by means of Cochran C and Hartley Fmax. Although normality assumption was violated, repeated measures ANOVA was used on the basis of balanced sample size in comparison because of its robustness. Duncan’s multiple range test was used for multiple comparisons. In the 2 x 2 contingency tables, Fisher’s exact test was used to determine the association between two categorical data. In addition, the strength of relationship between two categorical data was analyzed with relative ratio (RR) calculated by dividing prevalence in exposed group by prevalence in unexposed group. Data analyzed with ANOVA were plotted with means and whiskers denoting 95% confidence intervals. All statistical analyses were performed with the Statistica 8 software package (StatSoft, Inc., Tulsa, Ok). All tests
were considered significant at a probability of $p < 0.05$.

3.4 RESULTS

Vitamin A status did not affect the pattern of antibody titer and frequency of calves that seroconverted to inactivated BRV vaccine. To determine the BRV Ab isotype responses to inactivated BRV vaccine, serum BRV Ab isotype titers IgG1, IgG2, IgA, IgM and fecal IgA (fIgA) at PAD 112 (before vaccination) and 140 (after vaccination) were measured and analyzed according to the vitamin A treatments (Figure 3.1). In addition, 2 x 2 contingency tables were constructed for each Ab isotype to calculate the strength of the relationship between vitamin A treatments and seroconversion (Table 3.1).

(i) **Serum IgG1 antibody.** Serum IgG1 BRV Ab titers in both vitamin A treatment groups were significantly higher at PAD 140 than at PAD 112 (GMTs, 10,159 at PAD 112 to 119,453 at PAD 140 in the HVA and 3,616 at PAD 112 to 102,400 at PAD 140 in the LVA group) ($p < 0.05$ in the HVA and $p < 0.005$ in the LVA group) (Figure 3.1.A and Table 3.1). However, there were no statistically significant differences between the HVA and LVA groups at either PAD (Figure 3.1.A). According to the 2 x 2 contingency table, vitamin A dietary regimen was not significantly associated with IgG1-BRV Ab seroconversion after vaccination, characterized by $p > 0.05$, Fisher’s exact test and 0.78 relative risk (RR) (0.47-1.32 95% confidence intervals).

(ii) **Serum IgG2 antibody.** Serum IgG2 BRV Ab titers in both vitamin A treatment groups were significantly higher at PAD 140 than at PAD 112 (GMTs, 159 at PAD 112 to 2,352 at PAD 140 in the HVA and 150 at PAD 112 to 1,883 at PAD 140 in
the LVA group) \( (p < 0.005) \) (Figure 3.1.B and Table 3.1). However, there were no statistically significant differences between the HVA and LVA groups at either PAD (Figure 3.1.B). According to the 2 x 2 contingency table, vitamin A dietary regimen was not significantly associated with IgG2-BRV Ab seroconversion after vaccination with \( p > 0.05 \), Fisher’s exact test and 0.87 RR (0.57-1.33 95% confidence intervals).

(iii) **Serum IgA antibody.** Serum IgA BRV Ab titers in both vitamin A treatment groups were significantly higher at PAD 140 than at PAD 112 (GMTs, 22.4 at PAD 112 to 645 at PAD 140 in the HVA and 33.3 at PAD 112 to 739 at PAD 140 in the LVA group) \( (p < 0.001) \) (Figure 3.1.C and Table 3.1). However, there were no statistically significant differences between the HVA and LVA groups at either PAD (Figure 3.1.C). According to the 2 x 2 contingency table, vitamin A dietary regimen was not significantly associated with IgA-BRV Ab seroconversion after vaccination with \( p > 0.05 \), Fisher’s exact test and 0.94 RR (0.67-1.31 95% confidence intervals).

