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# Investigation of Sheep-Associated Malignant Catarrhal Fever Virus Infection in Ruminants by PCR and Competitive Inhibition Enzyme-Linked Immunosorbent Assay

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**Development of control measures for the gammaherpesviral disease of cattle known as sheep-associated malignant catarrhal fever (SA-MCF) has been hampered by a lack of accurate diagnostic tests either for the causative virus or for antibody against that virus. A recently developed competitive-inhibition enzyme-linked immunosorbent assay (CI-ELISA) for the detection of antibody to malignant catarrhal fever (MCF) virus (MCFV) in ruminants based on a monoclonal antibody to a widely conserved epitope of MCFV (H. Li, D. T. Shen, D. P. Knowles, J. R. Gorham, and T. B. Crawford, J. Clin. Microbiol. 32:1674–1679, 1994) and a PCR assay based on previously reported primers (S. I. F. Baxter, I. Pow, A. Bridgen, and H. W. Reid, Arch. Virol. 132:145–159, 1993) were used to detect anti-MCFV antibody and SA-MCFV DNA in sheep and other ruminants. The PCR amplified a specific 238-bp SA-MCFV genomic DNA fragment from peripheral blood lymphocytes of adult sheep and other ruminants with clinical MCF. Of 144 samples from randomly selected healthy adult sheep, 143 (99%) were positive by PCR and 136 (94%) were positive by CI-ELISA. The agreement between the two assays exceeded 95%. Of nine samples collected from cattle and deer with clinical MCF of apparent sheep origin, seven were CI-ELISA positive and all 9 were PCR positive. Among 59 serum samples from presuckling lambs, none contained antibody detectable by CI-ELISA. After suckling, maternal anti-MCFV antibody was detectable for about 10 ± 3 weeks. Although all colostrum and milk samples from infected ewes were strongly PCR positive, the appearance of detectable SA-MCFV DNA in lambs was correlated generally with antibody patterns, which suggests that the natural infection event in sheep may not occur during the perinatal period but occurs sometime later in life.**

Malignant catarrhal fever (MCF) is a highly fatal disease of cattle and certain other ruminants that exists as two known epidemiologic entities. One is known as wildebeest-associated MCF (WA-MCF), reflecting the fact that the causative virus exists in nature as endemic subclinical infection in wildebeests; the other entity, known as sheep-associated MCF (SA-MCF), refers to cases of MCF in cattle, deer, or other susceptible ruminants which have no history of contact with wildebeests or other exotic ruminants (9, 22). SA-MCF occurs worldwide, usually sporadically, although significant herd outbreaks are occasionally seen, with losses of up to 50% of the herd (3, 21, 26). The etiologic agent of SA-MCF has not been isolated. A large body of evidence suggests, however, that sheep carry a gammaherpesvirus closely related to the relatively well characterized WA-MCF virus (WA-MCFV) (7–9, 22). Recent studies revealed a high degree of homology between DNA sequences in lymphoblastoid cell lines that originated from cattle with clinical SA-MCF and genomic WA-MCFV DNA (2). Moreover, the presence of such virus-specific DNA sequences in lymphocytes of healthy sheep and in cattle clinically affected by SA-MCF (1) further indicates that sheep are carriers of the virus.

Little is known about the transmission mechanisms of SA-MCF virus (SA-MCFV) among sheep. Earlier studies based on competitive-inhibition enzyme-linked immunosorbent assay (CI-ELISA) showed that a high percentage of adult sheep were seropositive but that antibody was rarely detected in lambs less than 1 year of age, suggesting that lambs may not be infected at an early age (15). This pattern is in sharp contrast to that seen in wildebeest calves, which are infected in the perinatal period (18). Whether the antibody pattern in lambs reflects a late infection or a delayed immune response to an earlier infection has not been determined.

Techniques to amplify specific DNA and RNA fragments have provided new approaches to the detection of pathogens (13, 27). Application of PCR to the detection of either WA-MCFV (10–12, 17) or SA-MCFV DNA (1, 32, 34) has been reported. In the present study, a newly developed CI-ELISA (15) and a PCR assay based on the previously reported primers (1) were used to examine the prevalence of the sheep-associated agent and antibody against it in sheep and in other ruminants with clinical MCF.

