Clinical sensitivity and specificity of a real-time PCR assay for *Campylobacter fetus* subsp *venerealis* in preputial samples from bulls

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Objective—To determine clinical sensitivity and specificity of a quantitative real-time PCR (qRT-PCR) assay for *Campylobacter fetus* subsp *venerealis* (Cfv) in preputial samples of bulls.

Animals—313 beef bulls.

Procedures—Preputial samples were collected from 300 virgin bulls and 13 Cfv-infected bulls. Specificity of the qRT-PCR assay, determined on the basis of results for samples collected from virgin bulls, was compared with specificity of bacteriologic culture performed with transport enrichment medium (TEM). Sensitivity of the qRT-PCR assay, determined on the basis of results for multiple samples collected at weekly intervals from infected bulls, was compared with sensitivity of the direct fluorescent antibody test (DFAT), bacteriologic culture, and bacteriologic culture with TEM.

Results—Specificity was 85% for the qRT-PCR assay and 100% for bacteriologic culture; results were significantly different. Mean sensitivity was 85.4% for the qRT-PCR assay, 82.3% for direct culture in blood agar, 72.1% for the DFAT, 32.7% for direct culture in Skirrow agar, 30% for bacteriologic culture with TEM and blood agar, and 38.1% for bacteriologic culture with TEM and Skirrow agar. Differences in sensitivity among tests varied with ambient outdoor temperature. Repeated sampling significantly increased sensitivity of the qRT-PCR assay.

Conclusions and Clinical Relevance—Use of the qRT-PCR assay as a screening test on direct preputial samples had comparable sensitivity to bacteriologic culture, and repeated sampling improved sensitivity. Although improved performance of the qRT-PCR assay, compared with direct bacteriologic culture, was dependent on temperature, transport times that allow direct culture are unlikely under field conditions. The qRT-PCR assay would provide a fast and sensitive screening method for Cfv in bulls. *(Am J Vet Res 2014;75:851–860)*

Bovine genital campylobacteriosis is a venereal disease caused by Cfv and characterized by temporary infertility, reduced pregnancy rates, and abortions.1–4 This disease occurs worldwide wherever natural service is used for breeding.1,3,5 In addition, bovine genital campylobacteriosis is a major limitation to the international trade of semen and embryos.6 Detection of Cfv is often focused on bulls because they are subclinical carriers.7

<table>
<thead>
<tr>
<th>ABBREVIATIONS</th>
<th>DESCRIPTION</th>
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<tbody>
<tr>
<td>BA</td>
<td>Blood agar</td>
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<tr>
<td>Cff</td>
<td><em>Campylobacter fetus</em> subsp <em>fetus</em></td>
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<tr>
<td>Cfv</td>
<td><em>Campylobacter fetus</em> subsp <em>venerealis</em></td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>CSA</td>
<td><em>Campylobacter</em> selective agar</td>
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<tr>
<td>C</td>
<td>Cycle threshold</td>
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<td>DFAT</td>
<td>Direct fluorescent antibody test</td>
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<td>qRT-PCR</td>
<td>Quantitative real-time PCR</td>
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<td>RFU</td>
<td>Relative fluorescent units</td>
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<td>ROC</td>
<td>Receiver-operating characteristic</td>
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<td>SKA</td>
<td>Skirrow selective agar</td>
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<td>TEM</td>
<td>Transport enrichment medium</td>
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As a result, most diagnostic tests have been designed to identify Cfv in preputial samples. Current diagnostic techniques available for the detection of Cfv in preputial samples include bacteriologic culture,6,7 DFAT,7 ELISA,8 and PCR assay.6,11 Bacteriologic culture with subsequent phenotypic identification remains the gold standard for detection of Cfv.11 However, isolation of Cfv is challenging in most field situations because of its fastidious nature and sensitivity to environmental conditions.13 As a result, the accuracy of bacteriologic culture depends on the inoculum size,
the presence of competing microbiota in the sample, environmental conditions during transport, and laboratory techniques.\textsuperscript{16,17} Cattle management conditions often result in samples being collected in distant locations where samples must undergo transport times > 24 hours. Prolonged transport times can make temperature control extremely challenging, especially when using regular courier services.\textsuperscript{16} Provision of a microaerophilic environment is also required.

Because of these circumstances, special transport media have been developed to improve bacteriologic culture sensitivity by providing an appropriate environment and reduced oxygen tension for CfV and selectively inhibiting competing microbiota. Two of the most common TEM used are Clark’s TEM\textsuperscript{18} and modified Weybridge TEM.\textsuperscript{19} Unfortunately, the sensitivity of bacteriologic culture using TEM is quite variable, ranging from 25% to 90%.\textsuperscript{20–22} Moreover, the use of TEM requires special media that have a limited shelf life, and the sample must be held at a temperature between 20° and 37°C until analysis. Maintaining these conditions during transport can be difficult, depending on the geographic location and season.

