Serologic evaluation of five unvaccinated heifers to detect herds that have cattle persistently infected with bovine viral diarrhea virus

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Objective—To determine whether serologic evaluation of 5 unvaccinated 6- to 12-month-old heifers is a valid method for identifying herds that contain cattle persistently infected (PI) with bovine viral diarrhea virus (BVDV).

Animals—14 dairy herds with a history of BVDV infection, with health problems consistent with BVDV infection, or at risk for contracting BVDV infection.

Procedure—5 unvaccinated 6- to 12-month-old heifers were randomly selected from each herd. Neutralizing antibody titters for type-I and -II BVDV were determined. A herd was classified as likely to contain PI cattle when at least 3/5 heifers had antibody titters $\geq 128$. Virus isolation was performed on all cattle to identify PI cattle. Genotype of isolated viruses was determined by nested multiplex polymerase chain reaction.

Results—6 of 14 herds contained PI cattle. Sensitivity and specificity of serologic evaluation of 5 heifers for identifying these herds were 66 and 100%, respectively. In herds that contained PI cattle, the predominant BVDV titer in the tested heifers corresponded to the genotype of the isolated virus.

Conclusions and Clinical Relevance—Serologic evaluation of unvaccinated 6- to 12-month-old heifers is an accurate method for identifying herds containing PI cattle. Both type-I and -II BVDV antibody titters should be determined to prevent herd misclassification. The genotype of BVDV found in PI cattle can be predicted by the predominant neutralizing antibody titers found in tested heifers. Serologic evaluation of 5 unvaccinated heifers can be used to determine whether a herd is likely to contain PI cattle.

Disease caused by infection with bovine viral diarrhea virus (BVDV) results in an estimated annual loss of $10$ million to $40$ million for every $1$ million calves born. Because of the complexity of the virus and its many clinical manifestations, BVDV is also one of the most difficult diseases to diagnose and control.

Cattle persistently infected (PI) with BVDV are the major reservoir for viral transmission within and between herds. Persistently infected cattle are immunotolerant to BVDV and continuously shed large amounts of virus into the environment throughout their lives. A key to eradicating BVDV is the identification and removal of PI cattle from each herd. This requires testing all cattle in each herd for the virus, which is a major investment of time and money for producers. Development of a quick, inexpensive, and accurate way to determine whether a herd contains PI cattle prior to committing the resources needed for whole-herd testing would be useful.

Several strategies have been developed to identify herds infected with BVDV. Detection of BVDV antibodies in bulk-tank milk can be used to identify dairy herds exposed to BVDV and is part of the national eradication program in many European countries. However, use of bulk-tank milk for detection of BVDV antibodies in the United States is limited because of the widespread use of vaccines against BVDV in lactating cows. Thus, it is impossible to determine whether BVDV antibodies in bulk-tank milk samples are the result of vaccination or natural exposure.

Detection of BVDV RNA in bulk-tank milk samples, using a polymerase chain reaction (PCR) technique, has been described, but usefulness of the PCR as a herd-screening test has not been documented. The advantage of the PCR over the antibody test for bulk-tank milk samples is that PCR detects actual virus rather than antibodies of cattle that have been exposed to or vaccinated against BVDV. It has been reported that PCR can detect 1 PI cow in a bulk-tank milk sample containing milk from 162 cows. However, similar to the antibody test of bulk-tank milk samples, it only evaluates cattle whose milk contributes to the tank sample tested. Thus, results cannot be extrapolated to the rest of the cattle in the herd, specifically young replacement heifers. Additionally, its usefulness in larger herds is unknown.

Serologic evaluation of a small group of young unvaccinated calves has been proposed as another method for identifying herds with PI cattle. The basis for this strategy is the fact that a high prevalence of seropositive calves in a herd is indirect evidence that a PI animal exists in that herd. Young calves essentially serve as sentinels for detecting circulating virus, which is generally the result of a PI herdmate.