## MATERIALS AND METHODS

**Virus and cells.** Fetal mouflon sheep kidney (FMSK) cells and the Minnesota isolate of MCFV (MN-MCFV), originally derived from an outbreak of MCF in cattle in Minnesota (6), were kindly provided by W. Heuschele, Center for Reproduction of Endangered Species, Zoological Society of San Diego, San Diego, Calif. A putative SA-MCFV isolate from Austria (29) (designated here as Au-732) was kindly provided by J. Pearson, National Veterinary Service Laboratory, Animal and Plant Inspection Service, U.S. Department of Agriculture, Ames, Iowa. The viruses were propagated on FMSK cells in high-glucose Dul-

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TABLE 1. Comparison of CI-ELISA and PCR for detection of MCFV infection in healthy adult sheep from diverse locations

| Location | No. of sheep tested | No. (%) of sheep positive by | |
|---|---|---|---|
| | | CI-ELISA | PCR[a] |
| Idaho | 30 | 28 | 30 |
| Idaho | 60 | 57 | 59 |
| North Dakota | 14[b] | 13 | 14 |
| Montana | 20[b] | 20 | 20 |
| Washington | 10 | 8 | 10 |
| Wyoming | 10[b] | 10 | 10 |
| Total | 144 | 136 (94) | 143[c] (99) |

[a] Bands were visualized by ethidium bromide staining only.
[b] Associated with an MCF outbreak.
[c] Specificity of amplified bands confirmed by *Rsa*I digestion or Southern blot hybridization of 20% of randomly selected samples.

becco's modified Eagle medium with 10% fetal bovine serum and were purified by sucrose gradient centrifugation (14).

**Sera, peripheral blood lymphocytes, and other samples.** Sera and peripheral blood lymphocytes (PBLs) were collected from 144 adult sheep in five states (Table 1). In order to examine the kinetics of appearance of antibody and viral DNA in lambs, a series of serum, lymphocyte, and amniotic fluid samples were collected aseptically from ewes and lambs ($n = 12$; ranging from presuckling to 34 weeks of age; these lambs were weaned at about 3 months of age and were separated from the ewes at 4 months of age) at the University of Wyoming. In addition, serum and PBL samples were collected from lambs at the University of Idaho and the Sheep Experimental Station, U.S. Department of Agriculture, Dubois, Idaho, both before and after nursing. Colostrum and milk samples were also obtained from above sheep from the same locations. Sera and PBLs were collected from cattle and deer with MCF in Wyoming, Idaho, Washington, and Montana. The initial diagnoses of MCF on the basis of clinical signs were confirmed by histopathology by using established diagnostic criteria, such as the presence of disseminated necrotizing lymphoid vasculitis, lymphadenopathy, ulcerative enteritis, and panophthalmitis (22). Sera and PBL samples were also obtained from a cow in Wyoming that had recovered from acute MCF (20), her clinically healthy calf, and 10 randomly selected healthy cows 1 to 3 years of age. The lymphocytes were purified by isodensity centrifugation by using Accu-Paque Lymphocytes (Accurate Chemical & Scientific Corp., Westbury, N.Y.) and were stored in Tris-EDTA buffer at −20°C. DNA was purified from PBLs by proteinase K digestion and phenol-chloroform extraction (16, 30), dissolved in water, and quantified by measuring the optical density at 260 nm.

**CI-ELISA.** Monoclonal antibody 15-A, which defines a conserved epitope present on all isolates of MCFV tested but on none of 13 other common sheep and cattle viruses (15), was used in the CI-ELISA. The procedures and data interpretation for the assay were described previously (15).

