Bovine viral diarrhea virus (BVDV) is a significant infectious viral agent of cattle (1, 8). The primary sources of virus transmission within and between cattle herds are cattle that are persistently infected with BVDV (13). Persistent infection occurs with the development of immunotolerance to BVDV, which can result if fetal infection with BVDV occurs prior to the fourth month of gestation (14, 16). A key management strategy that is used to control and prevent BVDV infection is the detection and elimination of cattle that are persistently infected with BVDV. Accurate and inexpensive methods for the detection soon after birth of cattle persistently infected with BVDV would be ideal, since it would allow prevention of BVDV dissemination on farms. Many methods have been used to screen cattle for persistent infection, including virus isolation (VI), enzyme-linked immunosorbent assay (ELISA), and PCR analysis of blood samples (5, 7). However, the detection of BVDV in neonatal calves is complicated by the presence of colostrum-derived passive antibodies which can neutralize virus in serum for up to 4 months (6, 19), thus making the use of VI and an ELISA using serum unreliable prior to this point (5, 7). VI from and PCR analysis of white blood cell preparations from neonatal calves have been used successfully to detect BVDV in neonatal calves (5, 7), but the costs of these procedures for routine screening may be prohibitive. Immunohistochemical staining of skin biopsy samples has recently been shown to be a useful method for screening cattle for persistent BVDV infection (11, 18, 20) and has the potential to be a relatively inexpensive and accurate method for screening cattle for persistent BVDV infection. The use of immunohistochemistry (IHC) for detecting neonatal calves with persistent infection has not been evaluated. The objective of this study was to evaluate IHC as a tool for detecting neonatal calves persistently infected with BVDV.

MATERIALS AND METHODS
Calves. Holstein calves between 1 and 4 weeks of age from four different dairy farms were used in this project. Samples were collected between June and August 2000. These farms routinely screened neonatal calves for BVDV by VI from white blood cell preparations as part of a BVDV control program.

Samples. Triangular skin biopsy samples (ear notches) measuring approximately 12 by 5 mm were obtained from the ventral pinna of each calf by using a pig ear notcher. Hemostasis at the biopsy site was accomplished by applying a spring paper clip over cotton gauze for 5 min. All tissue specimens were fixed in 10% buffered formalin for 24 to 48 h, followed by embedding in paraffin blocks. Concurrently with tissue sampling, 10 ml of whole blood was collected from each animal in EDTA Vacutainer tubes. Buffy coat cells were isolated from the whole-blood samples, washed three times in 0.1 M phosphate-buffered saline (pH 7.4), and lysed by freezing at —70°C (21). White blood cell lysates were inoculated onto bovine turbinate cell cultures and incubated for 3 days in 5% CO2 at 37°C. Virus was detected in cell monolayers with an immunoperoxidase monolayer assay (17) using polyclonal antibody to BVDV (National Veterinary Services Laboratory, Ames, Iowa) of swine origin as the detection antibody.

IHC. Five-micrometer tissue sections were mounted on silane-treated slides and stained for BVDV by using an automated procedure adapted from a previously described technique (10). The preparation of samples for staining included warming in an oven at 56°C for 30 min and deparaffinization in 4 changes of limonene-based solvent for 5 min each. Samples were rehydrated in decreasing concentrations of ethanol and treated with a 3% H2O2 in methanol solution to inactivate any endogenous peroxidase. Tissue was digested with protease for 10 min and then treated with 5% horse serum to block nonspecific antibody binding sites. Antigen detection was achieved by using the BVDV monoclonal antibody 15C5 (Ed Dubovi, Cornell University) as the primary antibody and a commercially available immunoperoxidase detection system (Vector Elite ABC; Vector Laboratories, Burlingame, Calif.). Control slides consisting of known BVDV-positive and -negative samples were stained concurrently in each batch to

Screening of Neonatal Calves for Persistent Infection with Bovine Viral Diarrhea Virus by Immunohistochemistry on Skin Biopsy Samples

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Received 2 January 2002/Returned for modification 11 March 2002/Accepted 8 April 2002

Detection and elimination of cattle that are persistently infected with bovine viral diarrhea virus (BVDV) is important for controlling the transmission of this virus. Colostrum-derived antibodies make the detection of persistently BVDV-infected neonatal calves cumbersome and expensive. The objective of this study was to evaluate the use of immunohistochemical staining of skin biopsy samples from neonatal calves as a method for the early detection of persistent BVDV infection. Three hundred thirty-two 1- to 4-week-old dairy calves were screened for BVDV as part of a routine control program. Formalin-fixed skin biopsy samples were stained for BVDV antigen by immunohistochemistry (IHC), and the results were compared to those of virus isolation (VI) from white blood cell preparations. Six calves were positive by both IHC and VI and remained positive for BVDV upon subsequent follow-up testing; thus, they were classified as persistently infected with BVDV. One calf was positive by VI but negative by IHC. On subsequent testing, the calf was negative by VI, suggesting that the initial VI result was due to an acute BVDV infection. One calf was positive by IHC but negative by VI. This calf remained negative by VI on follow-up testing. Immunohistochemical staining of skin biopsy samples is a reliable method for screening neonatal calves for persistent BVDV infection and would be a useful management tool as an aid for controlling and preventing BVDV infection.

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monitor quality control. To distinguish nonspecific binding, serial sections of each block were treated with an irrelevant monoclonal antibody directed against bovine coronavirus. Blinded to the VI results, each author independently evaluated every sample. A skin sample was declared positive for BVDV if cell-specific cherry-red staining was present.

