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Antibody Responses of Cattle with Respiratory Coronavirus Infections during Pathogenesis of Shipping Fever Pneumonia Are Lower with Antigens of Enteric Strains than with Those of a Respiratory Strain

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The serum antibody responses of cattle with respiratory coronavirus infections during the pathogenesis of shipping fever pneumonia were analyzed with different bovine coronavirus antigens, including those from a wild-type respiratory bovine coronavirus (RBCV) strain (97TXSF-Lu 15-2) directly isolated from lung tissue from a fatally infected bovine, a wild-type enteropathogenic bovine coronavirus (EBCV) strain (Ly 138-3), and the highly cell culture-adapted, enteric prototype strain (EBCV L9-81). Infectivity-neutralizing (IN) and hemagglutinin-inhibiting (HAI) activities were tested. Sequential serum samples, collected during the onset of the respiratory coronavirus infection and at weekly intervals for 5 weeks thereafter, had significantly higher IN and HAI titers for antigens of RBCV strain 97TXSF-Lu15-2 than for the wild-type and the highly cell culture-adapted EBCV strains, with *P* values ranging from <0.0001 to 0.0483. The IN and HAI antibody responses against the two EBCV strains did not differ significantly, but the lowest titers were detected with EBCV strain L9-81.

The role of coronaviruses as respiratory pathogens in cattle was recently recognized when numerous coronavirus strains were isolated from the nasal secretions and lung tissues from cattle with fatal cases of shipping fever pneumonia (SFP). These isolates are referred to as respiratory bovine coronaviruses (RBCV) (18, 19). The enteropathogenic potential of coronaviruses was identified previously when these viruses were isolated from diarrhea fluid or intestinal samples from neonatal calves with severe diarrhea (7, 13). In addition, coronaviruses were implicated in winter dysentery of adult cattle (14). These coronaviruses are referred to as enteropathogenic bovine coronaviruses (EBCV). Significant phenotypic, antigenic, and genetic differences separate the newly recognized RBCV from EBCV (2, 3, 11, 18). Furthermore, infectivity-neutralizing (IN) monoclonal antibodies (MAb) distinguished between the EBCV wild-type strain LY138-3 and the highly cell culture-adapted EBCV strain L9-81, as well as the Norden vaccine strain (8).

Bovine coronaviruses are enveloped, have a positive single-stranded RNA genome of about 31 kb, and belong to the *Coronaviridae* family of the order *Nidovirales* (6). The spike (S) and the hemagglutinin esterase (HE) glycoproteins of the five structural proteins function in the infectious process through adsorption and coadsorption to the receptors on susceptible host cells (5, 6). The S and HE glycoproteins are both capable of hemagglutinating rodent and chicken red blood cells (RBC). The HE but not S glycoprotein facilitates the release of viruses adsorbed to RBC in the receptor-destroying enzyme (RDE) test mediated by an acetylesterase (AE) component of the HE (15–17, 20, 21). These two glycoproteins induce IN and hemagglutinin-inhibiting (HAI) antibodies. The objectives of the present investigations were to assess the antibody responses of cattle during a naturally occurring and experimentally monitored infection that had induced severe pneumonic signs, including SFP. The kinetics of IN and HAI antibody responses of these cattle were analyzed with antigens of an endogenous RBCV strain, which were isolated from lung tissue from a fatally infected bovine from this SFP epizootic, and with antigens of the wild-type and the highly cell culture-adapted EBCV strains. The analysis was conducted with sera sequentially collected from cattle which had primary and secondary immune responses to the RBCV infection.