In a study in Denmark, the probability of obtaining $\geq 2$ of 5 BVDV-seropositive calves 6 to 18 months old in a herd was calculated by use of a hypergeometric probability function for herds with and without PI cattle. The probability of $\geq 2$ of 5 animals being seropositive was $>0.977$ in 10 herds with PI cattle, whereas in 9 herds without PI cattle, the probability of obtaining 2 seropositive animals was $<0.048$. The same hypergeometric probability function was applied...
to data from 10 dairy herds in Michigan to determine the optimum sample size and cutoff value for neutralizing-antibody titers needed to accurately identify herds with and without PI cattle. The probability of a PI animal being detected in a herd was 0.994 when 3 of 5 heifers 9 to 18 months old had BVDV antibody titers ≥ 1:128. Conversely, when 3 of 5 heifers had titers ≤ 1:64, the probability of a PI animal in the herd was < 0.01. In both of those studies, all cattle had been tested for BVDV and BVDV-neutralizing antibodies prior to the calculations. This technique has yet to be validated in herds with unknown BVDV status.

The main objective of the study reported here was to determine whether serologic analysis of 5 unvaccinated heifers from each herd is a valid and accurate method for identifying dairy herds with PI cattle. The genotype of BVDV isolated from PI cattle was determined and compared with the genotype of the predominant BVDV-neutralizing antibodies found during the serologic analysis of the heifers in the respective herds. A PCR test was also performed on bulk-tank milk samples obtained from each herd, and a questionnaire was used to evaluate basic herd management practices.

Materials and Methods

Selection of herds—Dairy herds in which the current BVDV status was unknown were asked to voluntarily participate in the study. It was required that all participating herds consist of ≤ 200 lactating cows and that young stock in those herds were not vaccinated against BVDV prior to testing. Herds for the study were selected on the basis of a recent diagnosis of BVDV infection, health problems consistent with BVDV infection, or they were deemed to be at risk for contracting BVDV infection (herds that were introducing new cattle into the herd or that did not routinely vaccinate against BVDV).

Determination of sample size—A statistical computer program was used to determine the sample size needed for this study. Given the criteria by which herds were selected for inclusion in the study and the identified risk factors for herd infection with BVDV, it was assumed that the number of herds with PI cattle would equal the number of herds without PI cattle. Other assumptions used to determine sample size were that the specificity for herds with a negative serologic evaluation was 95%, whereas the sensitivity for herds with a positive serologic evaluation was 90%. The desired confidence interval (CI) was 95% with 80% power. On the basis of the aforementioned requirements, it was determined that a minimum of 6 herds with PI cattle and 6 herds without PI cattle were needed.

Collection of blood and bulk-tank milk samples—Blood samples were collected from all cattle in each herd for virus isolation. Serum samples were obtained from all cattle ≥ 6 months old, and blood samples were collected from calves < 6 months old. Serum is the easiest and least expensive sample to process; therefore, it was the preferred sample for most of the cattle. However, collostral antibodies may neutralize free BVDV in the serum of young calves, resulting in false-negative results on virus isolation. To increase the chance of identifying PI calves that were < 6 months old, virus isolation was performed on buffy coats obtained from blood samples.

Each herd veterinarian collected follow-up blood samples from all cattle that had positive results for virus isolation. Cattle were classified as PI cattle when they had positive results for BVDV in both samples or when they had positive results on the first test but were removed from the herd (culled or died) as a result of disease consistent with BVDV infection before the follow-up sample could be obtained.

Samples were placed on ice, transported to our laboratory, and processed immediately. Serum samples were stored frozen at -80 C. Following hypotonic lysis of RBC by use of a 0.83% solution of ammonium chloride in distilled water, WBC were removed from blood samples. The WBC were washed once with 10 ml of 0.01M PBS solution (pH 7.6), and cells then were resuspended in 1 ml of Eagle’s minimum essential medium (EMEM) with 1% l-glutamine and stored at -80 C.

An aliquot (250 ml) of milk was obtained from the bulk tank of each herd and used for PCR analysis. Milk samples were obtained from each herd on the same day that blood samples were collected from the entire herd. Somatic cells were isolated from each milk sample by centrifugation of the samples (2,000 X g for 30 minutes at 4 C). Supernatant was removed, and the cell pellet was resuspended with 2 ml of RNase free water, which was followed by centrifugation at 2,000 X g for 30 minutes. Supernatant again was removed, and the process was repeated 2 more times. The cell pellet was then allowed to thoroughly dry and was stored at -80 C.