Following arrival at the diagnostic laboratory, the samples must be processed and placed onto a culture medium with a modified atmosphere. Several selective agar media, such as CSA\textsuperscript{12} and SKA,\textsuperscript{24} are available for the isolation of CfV. These selective media contain polymixin B, among other antimicrobials, and susceptibility of some CfV strains to antimicrobials has been reported.\textsuperscript{21} Overgrowth of bacteriologic culture plates by non-CfV organisms is also common.\textsuperscript{8,22} An alternative bacteriologic culture method has been the use of filtration and nonselective agar media.\textsuperscript{22,23,26} Upon isolation, subsequent identification needs to be performed to confirm that the isolate is CfV. The introduction of molecular diagnostic techniques provided the necessary tools to develop a rapid and reliable test that would be useful in field investigations. Although several PCR assays have been developed for detection of Campylobacter fetus, few permit identification of CfV specifically.\textsuperscript{10,27–29} Primers VenSF and VenSR, developed by Hum et al\textsuperscript{10} as part of a multiplex PCR assay, amplify a 142-bp region of the parA gene in a genomic island present only in CfV.\textsuperscript{30} These primers have been the most extensively used and have been validated with hundreds of isolates from different countries.\textsuperscript{11} However, these primers have been mainly used for identification of isolates, and their use in field samples has been limited. A recent study\textsuperscript{11} adapted primers VenSF-VenSR to a HotSYBR Green qRT-PCR platform and optimized the sample processing for direct preputial sample testing. In addition, the analytic sensitivity of this assay was determined to be 10\textsuperscript{6} CFUs/mL of sample, which translates to a single cell per reaction. This test provides the possibility of a sensitive, simple, and cost-effective procedure that eliminates the need for special transport media and would be suitable for screening large numbers of bulls.\textsuperscript{31}

Currently, little is known about the diagnostic sensitivity and specificity of this qRT-PCR assay in clinical samples and how it compares with other available tests. Thus, the objective of the present study was to determine the clinical sensitivity and specificity of a published qRT-PCR assay for the detection of CfV in direct preputial samples, its performance compared with bacteriologic culture with or without TEM and DFAT, and the effect of ambient temperature at the time of sample collection on the sensitivity of different diagnostic tests.

Materials and Methods

Animals—Preputial samples were collected from 300 virgin beef bulls housed at the Agri-Environment Service Branch Maple Creek and Spring Creek bull stations in Saskatchewan, Canada. This group represented 3 breeds of beef cattle as follows: Angus, 57% (172/300); Charolais, 38% (114/300); Gelbvieh, 2.3% (7/300); Simmental, 1.7% (5/300); and Limousin, 0.7% (2/300). Of 300 samples collected, 36% (109/300) were from 1-year-old bulls, and 33% (99/300) were from 2-year-old bulls. The exact age was not reported for 31% (92/300); however, all bulls were ≤ 2 years old. Samples originated from a first or second preputial scrape in 69% (207/300) and 31% (93/300) of the bulls, respectively. The mean ± SD ambient outdoor temperature for the days of sample collection was 5.3 ± 0.9°C.

Samples were also collected at regular intervals from 13 bulls infected with CfV housed at the University of Saskatchewan Animal Care Unit. Median age of the bulls was 5 years (range, 3 to 7 years). Four of the bulls were purchased from 2 infected herds in Saskatchewan and were determined to be CfV carriers by use of PCR assay,\textsuperscript{11} bacteriologic culture, and phenotypic evaluation.\textsuperscript{32} Isolates recovered from the naturally infected bulls were used to experimentally infect 9 additional bulls as described by Bier et al\textsuperscript{33} with minor modifications. Briefly, CfV cultures were grown on 5% sheep BA plates\textsuperscript{34} for 3 days and harvested into PBS solution with a sterile swab. The culture was diluted with PBS solution to an optical density (measured at a 600-nm wavelength) of 0.4, and 2 mL of this inoculum was deposited in the fornix of the prepuce with a sterile plastic pipette. The inoculum was also plated on BA plates\textsuperscript{34} to confirm that 10\textsuperscript{6} to10\textsuperscript{7} CFUs/exposure was delivered. Eight of the experimentally challenged bulls were successfully infected with 1 series of 2 exposures approximately 1 week apart, whereas the remaining bull required a second series of 2 exposures. The mean ± SD interval between first exposure and the first culture-positive sample obtained from the experimentally infected bulls was 14.5 ± 11.2 days. All animal procedures were performed in accordance with the Canadian Council on Animal Care\textsuperscript{34} and approved by the University of Saskatchewan Protocol Review Committee.