**PCR and Southern hybridization.** A two-step PCR amplification was used with previously described (1) primer sets: primer set 556 (5′-AGTCTGGGGTATATGAATCCAGATGGCTCTC-3′) and primer set 775 (5′-AAGATAAGCACCAGTTATGCATCTGATAAA-3′) in the first step and primer sets 556 and 555 (5′-TTCTGGGGTAGTGGCGAGCGAAGGCTTC-3′) in the second step. All amplification reactions were performed in a 100-μl volume containing 10 mM Tris-HCl (pH 8.0); 2 mM $MgCl_2$; 200 μM (each) dATP, dCTP, dGTP, and dTTP (Boehringer Mannheim Co., Indianapolis, Ind.); 20 pM (each) primer; and 2 U of *Taq* DNA polymerase (Boehringer Mannheim Co.). Thermal cycling conditions for both steps were 5 min at 95°C and then 34 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min; this was followed by a final 7 min at 72°C for extension. Ten microliters of the PCR mixture from the first step was used as target for the amplification in the second step. For product visualization, 10 μl of the amplified PCR products was analyzed by 2% agarose gel electrophoresis and ethidium bromide staining.

In order to confirm the specificities of the PCR-amplified products, the 238-bp DNA fragment (see Results) purified from an agarose gel with GENCLEAN (Bio 101, Inc., La Jolla, Calif.) was cloned into plasmid pUC18 by using *Eco*RI adapters and was sequenced by standard procedures (28).

For Southern hybridization, a 175-bp DNA fragment for the probe was amplified from pUC18 containing the 238-bp DNA insert by using primer pairs 5′-AGGGTATCCGAAAGCAG-3′ and 5′-CGGGGGTCTTGGACTGA-3′, which amplify the fragment included between nucleotide positions 73 and 247 (1). The amplified fragments were electroeluted from an agarose gel and were labeled with [$^{33}$P]dATP (New England Nuclear, Boston, Mass.) by random priming according to the manufacturer's instructions (GIBCO BRL, Grand Island, N.Y.). Southern hybridization was performed by standard procedures (31), whereby the separated DNA fragments in an agarose gel were transferred to nylon membranes, hybridized for 8 h at 68°C in the presence of $1 \times 10^6$ cpm of the $^{33}$P-labeled probe, washed in 0.1× standard saline citrate at 68°C, and exposed to X-ray film (16).

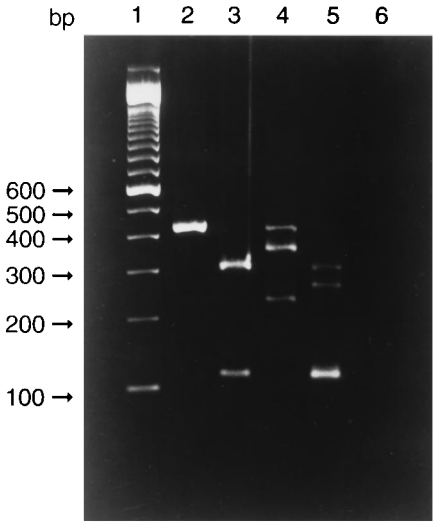


FIG. 1. Specificities of the PCR products. *Rsa*I restriction analysis of the 422-, 340-, and 238-bp PCR-amplified DNA fragments specified by primer pairs 556-755 and primer pairs 556-555 from PBLs of a cow with clinical MCF. Lane 1, 100-bp ladder; lanes 2 and 4, undigested DNA fragment; lanes 3 and 5, DNA fragment digested with *Rsa*I; lane 6, control containing no target DNA.

## RESULTS

**Specificity and sensitivity of PCR.** The amplification of PBL DNA from a sheep that was seropositive by CI-ELISA and immunoprecipitation with primer pairs 556-755 and 556-555 yielded two fragments that were similar in size (422 and 238 bp) to those previously reported by Baxter et al. (1). The base sequence of the 238-bp DNA fragment was identical to the reported sequence of the DNA cloned from a lymphoblastoid cell line derived from a cow with clinical SA-MCF (1) (data not shown).

As shown in Fig. 1, a single 422-bp DNA fragment was amplified from PBL DNA of a cow with SA-MCF by primer pair 556-755 (Fig. 1, lane 2). On the other hand, primer pair 556-555 amplified three DNA fragments with distinct sizes (one of 238 bp and two others of approximately 340 and 420 bp) (Fig. 1, lane 4). The specificities of these fragments were analyzed by both *Rsa*I restriction digestion and Southern hybridization. *Rsa*I restriction digestion reduced the sizes of the 420-, 340-, and 238-bp DNA fragments by about 110 bp, indicating that the *Rsa*I site was located in the same position of each fragment (Fig. 1, lanes 3 and 5). All three fragments (420, 340, and 238 bp) hybridized with the $^{33}$P-labeled DNA probe (data not shown).