Herd management—A questionnaire that covered basic herd management practices was administered to the herd owner or primary herdsman. The questionnaire was always administered by the principle investigator (RBP) on the same day on which blood samples were collected from the entire herd.

Analysis of samples—Virus isolation, serologic analysis, and PCR were used to evaluate samples collected for the study.

Virus isolation—Virus isolation was performed on all samples, using an immunoperoxidase monolayer assay similar to that described elsewhere. Briefly, 15 µl of each sample (serum or buffy coat suspension) was inoculated into 96-well microtiter plates that contained a monolayer of bovine turbinate cells in EMEM containing 10% equine serum and 1% l-glutamine. Plates were incubated for 3 days at 37 C in humidified air containing 5% CO2. Samples were transferred to new monolayers of bovine turbinate cells in 96-well plates on the third day. This was accomplished by inoculating the new wells with 15 µl of the supernatant from each well of the original plates. New plates were incubated for 3 days, after which they were drained and the cells rinsed with PBS solution. Cells were fixed in 35% acetone in PBS which they were drained and the cells rinsed with PBS solution. Samples were then allowed to dry at 20 C. Cells were incubated for 30 minutes at 20 C with 100 µl of porcine-origin polyclonal BVDV antibody diluted 1:100 in binding buffer (PBS solution that contained 0.05% Tween and 2.95% NaCl). The polyclonal antibody was removed, and cells were washed 3 times with wash buffer (PBS solution containing 0.05% Tween). A volume (50 µl) of protein G-horseradish peroxidase diluted 1:2,000 with binding buffer was added to each well, and cells then were incubated for another 30 minutes at 20 C. Cells again were washed and then were incubated in the dark for 1 hour with 100 µl of substrate solution consisting of 3 amino-9 ethylcarbazole in 0.05M sodium acetate buffer (pH 5.0) and 30% hydrogen peroxide. Two positive-control samples, 1 for BVDV type I and the other for BVDV type II, and 2 negative-control samples on virus included on each plate. A sample was considered to have positive results when distinct red cytoplasmic staining was evident in infected cells. Samples were classified as negative when red cytoplasmic staining was not detected.
Results

Herds—Fourteen dairy herds were included in the study. Eight of the herds did not contain PI cattle, and 6 herds contained PI cattle; thus, herd prevalence for this study was 43%. The number and prevalence of PI cattle in each herd were summarized (Table 1). These numbers were calculated on the basis of all cattle tested in each herd, which included calves, heifers, and cows.

Results of the questionnaire were used to compare herd management practices between herds with PI cattle and herds without PI cattle. Only 1 herd (a herd without PI cattle) was a totally closed herd. The other 13 herds had all purchased cattle within the preceding 5 years. Herds without PI cattle had purchased the replacement cattle predominantly from private herds (5 herds) but also from a stockyard-auction market (2), whereas the herds with PI cattle had purchased replacement cattle predominantly from a stockyard-auction market (3) as well as a private herd (1). Only 1 herd with PI cattle screened purchased cattle for BVDV, and none of the herds without PI cattle screened purchased cattle for BVDV. Only 2 herds (1 with PI cattle and 1 without PI cattle) segregated purchased cattle at the time of arrival on the farm before introduction to the rest of the herd.

Cows in 6 of 8 herds without PI cattle were vaccinated against BVDV. One herd was vaccinated with a modified-live virus (MLV) vaccine, whereas the other 5 herds were vaccinated with a killed-virus vaccine 1 time (2 herds), 2 times (1), or 3 times (2) each year. Cows in 5 of 6 herds with PI cattle were vaccinated against BVDV. However, these herds were all vaccinated with a killed-virus vaccine once (1) or twice (4) each year.

In 5 of 8 herds without PI cattle, calves and heifers were vaccinated against BVDV, using a MLV vaccine (4 herds) or killed-virus vaccine (1). In 4 of 6 herds with

<table>
<thead>
<tr>
<th>Herd</th>
<th>PI cattle in herd</th>
<th>No. of cattle in herd</th>
<th>PI cattle in herd</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Yes</td>
<td>236</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>198</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>379</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Yes</td>
<td>261</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
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<td>6</td>
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<td>6</td>
</tr>
<tr>
<td>Subtotal</td>
<td>1,675</td>
<td>22</td>
<td>1.3</td>
</tr>
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</table>

Table 1—Prevalence of cattle persistently infected (PI) with bovine viral diarrhea virus (BVDV) in 14 dairy herds.
PI cattle, calves and heifers were vaccinated against BVDV, using a MLV vaccine (1 herd) or killed-virus vaccine (3).