Data analysis. Calves were classified as persistently infected with BVDV if they remained positive by VI for 2 weeks following the initial test. Sensitivity, specificity, positive predictive value, negative predictive value, and their respective confidence levels were calculated for the skin IHC assay to detect calves persistently infected with BVDV. The correlation between the two tests was determined by using the kappa statistic.

## RESULTS

The diagnostic accuracy of skin IHC as a screening test for persistent BVDV infection in neonatal calves is compared in Table 1 to that of VI from white blood cells. A total of 332 calves were tested during the course of the study. Of these, 6 (1.8%) were found to be persistently infected with BVDV. Infection in each of these calves was detected by both VI and IHC. At the initial screening, virus was detected in the white blood cells of one calf but was not detected in that calf by skin IHC. On follow-up 2 weeks later, virus could not be detected in the white blood cells. In another calf, BVDV was detected by skin IHC but could not be detected by VI from white blood cells. Several attempts to isolate virus from this calf over the next 3 weeks yielded negative results.

## DISCUSSION

Early detection of cattle that are persistently infected with BVDV would be of significant benefit to cattle producers who wish to implement BVDV control programs. Control programs that screen neonatal calves soon after birth have been advocated (4, 12). By strategically identifying and removing calves that are persistently infected with BVDV as early as possible, the length of time that these virus reservoirs are on a farm, serving as a source of virus dissemination, is significantly reduced. The cost to raise calves is significant, and it would be economically beneficial if a BVDV control strategy could be implemented in which cattle that are persistently BVDV infected were identified and eliminated at an age as young as possible, i.e., prior to the investment of significant resources in them. The most commonly used technique to screen neonatal calves for BVDV is VI from white blood cells. However, the cost of the routine use of this technique as a screening tool for BVDV may be prohibitive. The use of less costly screening assays such as ELISA or VI from serum is not reliable in neonatal calves because of the presence of colostrum-derived passive antibodies to BVDV that neutralize free virus. These assays can be used as passive antibodies decay and free virus begins to reappear in serum. The reappearance of virus in serum is variable and may occur in cattle that range from 2 to 6 months of age (6, 19). Many BVDV control programs screen calves at 4 to 6 months of age. However, by this time producers have incurred a significant cost in raising these calves, and any calf that is persistently infected with BVDV is a potential source of virus transmission.

In this study, the use of IHC to detect BVDV in skin samples from neonatal calves was shown to be an accurate method for identifying persistent BVDV infection. We sampled calves between 1 and 4 weeks of age. It would be advantageous to sample calves immediately after birth and eliminate persistently infected calves as soon as possible. However, as was the case in this study, it may be logistically simpler to regularly sample all new calves born during a set interval. Virus can readily be identified in most tissues of cattle that are persistently infected with BVDV, including the skin (2, 3, 18, 20). The use of skin biopsy samples has been reported to be an accurate method for identifying cattle persistently infected with BVDV (18). However, these studies did not focus on neonatal calves, in which passively derived antibodies to BVDV are likely to be present.

In this study, the results of IHC and VI from white blood cells for two calves were in disagreement. In one calf, virus antigen was detected by IHC but not by VI. Staining was limited to small areas in the epidermal layer of the skin only. Several attempts to isolate virus from this calf over the next 3 weeks were unsuccessful. Staining by IHC was still present on a skin biopsy sample taken 1 week after the initial test. It is likely that this calf had undergone an acute infection prior to the initial test and that residual viral antigen was still detectable in tissues. A cohort of calves that was persistently infected with BVDV was identified the previous week, thus providing a virus reservoir for the infection of susceptible calves. Viral antigen may be detectable in tissues for an extended time after the virus has been cleared from the blood (10, 15, 21). In addition, virus can be detected in the skin of cattle that have undergone an acute infection, although the amount and distribution of staining are reported to be different than those found in cattle persistently infected with BVDV (9, 18). In a second calf, BVDV was isolated from white blood cells on initial screening but was not detected by IHC on the skin. On follow-up testing 1 and 2 weeks later, virus was not isolated from white blood cells or found by IHC on the skin. Again, it is likely that this calf was undergoing an acute BVDV infection and was viremic at the time of initial testing. The detection of BVDV in skin by IHC following acute infection has been reported to be inconsistent (9, 18), so the failure to detect virus in this calf is not surprising. These two cases illustrate the importance of follow-up testing to confirm positive results that are found in the initial screening of calves for persistent BVDV infection.

The screening of neonatal calves for persistent BVDV infection when implementing a BVDV control program is economically and strategically advantageous. In this study, IHC on skin samples was shown to be an effective method for screening neonatal calves for persistent BVDV infection and could be used as a management tool to control and prevent BVDV infection in cattle herds.

### TABLE 1. Comparison of IHC with VI from white blood cells as a diagnostic test for detecting neonatal calves persistently infected with BVDV

| Parameter          | Sensitivity (%) | Specificity (%) | Positive predictive value (%) | Negative predictive value (%) | Kappa value |
|--------------------|----------------|----------------|------------------------------|-----------------------------|-------------|
| Value              | 100.00         | 99.69          | 85.71                        | 100.00                      | 0.92        |
| Lower confidence limit | 100.00    | 99.09          | 59.79                        | 100.00                      | 0.77        |

* The values given are expressed relative to those of VI (100%).
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