Data collection—Data on breed, age, order of the samples collected, and time between collection and sample processing at the laboratory were recorded for all bulls. In addition, ambient outdoor temperature at the time of sample collection was obtained from the National Climate Data and Information Archive, generated by the Weather Office of Environment Canada.\textsuperscript{35} Data were obtained from the weather station closest to where the bulls were sampled. During the winter
months, the temperature value used was adjusted for the effect of wind as provided in the wind chill index. For the virgin bulls, temperature data were obtained for every hour spent during sample collection on a given day, and a mean value was calculated. For the Cfv-infected bulls, temperature data were recorded for each sample collection day at the start of the procedure.

**Sample collection and transport**—Preputial samples were collected by means of the aspiration method. Briefly, an individually wrapped 25-inch plastic pipette attached to a 20-mL syringe was inserted into the prepuce, and the plastic sheath was pulled back. The scrape was performed by moving the pipette backward and forward 10 times while applying suction by moving the syringe plunger to the 15-mL mark. The prepucial material was transferred to the appropriate media by rinsing the pipette with media. Two samples were collected from all virgin bulls; however, only 1 was used for the purpose of this study. Samples collected from virgin bulls were rinsed into 2 mL of PBS solution. Immediately, a 300-µL aliquot was transferred into 10 mL of modified Weybridge TEM. Direct preputial samples collected into PBS solution were placed in a Styrofoam box containing ice packs, and samples in TEM were transported in an insulated container with warm water bag at approximately 25°C and no special atmospheric conditions. Samples were transported to the laboratory for further processing within 24 hours.

Two preputial samples were collected at weekly intervals from 13 Cfv-infected bulls by the described method. One sample was placed in 5 mL of a neutral-buffered 1% formaldehyde solution for DFAT; the remaining sample was placed in 2 mL of PBS solution, and an aliquot was placed immediately into TEM, as described. The order in which samples for either media were collected was alternated for each bull weekly. Both samples were placed in a Styrofoam container with warm water bags at 27.6 ± 2.1°C (mean ± SD). All samples collected from infected bulls were transported to the laboratory within 2 hours; those for DFAT were placed in a refrigerator at 4°C, and those in TEM were left at room temperature (mean ± SD, 24.6 ± 1.1°C) for 24 hours.

**Bacteriologic culture procedures**—Samples collected in TEM from virgin bulls were plated onto SKA (n = 191) or CSA (109). Campylobacter selective agar was prepared as described by Clark et al., and 2 plates were made with 1.5% and 4% agar, respectively. A 300-µL aliquot was placed on the edge of the plate and spread with a disposable loop.

A 300-µL aliquot of direct preputial material collected from Cfv-infected bulls was plated onto SKA and 5% sheep BA plates. The sample was placed on the edge of the plate and spread with a disposable loop for SKA plates versus being applied to a 0.65-µm mixed cellulose ester membrane filter overlay on the BA plates. The BA plates with the filter were incubated aerobically at 37°C for 30 minutes to allow motile cells to migrate through the membrane, after which the filters were removed and both the BA and SKA plates were incubated at 37°C for 72 hours in microaerophilic conditions by use of a commercially available pouch system. Samples placed in TEM were cultured in an identical fashion after 24-hour incubation at room temperature.

The Cfv colonies were identified as smooth, 1 to 2 mm in diameter, convex, white-gray, and translucent, and they were examined microscopically by use of Gram stain. Gram-negative cells with Campylobacter-like morphology (straight or curved thin rods) were confirmed as Cfv by a conventional multiplex PCR assay as described. Comparison of culture methods by use of a subset of the samples has been reported, and use of the data in the present study was for the purpose of comparison with the qRT-PCR assay.

**DFAT**—All samples were processed within 10 days of collection. The test was performed with a fluorescein isothiocyanate–conjugated polyclonal antibody against Cfv. The technique was performed as previously described with minor modifications. Briefly, samples were centrifuged at 600 × g for 10 minutes, and the supernatant was centrifuged at 2,700 × g for 30 minutes. The pellet was resuspended in 500 µL of PBS solution, and 20 µL of the suspension was applied to a 12-well slide in duplicate. Samples were air-dried and fixed in ethanol for 15 minutes. The labeled antibody was applied to each well and incubated at 37°C in a dark humid chamber for 30 minutes. The slides were then washed 3 times in PBS solution for 10 minutes each. For each batch of samples processed, single suspensions of Cfv and Campylobacter jejuni were included as positive and negative controls, respectively. Samples with at least 1 fluorescent organism with morphology consistent with Campylobacter spp in either one of the duplicates were considered to have positive results.