The sensitivity of the PCR was assessed by using as targets a series of dilutions of pUC18 containing the 238-bp DNA fragment. Two methods of detecting the fragments were compared: ethidium bromide staining and Southern hybridization. Ten femtograms of DNA from the pUC18 construct containing the 238-bp fragment was the minimum required to obtain a positive signal by ethidium bromide staining (data not shown). The sensitivity of detection was enhanced approximately 25-fold by Southern hybridization with the $^{33}$P-labeled DNA probe, which detected down to 0.4 fg of the recombinant plasmid DNA (Fig. 2). A comparison of the sensitivity of PCR with purified PBL DNA and that with solubilized cells as targets showed that prior purification of the DNA from PBLs improved the sensitivity of the assay about fourfold (data not shown). However, because of the advantages of speed and economy, the use of solubilized whole PBLs without the DNA

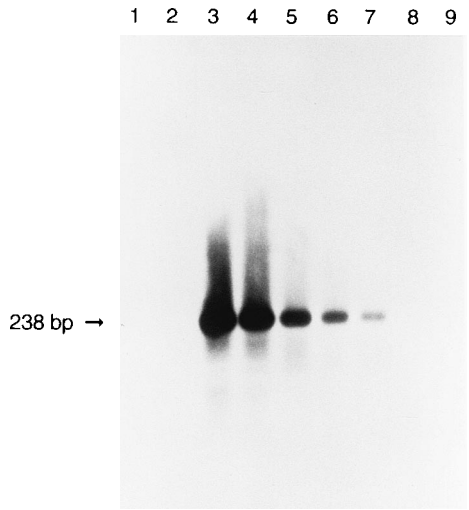


FIG. 2. Enhancement of sensitivity of detection of PCR products by Southern blot analysis with a $^{33}$P-labeled probe. The pUC18 plasmid containing the 238-bp SA-MCFV fragment was used as a target DNA. All reaction mixtures contained 3 μg of salmon sperm DNA. Lane 1, control containing no target DNA; lane 2, blank; lanes 3 to 9, 1 pg and 100, 10, 2, 0.4, 0.08, and 0.016 fg of plasmid DNA, respectively.

extraction step and ethidium bromide staining of PCR products rather than probing of the gels were the methods of choice, because they were adequately sensitive for the routine detection of MCFV DNA in adult sheep and in ruminants with clinical SA-MCF.

**SA-MCFV DNA in adult sheep and lambs.** A group of 144 PBL DNA samples from adult sheep were subjected to PCR amplification. One hundred forty-three (99%) of them were positive (Table 1 and Fig. 3A). Specificity was further confirmed by either *Rsa*I analysis or Southern hybridization of amplified products from 30 randomly selected sheep (data not shown). Strong PCR signals were observed in all of the five colostrum and 28 milk samples collected from infected ewes (Fig. 3A). No fragments were amplified from cells or from the cell-free, high-speed pellet of amniotic fluid collected by aseptic amniocentesis (Fig. 3A). Of the DNA samples extracted from the PBLs of 16 presuckling lambs, only one was positive by PCR. Of 57 DNA samples prepared from different individual lambs ranging from 1 to 4 weeks of age, 22 were PCR positive. Sequential PBL samples from 12 lambs born to infected ewes were collected and examined by PCR in order to determine the relationship between age and the presence of viral DNA in leukocytes. To enhance the sensitivity of detection, amplified products were analyzed by Southern hybridization with the $^{33}$P-labeled 157-bp DNA probe. As shown in Fig. 4A, no detectable bands were amplified from 11 of 12 samples from presuckling lambs. At 2 weeks of age, 3 of 12 samples from lambs were strongly positive (Fig. 4B). These PCR signals were detected, although inconsistently, in some individuals until about 10 weeks of age. From 12 (Fig. 4C) to 34 weeks of age (at the time of this writing), only 3 of 84 samples yielded detectable signals. These three were from different individuals.