(iv) **Serum IgM antibody.** Serum IgM BRV Ab titers in both vitamin A treatment groups were significantly higher at PAD 140 than at PAD 112 (GMTs, 40.3 at PAD 112 to 1,024 at PAD 140 in the HVA and 69.4 at PAD 112 to 1,024 at PAD 140 in the LVA group) \( (p < 0.001) \) (Figure 3.1.D and Table 3.1). However, there were no statistically significant differences between the HVA and LVA groups at either PAD (Figure 3.1.D). According to the 2 x 2 contingency table, vitamin A dietary regimen was not significantly associated with IgM-BRV Ab seroconversion after vaccination with \( p > 0.05 \), Fisher’s exact test and 1.18 RR (0.82-1.71 95% confidence intervals).

(v) **Fecal IgA antibody.** There were no statistically significant changes between PAD 112 and 140 in either vitamin A treatment group (Figure 3.1.E and Table 3.1).
addition, no significant differences in fIgA BRV Ab titers were observed between the HVA and LVA groups at either PAD (Figure 3.1.E). According to the 2 x 2 contingency table, vitamin A dietary regimen was not significantly associated with fIgA-BRV Ab seroconversion after vaccination with $p > 0.05$ in Fisher’s exact Test and 1.24 RR (0.93-1.64 95% confidence intervals).

In summary, all serum BRV Ab isotype titers were significantly higher at PAD 140 than at PAD 112. However, serum BRV Ab titers did not differ significantly between HVA and LVA groups in which the patterns of serum BRV Ab titers in the HVA and LVA groups were similar after vaccination, indicating that vitamin A dietary regimen did not affect Ab titers in response to inactivated BRV vaccine (Figure 3.1.A~E). In addition, there was no statistically significant association between vitamin A treatment and frequency of calves with seroconversion (Table 3.1).

**BRV vaccination induced different antibody responses than those induced by BCoV vaccination.** Levels of serum IgA, IgM and fIgA to BCoV were too low to allow comparison of isotype responses to BCoV and BRV vaccines. However, both serum IgG subclasses (IgG1, IgG2) were induced at higher levels after both BCoV and BRV vaccination. The serum BCoV and BRV IgG1 Ab titers in the HVA group were significantly higher at PAD 140 than at PAD 112 ($p < 0.005$ for BCoV and $p < 0.05$ for BRV) (Figure 3.1.A and F). However, serum IgG1 Ab titers induced by each inactivated virus showed distinctive patterns in the LVA group. Serum IgG1 BRV Ab titers were significantly higher after vaccination ($p < 0.005$), whereas serum IgG1 BCoV Ab titers were not (Figure 3.1.A and F). Patterns of serum IgG2 BCoV and BRV Ab titers were similar for both vitamin A dietary regimens (Figure 3.1.B and G). The serum IgG2
BCoV Ab titers in the HVA and LVA groups tended to be lower at PAD 140 than at PAD 112; however, they did not differ statistically (Figure 3.1.G). In contrast, the serum IgG2 BRV Ab titers in the HVA and LVA groups were significantly higher at PAD 140 than at PAD 112 ($p < 0.005$) (Figure 3.1.B). In addition, the ratios of IgG1 to IgG2 Abs were used to determine relative amount of IgG subclasses, resulting in distinct ratio patterns between inactivated BCoV and BRV vaccine according to vitamin A treatment groups (Figure 3.2). In the HVA group, the ratios of IgG1 to IgG2 BCoV Abs were significantly higher at PAD 140 than at PAD 112 ($p < 0.005$), whereas the ratios of IgG1 to IgG2 BRV Abs did not differ significantly between PAD 112 and 140 (Figure 3.2.A and B). In the LVA group, the ratios did not differ between PAD 112 and 140 for both inactivated BCoV and BRV vaccines (Figure 2.2.A and B). Vitamin A effects on Ab responses to inactivated BCoV vaccine was characterized by predominant IgG1 in the HVA group and compromised IgG1 responses in the LVA group (Figure 3.2.B). Unlike the response to the inactivated BCoV vaccine, calves showed dominant IgG1 Ab responses before and after BRV vaccination, regardless of vitamin A dietary regimen, indicating that vitamin A status may not affect Ab responses to inactivated BRV vaccine (Figure 3.2.A).