MATERIALS AND METHODS

Experimental design. One hundred five 6- to 8-month-old cattle were involved in this specific epizootic and were subjected to nasal swab and blood sampling and testing at the time of assembly at the order-buyer's barn (day 0), after transport on day 7, and thereafter on days 14, 21, 28, and 35, during the pathogenesis of and recovery from SFP (10, 19). Nasal swab samples were taken for virological and bacteriological studies, while blood samples for serum harvest were collected for immunological investigations. The cattle were separated into five response groups based on clinical signs of respiratory tract diseases and results of RBCV isolations (10, 12, 18, 19). Response groups 1 and 5 were utilized in the present analysis. Response group 1 included 72 cattle that exhibited clinical signs of respiratory tract disease and that nasally shed RBCV on day 0, day 7, or both. Seven randomly selected cattle of the group shedding RBCV on day 7 were investigated in detail. Seven calves in response group 5 were also included in these serological investigations because they remained clinically healthy and RBCV isolation negative during the epizootic and the 5-week follow-up period. To analyze sequential serum samples from the 14 cattle selected for their IN and HAI antibodies, we used antigens of three specific strains of bovine coronaviruses.

Cell line, virus isolates, and virus purification. Clone G of human rectal tumor (HRT-18) cells was used at the 24th passage level for RBCV and EBCV prop-

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agation (19). The wild-type RBCV strain 97TXSF-Lu15-2 was used at its second passage in clone G cells after initial isolation from lung tissue from a calf that died on day 8 of the epizootic (18). The highly cell culture-adapted EBCV strain L9-81 was originally isolated from bovine fetal kidney cell cultures as the EBCV Mebus strain (10, 13, 14). The EBCV L9-81 strain was propagated in 75 sequential passages in different bovine cell cultures, and the final six passages were done in clone G cells. The virulent wild-type EBCV strain LY138-3 from intestinal samples of a diarrheic calf was initially maintained by oral inoculation of conventional calves with induction of enteritis (7). It was used as an antigen in its third clone G passage. Partially purified preparations of RBCV strain 97TXSF-Lu15-2 and EBCV strains L9-81 and LY138-3 were employed for serological tests (10, 12).

IN assay. Serum samples were prepared in quadruplicate and tested for IN activities according to a previously reported method (10, 11). Serum 1745 with an IN titer of 128 and BCV antibody-free serum from a normal calf with an IN titer of <8 were included as positive and negative controls, respectively (22, 24). The serum IN titers were expressed as the reciprocal of the highest serum dilution that completely inhibited cytopathic changes in 50% of the wells.

Assays for HA and RDE. The assays for hemagglutinin (HA) and RDE were performed as reported previously (20, 21) with washed rat RBC prepared as 0.5% suspensions with phosphate-buffered saline (pH 7.4) containing 0.05% bovine serum albumin.

HAI test. The HAI titers of serum samples were determined with partially purified virus stocks, which were diluted to contain 8 to 16 U of both HA and RDE as antigens (20, 21). Again, serum 1745 with an HAI titer of 128 was included as a positive control, while BCV antibody-free serum from a normal calf with an HAI titer of <8 was used as a negative control (22, 24). The serum HAI titers were expressed as the reciprocal of the highest dilution of serum sample that completely inhibited the aggregation of rat RBC.

Data analysis and statistical methods. The serum IN and HAI titers were transformed to base 2 logarithms for statistical analysis. All data were presented as means ± standard errors of the means. The IN and HAI activities of the two response groups were compared by an analysis of variance of repeated measures designed with a split-plot arrangement of treatments. Pairwise comparisons of treatment and day differences were assessed with Scheffe's test. Interaction effects were examined with pairwise *t* tests of least-squares means for preplanned comparisons of treatments at specific day levels. All tests were considered significant at a probability of <0.05.