**SA-MCFV antibody in adult sheep and lambs.** Sera from 144 adult sheep from five states were tested by CI-ELISA. One hundred thirty-six (94%) were positive for MCFV antibody (Table 1). Antibody levels in sera collected monthly from 14 adult sheep for more than 1 year were stable (Fig. 5A). No antibody was detectable in 59 serum samples from presuckling lambs. Examination of sera collected weekly from 12 lambs showed that, as expected, all lambs obtained anti-MCFV antibody from colostrum and that the antibody remained detectable for about 10 ± 3 weeks before falling below the threshold of the assay (Fig. 5B). No seroconversion was observed in any lambs followed for 34 weeks.

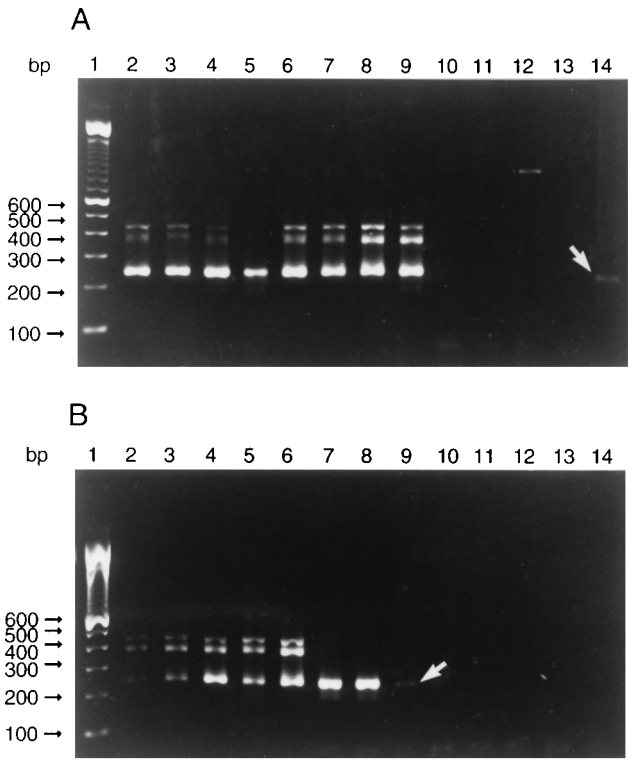


FIG. 3. Agarose gel electrophoresis of ethidium bromide-stained PCR products amplified from a variety of tissues from sheep (A) and cattle (B). (A) Sheep PBLs, milk, and amniotic fluid cells, as follows: PBLs from five adult sheep from diverse geographic locations (lanes 2 to 6); cells from colostrum (lanes 7 and 8); cells from milk (lane 9); cells or cell-free high-speed pellet of amniotic fluid (lanes 10 and 11); PBLs from an MCFV antibody-negative adult sheep (lane 12); control containing no target DNA (lane 13); minimal positive control (10 fg of the plasmid containing the 238-bp SA-MCFV fragment) (lane 14, faint band indicated by arrow). (B) Samples from cattle with clinical MCF, as follows: PBLs from clinically ill cattle with MCF from 4 different states (lanes 2 to 6, respectively); PBLs from a cow that recovered from clinical MCF and her calf (lanes 7 and 8, respectively); minimal positive control (lane 9; faint band indicated by an arrow); PBLs from a random healthy cow (lane 10); DNA from FMSK cell line, MN-MCFV, and MCFV Au-732 (lanes 11 to 13, respectively); control containing no target DNA (lane 14). DNA ladders of 100 bp are in lanes 1 of both panels.

**Detection of viral DNA and antibody in cattle and deer with clinical MCF.** Sera and PBL DNA samples were collected from seven cattle and two deer that had acute clinical MCF and a history of association with sheep. Among the samples from seven cattle, all were PCR positive (Fig. 3B, lanes 1 to 5) and five were CI-ELISA positive. Samples from both of the deer were positive by PCR and CI-ELISA (data not shown). Moreover, viral DNA was detected by PCR in a cow that had recovered from an episode of MCF about 6 months earlier and from her calf (Fig. 3B, lanes 6 and 7). The specificities of the PCR products from all cattle and deer samples were confirmed by both *Rsa*I digestion and Southern hybridization (data not shown). PCR did not amplify DNA purified from the isolates of MCFV from Minnesota or Austria, DNA from the FMSK cell line, or PBL DNA from healthy cattle ($n$ = 10) (Fig. 3B).