In herds without PI cattle, breeding of cows and heifers was accomplished by a combination of artificial insemination and natural service in 5 herds and by natural service alone in another herd. In herds with PI cattle, breeding of cows and heifers was accomplished by artificial insemination alone in 1 herd, natural service alone in 3 herds, and a combination of artificial insemination and natural service in 2 herds.

Owners of 3 herds without PI cattle reported that there was evidence of BVDV infection in their herds. Owners of 5 herds with PI cattle reported that there was evidence of BVDV infection in their herds. Evidence suggestive of BVDV included herds with reproductive problems, herds in which ill cattle did not respond as expected to treatment, or herds with recent results of diagnostic tests that indicated BVDV infection.

Serologic test of 5 randomly selected unvaccinated heifers—Results of the serologic testing of 5 heifers from each herd were compared with results for herd virus isolation (Table 2). Herd sensitivity for the serologic test was 66% (95% CI, 29 to 100%), and herd specificity was 100%. The $\kappa$ value for agreement between the 2 tests was 0.7 (95% CI, 0.20 to 1.20).

Correlation of virus genotype and predominant BVDV titer in herd—In all herds in which PI cattle were identified, the genotype of the virus isolated was the same as the genotype of the predominant BVDV-neutralizing antibody titers in the heifers tested. For example, when the heifers had higher titers for type-II BVDV than for type-I BVDV, type-II BVDV was isolated from the herd, and vice versa. Results of PCR analysis were used to identify type-I and -II BVDV isolates (Fig 1).

Analysis of bulk-tank milk samples—the PCR results for bulk-tank milk samples were compared with results of whole-herd virus isolation (Table 3). Herd sensitivity of the PCR for bulk-tank milk samples for identifying dairy herds with PI cattle was 33% (95% CI, 0 to 71%), and herd specificity was 100%. The $\kappa$ value was low (0.36; 95% CI, 0.04 to 0.77).

Discussion

Analysis of the results of the study reported here indicates that serologic evaluation of 5 unvaccinated heifers randomly selected from each herd is an accurate method for determining whether there is a PI animal in that herd. Defining the cutoff for a positive result on the serologic test as a herd in which 3 of 5 heifers have neutralizing antibody titers $\geq$ 128 to type-I or -II BVDV, whereas a negative result was defined as a herd in which at least 3 of 5 randomly selected unvaccinated 6- to 12-month-old heifers had neutralizing antibody titers $\geq$ 64 to both type-I and -II BVDV.

Table 2—Comparison of results of serologic testing of 5 heifers with results of whole-herd virus isolation as a method of preliminary screening to detect dairy herds with PI cattle

<table>
<thead>
<tr>
<th>Results of whole-herd virus isolation</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive*</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Negative</td>
<td>2</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>8</td>
<td>14</td>
</tr>
</tbody>
</table>

*Positive result of a serologic test was defined as a herd in which at least 3 of 5 randomly selected unvaccinated 6- to 12-month-old heifers had neutralizing antibody titers $\geq$ 128 to type-I or -II BVDV, whereas a negative result was defined as a herd in which at least 3 of 5 randomly selected unvaccinated 6- to 12-month-old heifers had neutralizing antibody titers $<$ 64 to both type-I and -II BVDV.

Table 3—Comparison of results of a polymerase chain reaction (PCR) analysis of a bulk-tank milk sample with results of whole-herd virus isolation as a method of preliminary screening to detect dairy herds with PI cattle

<table>
<thead>
<tr>
<th>Results of whole-herd virus isolation</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>8</td>
<td>14</td>
</tr>
</tbody>
</table>
Serologic evaluation of another herd with PI cattle included 1 PI heifer. However, the other 4 heifers that were tested in that herd all had extremely high titers such that the herd was correctly classified. Thus, the inclusion of PI heifers in the animals that are tested in a specific herd may influence results of the serologic test, depending on the number of PI animals tested and the cutoff values that are used.