RESULTS

Comparison of IN activities of serial serum samples with antigens of RBCV strain 97TXSF-Lu15-2 and EBCV strains L9-81 and LY138-3. The antibody responses with serum samples collected from cattle in response groups 1 and 5 (Fig. 1A and B) had significantly higher overall kinetics of IN activities against RBCV strain 97TXSF-Lu15-2 than against EBCV strain L9-81, with *P* values of 0.0001 and 0.0001, and EBCV strain LY138-3, with *P* values of 0.0001 and 0.0025, respectively. Significant differences in IN activities against RBCV strain 97TXSF-Lu15-2 and EBCV strain L9-81 were observed in serum samples collected from cattle in response group 1, with *P* values of 0.0439, 0.001, <0.0001, <0.0001, and <0.0001 for results on days 7, 14, 21, 28, and 35, respectively. Similarly, the cattle in response group 5 had significantly lower levels on days 7, 14, and 28, with *P* values of 0.0095, 0.0164, and 0.0164, respectively. Significant differences between serum IN activities against RBCV strain 97TXSF-Lu15-2 and these against the EBCV strain LY138-3 antigen were also detected for cattle in response group 1, with *P* values of 0.0029, <0.0001, 0.001, and <0.0001 for results for days 14, 21, 28, and 35, respectively, and for cattle of response group 5 on days 7, 14, and 28 (*P* values of 0.0446, 0.0399, and 0.0335, respectively). Interestingly, IN antibody titers against EBCV strains L9-81 and LY138-3 in cattle of response groups 1 and 5 did not differ significantly during this 5-week investigation.

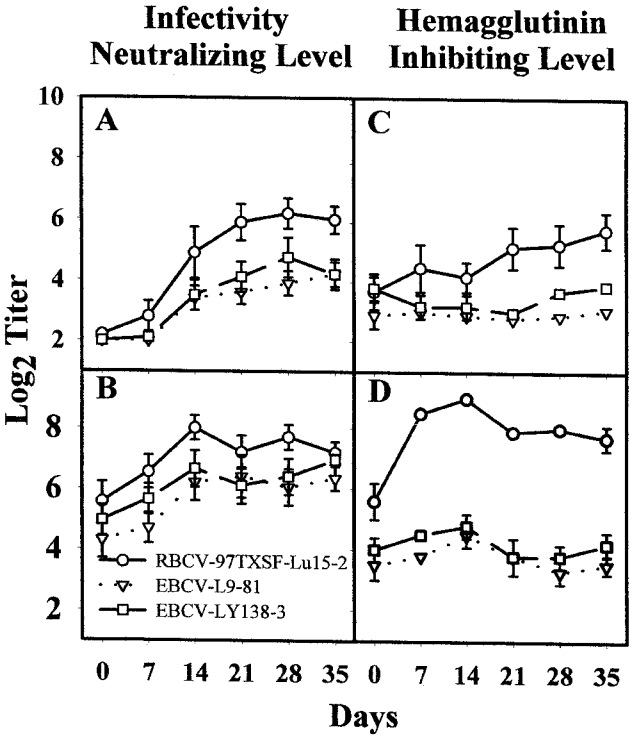

FIG. 1. IN (A and B) and HAI (C and D) antibody responses against RBCV strain 97TXSF-Lu15-2 (circles), EBCV strain L9-81 (triangles), and EBCV strain LY138-3 (squares) in serum samples from cattle. Cattle in response group 1 (A and C) shed RBCV on day 7 and had clinical signs of respiratory tract disease, while cattle in response group 5 (B and D) did not develop adverse clinical signs and remained virus isolation negative, despite exposure to the animals that were shedding virus (response group 1). Data are means ± standard errors of the means (error bars). Results are shown for seven cattle (A and C) and for seven cattle (B and D).