## DISCUSSION

Significant progress in understanding and controlling the complex syndrome of MCF requires much more knowledge of

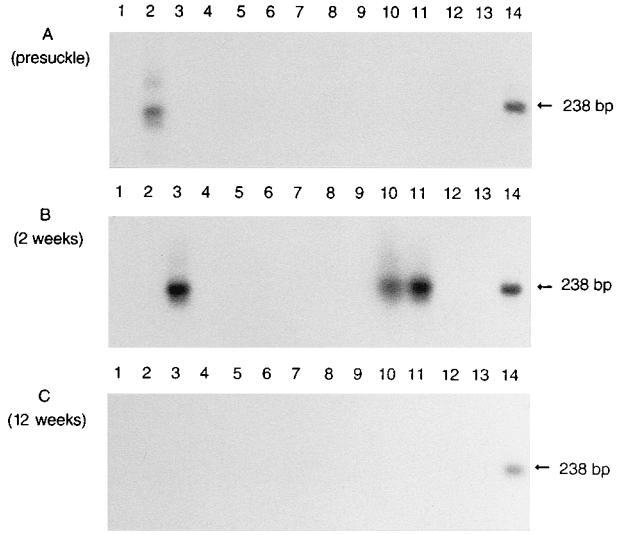


FIG. 4. Southern blot hybridization of PCR products with $^{33}$P-labeled probe from PBL DNAs of lambs of different ages: (A) presuckle; (B) 2 weeks; (C) 12 weeks. Lanes 1, control containing no target DNA; lanes 2 to 13, lambs WY 1-1, 2-1, 2-2, 4-1, 5-1, 6-1, 6-2, 7-1, 7-2, 8-1, 10-1, and 10-2, respectively; lanes 14, 10 fg of the plasmid containing the 238-bp SA-MCFV fragment as positive controls.

the fundamental properties of the causative agents, their epidemiology, and their interactions with their hosts than currently exists. For many years, the inability to propagate the sheep-associated agent in vitro has been a major impediment to research progress. In spite of this limitation, however, valuable information is emerging about the agent and its ecology. This information has been gained from assays that are not dependent on in vitro infectivity. In the present study we used two recently developed assays, one measuring antibody against the sheep agent and the other detecting its genomic DNA. The results confirmed the specificities of the two assays for the detection of SA-MCF, confirmed earlier reports that SA-MCFV DNA can consistently be detected in peripheral blood mononuclear cells during acute illness, showed that virtually all adult sheep are subclinical carriers of the virus, and showed that lambs do not appear to become infected either in utero or at birth, but become infected sometime considerably later in life.

The PCR assay with previously reported primers (1) successfully amplified the 238-bp DNA fragment from PBLs of either infected adult sheep or cattle and deer with clinical SA-MCF. The base sequence derived from the 238-bp fragment was identical to the reported sequence of a DNA fragment cloned from a lymphoblastoid cell line derived from a cow with clinical SA-MCF (1). This suggests that the SA-MCF agent in North America is very closely related to the agents in Scotland and Indonesia (1, 34). PCR did not amplify bands from the isolates of MCFV from Minnesota (6) or Austria (29), which were isolated from cattle with clinical MCF that were reported to have had contact with sheep. This is evidence that these two isolates are not of sheep origin. These two isolates are readily detected by primers based on WC-11 DNA sequences (12), further suggesting that they belong to the AHV-1 group. However, further study is needed to rule out the possibility that SA-MCFVs that are not detected by the PCR primer sets used here may exist.