The $\kappa$ test statistic was 0.7, meaning there was substantial agreement between the serologic evaluation of 5 heifers and results for whole-herd virus isolation, the method used to definitively classify herds that contained PI cattle. The $\kappa$ statistic does not identify the test that provides correct information, only the amount of agreement between tests. However, if whole-herd virus isolation was the criterion-referenced standard by which herds were identified as containing PI cattle, then the $\kappa$ statistic is an indirect measure of the ability of the serologic evaluation of 5 heifers to identify herds with PI cattle.

The definition of a positive result for the serologic test in the study reported here was determined on the basis of results of another study. Changing the cutoff values used to define a positive result for the serologic test would alter herd sensitivity and specificity of the test. Generally, increasing the titer used as the cutoff value or the number of heifers tested with titers greater than a particular cutoff value would improve specificity but decrease sensitivity, and vice versa. Selecting a cutoff value is a subjective process and depends on the relative cost of false-negative and false-positive test results. For example, in this study, the cost of a false-positive test result was considered much greater than the cost of a false-negative result, because a false-positive result could lead to expensive testing to identify a nonexistent PI animal within a herd. Therefore, if another cutoff value for a positive result of a serologic test were chosen, a greater value would be placed on specificity, even though it would likely mean sacrificing sensitivity.

Overall sensitivity of the use of serologic evaluation of 5 unvaccinated heifers as a method to screen dairy herds for PI cattle could be improved by retesting those herds that had negative test results. Retesting should be performed at regular intervals. Had the herds with negative test results in this study been retested in 6 months, it is likely that the 2 herds with false-negative results would have yielded positive test results. In the 1 herd, the PI heifer would have been within the target population, and it is likely her penmates would have been seropositive. The age cohort of PI cattle in the other herd would no longer have been in the target population, and it is probable that subsequent heifers would have seroconverted.

Another way that the overall sensitivity of screening a sample of 5 heifers for BVDV could be improved is to concurrently conduct virus isolation and serum virus neutralization tests on the heifers. A positive result for a herd test would be defined as a positive result on the serologic test or an animal that had positive results for virus isolation. In this study, concurrent testing would have resulted in 1 of the herds with false-negative results being correctly identified with positive results, and the overall sensitivity would have improved from 66 to 83%. However, the other herd with false-negative results would still have been classified incorrectly as negative for PI cattle. Either of the aforementioned methods to improve sensitivity will increase the overall cost for screening of a herd, but this increase is minimal when compared to the time and money needed for testing all cattle in each herd.

In other studies, herd size did not impact results of the serologic test. Therefore, it is likely that serologic evaluation of 5 unvaccinated heifers will be effective for herds regardless of size as long as the herd is managed as 1 unit with the usual commingling of cattle of various ages. Serologic evaluation of 5 unvaccinated heifers will be less likely to be effective if the heifers are housed at another location some distance from the main farm or are contracted to a heifer raiser where they are commingled with heifers from other herds. In that instance, the serologic test would reflect the status of the heifer raiser’s herd rather than the herd from which the heifer originated.

A factor that does play an important role when serologic evaluation of 5 unvaccinated heifers is used to identify herds with PI cattle is that neutralizing antibody titers must be determined for type-I and -II BVDV. Some laboratories may only determine titers for type-I BVDV unless specifically requested to provide titers for type-I and -II BVDV. In the study reported here, had we determined titers for only type-I BVDV, all 4 of the herds that had a positive result on the serologic test would have been seronegative and misclassified.

We detected excellent correlation between the genotype of the virus isolated from a herd and the predominant genotype of BVDV-neutralizing antibody titers in the 5 heifers that were tested from that respective herd. Thus, if the titers for type-II BVDV were higher than the titers for type-I BVDV on the serologic test, type-II BVDV was isolated from the herd. Knowing the genotype of the virus infecting the herd may be useful when designing a vaccination program for that herd. Many vaccines used to control BVDV contain only type-I strains, although there are newer vaccines that contain type-I and -II strains. There is some evidence that type-I vaccines will cross-protect against some type-II strains, but this protection may be incomplete, especially for protecting against fetal infection. However, because of the antigenic variation between type-I and -II BVDV isolates, it may be wise to use a vaccine that specifically contains a type-II BVDV strain in herds in which type-II BVDV is known to be circulating.