Comparison of HAI activities of serial serum samples with antigens of RBCV strain 97TXSF-Lu15-2 and EBCV strains L9-81 and LY138-3. The kinetics of the responses in HAI antibodies against RBCV strain 97TXSF-Lu15-2 were significantly greater than those against EBCV strain L9-81, with *P* values of 0.0001 and 0.0001 for the results for cattle of response groups 1 and 5, respectively. The differences between RBCV strain 97TXSF-Lu15-2 and EBCV strain LY138 were significant at a *P* value of 0.0001 for both groups 1 and 5 (Fig. 1C and D). The HAI activities against RBCV strain 97TXSF-Lu15-2 and EBCV strain L9-81 were remarkably different in serum samples collected from cattle in response group 1 for days 7, 14, 21, 28, and 35, respectively, with *P* values of 0.0483, 0.0004, 0.0035, <0.0001, <0.0001, and <0.0001. The HAI activities showed similar differences among cattle in response group 5 from day 0 onward with a *P* value of <0.0001. Levels of HAI antibodies against RBCV strain 97TXSF-Lu15-2 and EBCV strain LY138-3 were also substantially different for the cattle in response group 1 for days 7, 14, 21, 28, and 35 (*P* values of 0.0035, 0.0483, <0.0001, 0.0001, and <0.0001, respectively), and for the cattle in response group 5 for days 0, 7, 14, 21, 28, and 35 (*P* values of 0.0024, <0.0001, <0.0001, <0.0001, <0.0001, and <0.0001, respectively). Significant differences between serum HAI antibody kinetics against EBCV strain

L9-81 and EBCV strain LY138-3 were not observed in cattle of either response group.

DISCUSSION

Prospectively designed and experimentally controlled sampling and testing of cattle during a naturally occurring SFP epizootic created fortuitous circumstances for analyzing the kinetics of antibody responses of cattle to a native, wild-type RBCV infection (18, 19). This experiment also provided an excellent opportunity to compare antibody reactivities with both the wild-type RBCV and the highly cell culture-adapted and wild-type EBCV strains in serum samples collected from cattle involved in a naturally occurring RBCV infection. These conditions differ from experimentally induced infections that routinely involve giving virus inocula adapted to, and propagated in, cell cultures. Importantly, the IN and HAI antibody responses against RBCV were significantly greater than those detected with the wild-type EBCV strain LY138-3 and the highly cell culture-adapted EBCV. The EBCV strain L9-81 is virtually identical to the Norden vaccine strain that is used in attempts to protect against intestinal coronavirus infections of neonatal calves. Antigens of the highly cell culture-adapted EBCV strains reacted minimally with antibodies induced by naturally occurring RBCV infections. These findings call for analysis and a search for appropriate antigens in efforts to immunize cattle against these currently prevailing RBCV infections.

Bovine coronaviruses contain a large, single-stranded RNA genome and may mutate in the natural host to generate quasispecies differences (2, 3). Phenotypic changes may result from adaptation to, and propagation, of the virus in cell cultures, effecting the selection of host cell virus mutants. Differences in infectivity between the highly cell culture-adapted EBCV strain L9-81 and the wild-type strains of RBCV and EBCV consisted of a greatly expanded range of permissive host cells. This EBCV strain replicates readily, particularly in the presence of trypsin and in cultured bovine fetal kidney, spleen, thyroid, or adrenal cells, as well as in Vero cells (23). In contrast, the wild-type RBCV strains had restricted host cell ranges, because only clone G cells were permissive for initial isolation from clinical samples at low passage levels (18, 19, 23).

Differences between the RBCV and EBCV strains also were observed in the HA patterns, cell fusion, and AE activities (11, 19). Antigenic differences between RBCV and EBCV could not be detected by tests such as immunodiffusion immunofluorescence, immunoblotting, or enzyme-linked immunosorbent assays with antigens that include all the structural proteins of these coronaviruses (10, 12, 21, 22). MAb specific for S glycoprotein with IN activity distinguished between EBCV strain L9-81 and several wild-type strains of EBCV (8). Differences between EBCV strain L9-81 and the wild type strain LY138-3 of EBCV in inhibition of AE activity with HE-specific MAb (25) and in HA (4) reactivities were also detected.