**3.5 DISCUSSION**

Calves receiving a high vitamin A dietary regimen showed different Ab responses to BRV and BCoV. Especially, from PAD 112 (before vaccination) to 140 (after vaccination), both IgG1 and IgG2 BRV Ab titers significantly increased. However, for BCoV, IgG1 Ab titers increased significantly, whereas IgG2 Ab titers did not. In cattle, the response of IgG subclasses to infectious antigens has been studied as a function of
distinct subsets of CD4+ T cells. Different subsets of CD4+ T cells functioned as helper T cells in response to Babesia bigemina rhoptry-associated protein-1 (RAP-1) and Fasciola hepatica (9, 10, 19). The helper T cells for Ab responses to B. bigemina RAP-1 were antigen-primed unpolarized CD4+ Th0 cells, characterized by co-expression of IFN-γ and IL-4 in parallel with enhancing both IgG1 and IgG2 Abs (10). Unlike B. bigemina, CD4+ Th2 cells were the major helper T cells in response to F. hepatica to produce Ab, especially IgG1 by strong expression of IL-4 but little IFN-γ (9, 10, 21).

The bovine immune responses to parasites may correlate with our findings on Ab responses to BRV and BCoV vaccination, suggesting that enhanced IgG1 and IgG2 BRV Ab titers may reflect BRV specific unpolarized CD4+ Th0 cell type responses assisting Ab production. Exposure of unprimed naïve T cells to BCoV antigen by antigen-presenting cells may induce differentiation into BCoV specific CD4+ Th2, producing predominantly IgG1 BCoV Ab. The characterization of cytokine profiles in response to BRV and BCoV vaccination and the association between Ab production and subset of helper T cell still need to be investigated.

In the low vitamin A dietary regimen, the patterns of IgG1 and IgG2 BRV Ab titers were significantly enhanced after vaccination, whereas BCoV IgG1 and IgG2 Ab titers did not change. Especially, at PAD 140 (after vaccination), the IgG1 BCoV Ab titers were compromised under the low vitamin A dietary regimen, but enhanced under high vitamin A dietary regimens, implying that Th2 cells induced by BCoV antigens are modulated by vitamin A status. Impaired Th2 cell activity by vitamin A deficiency has been demonstrated with compromised Ab responses and cytokine profiles. Vitamin A deficient mice in an asthma study showed reduced IgE and IgG1 levels in serum as well.
as IL-4 and IL-5 concentrations in bronchoalveolar lavage specimens (44). Strong Th2 cell responses in mice infected with *Trichinella spiralis* shifted to Th1 dominated response under vitamin A deficiency (13). It is difficult to interpret the increased IgG1 and IgG2 BRV Ab titers on calves fed low vitamin A concentrations, because to our knowledge, there are no available data on the effects of vitamin A deficiency on proliferation and function of antigen-primed unpolarized Th0 cells in cattle. The unpolarized Th0 cells may be less influenced by vitamin A status than Th2 cells. A study of bovine T helper subsets including Th0 cells are germane to understand bovine immune responses, because a variety of infectious antigens stimulated T cells that co-express IL-4 and IFN-γ in cattle (10, 11).

The nature of the antigen should also be considered when interpreting our study. The DCs derived from bovine peripheral blood mononuclear cells (PBMCs) have been reported to directly influence B cell activation and differentiation without T cell help (6). Resting B cells co-cultured with peripheral blood derived DCs along with IL-2 dramatically up-regulated IgG1 levels without enhancing IgG2 or IgA in parallel with high level Th2 cytokine transcripts, i.e., IL-13 and IL-10. In addition, the bovine PBMC derived DCs expressed CD40L on the surface, indicating that the DCs are potential regulators of Ab responses, because the engagement of CD40-CD40L is critical for B cell proliferation as well as isotype switching (17, 36). The effect of vitamin A on bovine PBMC derived DCs has been poorly studied. If low vitamin A dietary regimen has a negative effect on the DCs, it would explain the compromised BCoV IgG1 Ab response induced by intramuscularly administrated vaccine.