In most samples, primers 556 and 555 amplified two fragments of approximately 340 and 420 bp, in addition to the expected 238-bp fragment. All three fragments hybridized with the truncated 175-bp DNA probe constructed to eliminate the initial sequences of primers 556 and 555 (data not shown) and contained a *Rsa*I site at the same location in each primer (Fig. 1). Both results are strong evidence that the fragments are SA-MCFV specific. We suspected that the existence of three rather than one band was because primer 555 hybridized with other regions of SA-MCFV DNA. From the sequence analysis of the region of the SA-MCFV DNA reported by Baxter et al. (1), we found that in addition to the primary binding site, there were two other distinct 5- and 6-base regions (nucleotide positions 348 to 353 and positions 421 to 425, respectively [1]) near the 3′ terminus of the cloned SA-MCFV DNA fragment that were identical to positions 3 through 8 of the 3′ end of primer 555 (TCGGAA). Binding to these two alternate sites by primer 555 at a lower efficiency than at the primary site is the apparent explanation for the frequent appearance of the two additional, larger (340- and 420-bp) bands in the PCR. Both of these bands were SA-MCFV specific. In several tries, reduction of the target DNA concentration by simple dilution consistently eliminated the two larger (340- and 420-bp) bands (data not shown). Therefore, it was apparent that amplification of only the single 238-bp fragment was a reflection of a lower target DNA concentration.

Parallel testing of 144 random samples from healthy adult sheep revealed more than 95% agreement between the CI-ELISA and PCR. The relatively lower percentage (94%) of positive results by CI-ELISA may be a reflection of the fact that some infected sheep produce lower levels of antibody than do others. Of samples from seven cattle and two deer with confirmed acute MCF, all nine were PCR positive, but only seven of the nine animals were seropositive by CI-ELISA. This probably reflects a lack of sufficient time to mount an immune response in some animals with rapidly fatal cases of infection. Thus, PCR with these primers appears to be useful and reliable for detecting acute clinical MCF in susceptible species, somewhat more so than antibody detection by CI-ELISA.

The occurrence of WA-MCF largely parallels the wildebeest calving season, with calves less than 3 months of age serving as the main source for transmission (18, 19). It is also generally accepted that most cases of SA-MCF are associated with close contact between sheep and the clinically susceptible host, particularly during the lambing season (7, 23, 24), implying that perinatal lambs may play a role similar to that played by wildebeest calves. However, previous studies showed that lambs less than 1 year of age were rarely seropositive for MCFV (15), suggesting that lambs may not be infected. This indicates that the epidemiologic pattern in sheep is different from that in wildebeests (18, 22).

To determine whether the phenomenon in sheep reflects a late infection or a delayed immune response, samples were collected at weekly intervals from a group of lambs born to PCR- and CI-ELISA-positive ewes. Antibody and viral DNA were not found in samples from presuckling lambs, which suggests that transplacental transmission is a rare event, if it occurs at all. The lambs obtained maternal antibody from colostrum, as expected. Antibody titers reached a level similar to that in their dams during the first week and remained detectable for about $10 \pm 3$ weeks. All colostrum and milk samples examined from infected ewes were strongly PCR positive, suggesting the possibility of transmission by this route (25, 33), if lambs are susceptible during this period. Only 1 of 17 PBL samples from presuckling lambs was PCR positive. The sample from its twin, however, was negative. Although the reason for this was not clear, contamination during sample preparation seems the most likely explanation. After suckling, viral DNA sequences became detectable in the PBLs of about 30% of the