Genotypic differences consisted of changes in the regions of the polymerase and associated genes, in the genes of S and HE glycoproteins, and in other nonstructural genes (2, 3, 11). Nucleotide and deduced amino acid mutations were within the A and B immunoreactive domains of the S-I subunit of the spike protein of the RBCV strains but not the EBCV strains, which could be the basis for the observed diversity in antigenic stimulation during these naturally occurring infections (2).

Comparatively, the differences in the kinetics of the primary bovine antibody responses to naturally occurring and experimentally monitored RBCV infections with the antigens of the wild-type RBCV and EBCV strains as well as the highly cell culture-adapted EBCV appear to be similar to responses of chickens to infections with the coronavirus of infectious bronchitis. Early antibodies generated during a primary immune response distinguished several serotypes of infectious bronchitis in both IN and HAI tests (1, 9).

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REFERENCES

1. **Cavanagh, D., and S. A. Naqui.** 1997. Infectious bronchitis, p. 511–521. *In* B. W. Calnek (ed.), Diseases of poultry, 10th ed. Iowa State University Press, Ames, Iowa.
2. **Chouljenko, V. N., K. G. Kousoulas, X. Q. Lin, and J. Storz.** 1998. Nucleotide and predicted amino acid sequences of all genes encoded by the 3′ genomic portion (9.5kb) of respiratory bovine coronaviruses and comparisons among respiratory and enteric coronaviruses. Virus Genes **17:**33–42.
3. **Chouljenko, V. N., X. Q. Lin, J. Storz, K. G. Kousoulas, and A. E. Gorbalenya.** 2001. Comparison of genomic and predicted amino acid sequences of respiratory and enteric bovine coronaviruses isolated from the same animal with fatal shipping fever pneumonia. J. Gen. Virol. **82:**2927–2933.
4. **Dea, S., L. Michaud, and G. Milane.** 1995. Comparison of bovine coronavirus isolates associated with neonatal calf diarrhea and winter dysentery in adult dairy cattle in Québec. J. Gen. Virol. **76:**1263–1270.
5. **Deregt, D., G. A. Gifford, M. K. Ijaz, T. C. Watts, J. E. Gilchrist, D. M. Haines, and L. A. Babiuk.** 1989. Monoclonal antibodies to bovine coronavirus glycoproteins E2 and E3: demonstration of in vivo virus-neutralizing activity. J. Gen. Virol. **70:**993–998.
6. **De Vries, A. A. F., M. C. Horzinek, P. J. M. Rottier, and R. J. De Groot.** 1997. The genome organization of the *Nidovirales:* similarities and differences between arteri-, toro-, and coronaviruses. Semin. Virol. **8:**33–47.
7. **Doughri, A. M., J. Storz, I. Hajer, and H. S. Fernando.** 1976. Morphology and morphogenesis of a coronavirus infecting intestinal epithelial cells of newborn calves. Exp. Mol. Pathol. **25:**355–370.
8. **Hussain, K. A., J. Storz, and K. G. Kousoulas.** 1991. Comparison of bovine coronavirus antigens: monoclonal antibodies to the spike glycoprotein distinguish between vaccine and wild-type strains. Virology **183:**442–445.
9. **Kusters, J. G., H. G. M. Niesters, J. A. Leustra, M. C. Horzinek, and B. A. M. Van der Zeist.** Phylogeny of antigenic variants of avian coronavirus IBV. Virology **192:**217–221.
10. **Lin, X., K. L. O'Reilly, M. L. Burrell, and J. Storz.** 2000. Infectivity-neutralizing and hemagglutinin-inhibiting antibody responses of respiratory coronavirus infections of cattle in pathogenesis of shipping fever pneumonia. Clin. Diagn. Lab. Immunol. **8:**357–362.
11. **Lin, X. Q., V. N. Chouljenko, K. G. Kousoulas, and J. Storz.** 2000. Temperature-sensitive acetylesterase activity of haemagglutinin-esterase specified by respiratory bovine coronaviruses. J. Med. Microbiol. **49:**1119–1127.
12. **Lin, X. Q., K. L. O'Reilly, J. Storz, C. W. Purdy, and R. W. Loan.** 2000. Antibody responses to respiratory coronavirus infections of cattle during shipping fever pathogenesis. Arch. Virol. **145:**520–532.
13. **Mebus, C. A., E. L. Stair, M. B. Rhodes, and M. J. Twiehaus.** 1973. Neonatal calf diarrhea: propagation, attenuation, and characteristics of a corona-like agent. Am. J. Vet. Res.**34:**145–150.
14. **Saif, L. J., D. R. Redman, K. V. Brock, E. M. Kohler, and R. A. Heckert.** 1988. Winter dysentery in adult dairy cattle: detection of coronavirus in the feces. Vet. Rec. **123:**300–301.
15. **Schultze, B., and G. Herrler.** 1992. Bovine coronavirus uses N-acetyl-9-O-