For the study reported here, the target population for collection of samples was heifers that were 6 to 12 months old. Passively derived antibodies are generally undetectable by the time cattle are 6 months old and will no longer interfere with results of the test. Because the first cattle that become seronegative following the removal of all PI cattle from a herd are unvaccinated heifers that are just slightly more than 6 months old, it has been suggested that the most effective age group from which to obtain samples is those heifers that are 6 to 12 months old.
Another method that has been suggested for screening dairy herds for PI cattle is the use of a PCR test on a bulk-tank milk sample. This test was performed on all herds in this study (Table 3). When compared with results for whole-herd virus isolation, sensitivity of the PCR test of the bulk-tank milk sample for identifying herds with PI cattle was only 33%, but specificity of the test was 100%. A low \( K \) value (0.36) suggested that there was little agreement between the results of the PCR test of the bulk-tank milk sample and the criterion-referenced standard whole-herd virus isolation. Part of the reason that the PCR test of bulk-tank milk samples had such poor sensitivity was that adult PI cows were found in only 3 of the 6 herds with PI cattle. Most PI heifers are culled or die before they enter the lactating herd. In 1 study, PI calves were twice as likely to die or be culled (sold because of unthriftiness) during the first year of life, compared with non-PI herdmates. Results of the PCR test of a bulk-tank milk sample were negative for 1 of the herds that had a PI cow. Because it is not known whether the milk from that PI cow was added to the bulk tank on the day the sample was collected, it is not possible to determine whether the PCR truly yielded a false-negative test (BVDV in the sample but not detected by the PCR). Regardless, when compared with the serologic evaluation of 5 unvaccinated 6- to 12-month-old heifers, PCR testing of a bulk-tank milk sample was not an effective method for identifying dairy herds with PI cattle.

Only 1 of the herds in the study was a completely closed herd. The rest of the herds purchased cattle on a regular basis or had added cattle during the preceding 5 years. Of particular interest was the source of these purchased cattle. Five of the 6 herds with PI cattle had purchased cattle from a stockyard-auction market, compared with only 2 of the herds without PI cattle. That does not mean that cattle purchased from the stockyard-auction market were the source of BVDV infection for these herds, but it could certainly be expected that the stockyard-auction market would be a good place for herds to acquire BVDV infection because of stress and commingling of cattle from several sources. Many producers use the stockyards as a market for their unthrifty or sick cattle, some of which could be PI animals. Moreover, this observation is in agreement with that of another study suggesting that the herds in our study were typical of those that believe they have PI cattle. Therefore, our study population may well be an accurate reflection of the population in which the serologic evaluation of 5 unvaccinated 6- to 12-month old heifers would most likely be applied.

\[ Epi\ Info,\ Centers\ for\ Disease\ Control,\ Atlanta,\ Ga. \]
\[ 445-BDV, National\ Veterinary\ Services\ Laboratory,\ Ames,\ Iowa. \]
\[ rec-Protein\ G\ HRP\ Zymed\ Laboratories\ Inc,\ San\ Francisco,\ Calif. \]
\[ AEC\ Tablets,\ Sigma\ Chemical\ Co, St\ Louis,\ Mo. \]
\[ 140-BDV, National\ Veterinary\ Services\ Laboratory,\ Ames,\ Iowa. \]
\[ 143-BDV, National\ Veterinary\ Services\ Laboratory,\ Ames,\ Iowa. \]
\[ Rneasy\ Total\ RNA\ System,\ Qiagen,\ Valencia,\ Calif. \]
\[ Superscript\ One-Step\ RT-PCR,\ Gibco\ BRL/Invitrogen\ Corp,\ Carlsbad,\ Calif. \]
\[ Taq\ DNA\ Polymerase,\ Qiagen,\ Valencia,\ Calif. \]
\[ Episcope,\ Royal\ (Dick)\ School\ of\ Veterinary\ Studies,\ University\ of\ Edinburgh,\ Edinburgh,\ UK. \]

References