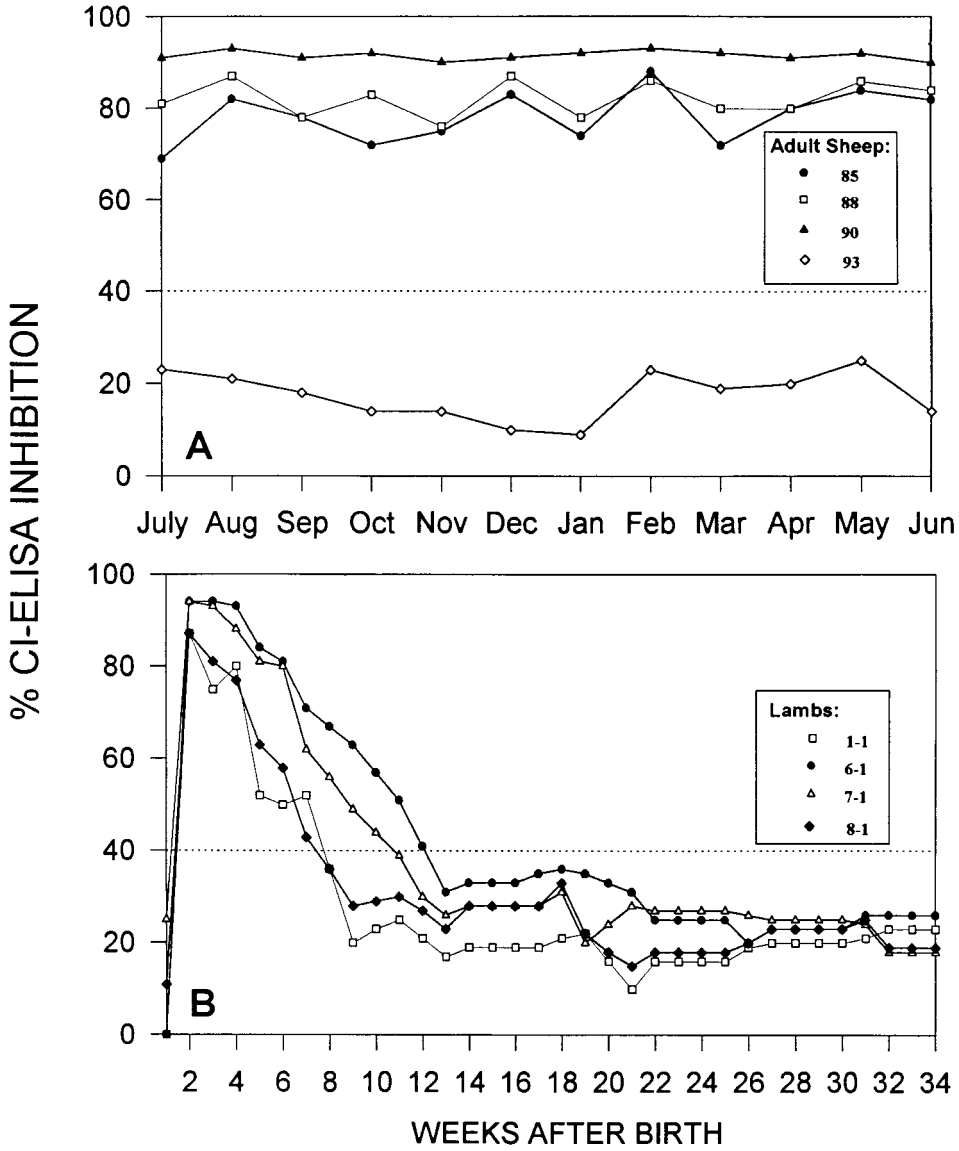


FIG. 5. Detection of MCFV antibody in adult sheep and lambs by CI-ELISA. (A) Sera collected monthly from 14 adult sheep associated with an MCF outbreak in North Dakota. The figure shows data for only 4 of the 14 sheep (sheep 85, 88, 90, and 93); (B) sera collected weekly from 12 lambs born to healthy MCFV-infected ewes in Wyoming. The figure shows data for panels 4 of the 12 lambs (lambs 1-1, 6-1, 7-1, and 8-1) (0 weeks = presuckle). The dotted lines in both panels indicate the statistical threshold for defining positive sera on the basis of mean percentage inhibition of negative sheep sera ($n = 6$) plus 3 standard deviations.

lambs. These positive signals, however, were somewhat sporadic and unpredictable. At 12 weeks of age, samples from all lambs in this experiment had become PCR negative. These data suggest that PCR signals during the first 10 weeks of life probably originate from transferred maternal leukocytes. The PCR patterns roughly correlated with the antibody patterns in lambs. The two most likely explanations are (i) that young lambs are infected but that the infection is latent with insufficient viral protein synthesis to stimulate seroconversion, or (ii) that lambs may not be susceptible to infection early in life. The latter would be reminiscent of the endemic natural infection of squirrel monkeys with *Herpesvirus saimiri*, another gammaherpesvirus. Monkeys are not infected with this virus early in life. Between 12 and 24 months of age, however, virtually all of the young squirrel monkeys become infected and seroconvert (4, 5). Studies to define the time of infection of lambs with SA-MCFV are under way.

## ACKNOWLEDGMENTS

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