Unlike Ab responses induced by BCoV vaccine, all serum Ab isotypes were
significantly enhanced after inactivated BRV vaccination. Because suppression of helper T cells by low vitamin A dietary regimen is believed to occur (48, 54), Ab production and isotype switching may occur using a T cell independent pathway to BRV vaccination. Viral double-stranded RNA (dsRNA) has been shown to trigger class switch DNA recombination (CSR) from IgM to IgG and IgA by engagement of TLR3 on human B cells, indicating that B cells rapidly undergo CSR through a T cell-independent pathway (55). Upon exposure to polyinosinic/polycytidylic acid (poly(I:C), TLR3 expressing B cells activate NF-κB, a transcription factor critical for CSR, resulting in expression of activation–induced cytidine deaminase (AID), a DNA-editing enzyme essential for CSR. It will be interesting to investigate whether or not TLR3 engagement on B cells activates NF-κB under low vitamin A concentrations or regardless of vitamin A status to explain the enhanced IgG BRV Ab titers in both vitamin A treatment groups. In addition, to our knowledge, TLR7 or 8 associated CSR stimulated by ssRNA has not been reported. Elucidation of the mechanisms involved may provide further explanations for the different Ab responses to BRV (dsRNA virus) and BCoV (ssRNA virus) vaccination.

According to our findings, vitamin A effects on Ab responses seems to be antigen-specific. Epidemiologic serological studies of natural infection with enteric and respiratory pathogens was investigated in low (< 200µg/L) and normal (> 200µg/L) serum vitamin A levels in Ecuadorian children (12). Consistent with our results, rotavirus specific IgG titers in low vitamin A children were not significantly different from those in normal vitamin A children; however, significantly lower titers of respiratory syncytial virus specific IgG were observed in low vitamin A children. In addition, pathogen-specific vitamin A effects on infection and clinical outcomes were
demonstrated with *Escherichia coli* (*E. coli*) strains in Mexican children (30). Vitamin A supplementation reduced the prevalence of enteropathogenic *E. coli* (EPEC) infection (0.52 RR and 0.23-0.86 95% confidence intervals), compared with a placebo group. However, there was no statistically significant association between vitamin A supplementation and the prevalence of enterotoxigenic *E. coli* (ETEC) infection. In terms of the duration of *E. coli* infection, vitamin A supplemented children had significantly reduced duration of EPEC and ETEC infections, compared with the placebo group. The EPEC associated diarrhea (0.41 RR and 0.16-1.00 95% confidence intervals) and fever (0.15 RR and 0.02-0.98 95% confidence intervals) were also significantly reduced in vitamin A supplemented children. Moreover, the duration of EPEC associated diarrheal episodes was significantly shorter in vitamin A supplemented children than in the placebo group. However, vitamin A status had no effect on ETEC associated diarrhea, duration of diarrheal episodes and fever.

Although BCoV and BRV have the same tissue tropism to intestinal epithelial cells, histopathological effects, e.g., villous atrophy and clinical signs, e.g., diarrhea (5, 14, 29, 34, 35, 37, 39, 43, 46), Ab responses to BCoV and BRV were differentially affected under the low vitamin A dietary regimen. In order to confirm whether or not the vitamin A effect on gut-immunity is virus-dependent, additional studies using various infectious agents causing the CD are required as well as determining the influence of vitamin A in the intestinal microbiota.

In conclusion, vitamin A status did not affect the Ab responses in feedlot calves intramuscularly vaccinated with inactivated BRV. Comparisons between BCoV and BRV vaccination revealed that vitamin A effects on Ab responses seems to be virus-
dependent, resulting in different IgG subclass responses, although these two viruses share common features in terms of tissue tropism, histopathological effects and clinical signs.


44. Schuster, G. U., N. J. Kenyon, and C. B. Stephensen. 2008. Vitamin A deficiency decreases and high dietary vitamin A increases disease severity in the


TABLE 3.1: Geometric mean BRV antibody isotype titers (GMTs) and frequency of calves with seroconversion between Post-Arrival Day (PAD) 112 (before vaccination) and 140 (after vaccination).