acetyl- neuraminic acid as a receptor determinant to initiate the infection of cultured cells. J. Gen. Virol. **74:**901–906.

16. **Schultze, B., H.-J. Gross, R. Brossmer, and G. Herrier.** 1991. The S protein of bovine coronavirus is a hemagglutinin recognizing 9-*O*-acetylated sialic acid as a receptor determinant. J. Virol. **65:**6232–6237.
17. **Schultze, B., K. Wahn, H. D. Klenk, and G. Herrler.** 1991. Isolated HE-protein from hemagglutinating encephalomyelitis virus and bovine coronavirus has receptor-destroying and receptor-binding activity. Virology **180:** 221–228.
18. **Storz, J., X. Lin, C. W. Purdy, V. N. Chouljenko, K. G. Kousoulas, F. M. Enright, W. C. Gilmore, R. E. Briggs, and R. W. Loan.** 2000. Coronavirus and *Pasteurella* infections in bovine shipping fever pneumonia and Evans' criteria for causation. J. Clin. Microbiol. **38:**3291–3298.
19. **Storz, J., C. W. Purdy, X. Q. Lin, M. Burrell, R. E. Truax, R. E. Briggs, and R. W. Loan.** 2000. Isolation of respiratory bovine coronavirus, other cytocidal viruses, and *Pasteurella* spp. from cattle involved in two natural outbreaks of shipping fever. J. Am. Vet. Med. Assoc. **216:**1599–1604.
20. **Storz, J., X. M. Zhang, and R. Rott.** 1992. Comparison of hemagglutinating, receptor-destroying, and acetylesterase activities of avirulent and virulent bovine coronavirus strains. Arch. Virol. **125:**193–204.
21. **Storz, J., G. Herrler, D. R. Snodgrass, K. A. Hussain, X. M. Zhang, M. A. Clark, and R. Rott.** 1991. Monoclonal antibodies differentiate between the haemagglutinating and the receptor-destroying activities of bovine coronavirus. J. Gen. Virol. **72:**2817–2820.
22. **Storz, J., and R. Rott.** 1981. Reactivity of antibodies in human serum with antigens of an enteropathogenic bovine coronavirus. Med. Microbiol. Immunol. **169:**169–178.
23. **Storz, J., R. Rott, and G. Kaluza.** 1981. Enhancement of plaque formation and cell fusion of an enteropathogenic coronavirus by trypsin treatment. Infect. Immun. **31:**1214–1222.
24. **Storz, J., und R. Rott.** 1980. Über die Verbreitung der Coronavirusinfektion bei Rindern in ausgewählten Gebieten Deutschlands: antikörpernachweis durch Mikroimmundiffusion und Neutralisation. Dtsch. Tierärztl. Wochenschr. **87:**252–254.
25. **Zhang, X. M., K. G. Kousoulas, and J. Storz.** 1991. The hemagglutinin/esterase glycoprotein of bovine coronaviruses: sequence and function comparison between virulent and avirulent strains. Virology **185:**847–852.