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Vitamin A&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GMTs</th>
<th>Frequency&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PAD</td>
<td></td>
<td>Seroconversion</td>
</tr>
<tr>
<td></td>
<td>112</td>
<td>140</td>
<td>Yes</td>
</tr>
<tr>
<td>IgG1</td>
<td>High</td>
<td>10159</td>
<td>119453</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>3616</td>
<td>102400</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>6151</td>
<td>110842</td>
</tr>
<tr>
<td>IgG2</td>
<td>High</td>
<td>159</td>
<td>2352</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>150</td>
<td>1883</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>155</td>
<td>2111</td>
</tr>
<tr>
<td>IgA</td>
<td>High</td>
<td>22.4</td>
<td>645</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>33.3</td>
<td>739</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>25.4</td>
<td>753</td>
</tr>
<tr>
<td>IgM</td>
<td>High</td>
<td>40.3</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>69.4</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>52.5</td>
<td>1024</td>
</tr>
<tr>
<td>fIgA</td>
<td>High</td>
<td>1.85</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>1.28</td>
<td>2.45</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1.55</td>
<td>1.74</td>
</tr>
</tbody>
</table>

<sup>a</sup> There were two different vitamin A dietary regimens; high (3300 IU/kg of dietary dry matter (DM)) and low vitamin A (1100 IU/kg of dietary DM).

<sup>b</sup> Seroconversion was defined as an increase in BRV specific-antibody titers of at least four-fold between PAD 112 and 140. Fisher’s exact test and relative risk (RR) were used to determine the association between vitamin A treatments and seroconversion.
Figure 3.1: Antibody responses to inactivated BCoV/BRV vaccine according to vitamin A status. The plots depict BRV- (right column, A~E) and BCoV-specific antibody responses (left column, F~J). Serum antibody isotype GMTs, i.e., IgG1 (A and F), IgG2 (B and G), IgA (C and H), IgM (D and I) and fecal IgA (fIgA) (E and J) are plotted according to PAD 112 (before vaccination) and 140 (after vaccination). Each virus-specific antibody titer between PAD 112 and 140 was evaluated with repeated measures ANOVA, followed by multiple comparisons using Duncan’s multiple range test. In the graph, ● depicts HVA and ○ depicts LVA group, and whiskers represent 95% confidence intervals.
Figure 3.1

Bovine Rotavirus (BRV) vs. Bovine Coronavirus (BCoV)

**A. IgG1**
- HVA: Black Circle
- LVA: White Circle

**B. IgG2**
- HVA: Black Circle
- LVA: White Circle

**C. IgA**
- HVA: Black Circle
- LVA: White Circle

**D. IgM**
- HVA: Black Circle
- LVA: White Circle

**E. fIgA**
- HVA: Black Circle
- LVA: White Circle

**F. IgG1**
- HVA: Black Circle
- LVA: White Circle

**G. IgG2**
- HVA: Black Circle
- LVA: White Circle

**H. IgA**
- HVA: Black Circle
- LVA: White Circle

**I. IgM**
- HVA: Black Circle
- LVA: White Circle

**J. fIgA**
- HVA: Black Circle
- LVA: White Circle

**Post-Arrival Day (PAD)**

Virus-specific antibody titers (Log10 GMTs)
Figure 3.2: The comparison of ratios of IgG1 to IgG2 antibody geometric mean titers (GMTs) between inactivated BCoV and BRV vaccines according to vitamin A status. The ratios of IgG1 to IgG2 antibody responses to inactivated BRV (A) and BCoV (B) are plotted according to PAD 112 (before vaccination) and 140 (after vaccination), and classified with vitamin A dietary regimens. Differences in the ratios between PAD 112 and 140 were evaluated with repeated measures ANOVA, followed by multiple comparisons using Duncan’s multiple range test. For the ratios of IgG1 to IgG2 antibody titers, ● depicts the HVA and ○ depicts the LVA group, and whiskers represent 95% confidence intervals. Significant differences between the HVA and LVA group according to PADs is indicated with asterisk; *, p-value < 0.05.
LIST OF REFERENCES


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parameters determining antibody response induced by vaccination against bovine respiratory syncytial virus. Vaccine 24:4007-16.


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