**qRT-PCR assay**—Extraction of DNA from direct preputial samples was performed by the heat lysis method as described, and all samples were tested in duplicate. Real-time PCR assay reactions were carried out as described with primers VenSF and VenSR. Data were analyzed with computer software. Endpoint analysis was performed by calculating the mean RFU for the last 3 cycles. Negative controls (no template and extraction control containing PBS) were used to define unknown samples. Samples were considered to have a positive result if at least one of the duplicates had a mean RFU value that was greater than the RFU value of known negative samples plus a tolerance value. The optimal tolerance value was determined by use of an ROC curve. In addition, all samples with positive results were evaluated to determine whether the correct peak signal was generated at 78.5 ± 0.5°C (mean ± SD). To ensure that spurious molecules, such as primer dimers, were not recorded, the RFU value was obtained after each PCR assay cycle by pausing at 76°C for 10 seconds between the PCR extension and denaturing steps. By obtaining the value at a temperature just less than the assay target’s melting temperature, any smaller non-target molecules would be denatured and would not generate a signal. Quantification of the number of organisms per milliliter of preputial sample was determined by use of a standard curve of known amounts of the target sequence plotted against C values. Target copy numbers were adjusted for dilution of the sample dur-
ing processing and expressed as CFUs per milliliter on the basis of the presence of a single copy of *parA*.

**Statistical analysis**—To determine the optimal tolerance level or cut point for the qRT-PCR assay, a nonparametric ROC curve was developed. The values used as the cut point were defined as the tolerance greater than the known negative samples and expressed as the percentage of the range of RFU for a given qRT-PCR assay run. The percentage of the range greater than the known negatives was calculated by the formula PR = (RFUXS - RFUXN) / RFUrange, where RFUXS is the mean RFU for the sample, RFUXN is the mean RFU for the known negatives, and RFUrange is the highest RFU value of the run minus the RFUXN. In those samples for which only one of the duplicates had a Cₜ value, only the RFU value of that duplicate was used in the calculation of the PR. The ROC curve was obtained by use of a commercial statistical analysis package. In addition, a plot of sensitivity and specificity against different cut points was produced. The optimal cut point was determined as the value that would maximize both sensitivity and specificity.

Crude specificity estimates for the qRT-PCR assay and bacteriologic culture were determined by calculating the proportion of virgin bulls that tested negative for each assay. Confidence intervals were calculated on the basis of the Wilson score method. The bulls tested for specificity determination (the virgin bulls) had never been exposed to breeding and were assumed to be true negatives and not infected with Cfv. Potential differences in the specificity between tests were evaluated by use of exact logistic regression. The order of the sample collected and the breed of the bulls were evaluated as covariates in the model.

Sensitivity estimates for each test were determined by calculating the proportion of samples collected from Cfv-infected bulls that tested positive. Samples from Cfv-infected bulls that were considered Cfv-positive were defined as those obtained within the first sampling that tested positive by bacteriologic culture (the gold standard) and the last culture positive sample obtained or the completion of the study. Samples collected during that period were considered to originate from a known Cfv-infected bull and were included regardless of individual test results. As a result, all samples originating from a known infected bull, as defined, were considered to be samples containing Cfv. Clearance of infection was determined when 4 consecutive samples were determined to have negative results of bacteriologic culture. Confidence intervals were determined by the Wilson score method. Potential differences in sensitivity among tests were evaluated by use of generalized linear mixed models with a binomial distribution and logit link function, considering each sample...
as the experimental unit. Within-bull clustering and sampling within bull clustering were accounted for with random intercepts. The proportion of total variance explained by differences between bulls ($\rho = \sigma_b^2 / \left( \sigma_b^2 + \sigma_s^2 + \pi^2 / 3 \right)$) and sampling within bull ($\rho = \left( \sigma_b^2 + \sigma_s^2 \right) / \left( \sigma_b^2 + \sigma_s^2 + \pi^2 / 3 \right)$) was also estimated. The ambient outdoor temperature at the time of sample collection was included as a fixed effect to evaluate its influence in test sensitivity, and first-order interaction was evaluated. In addition, a series of potential risk factors were evaluated, including age, breed, order of sample collection, source of infection (natural or experimental) and transport time ($\leq 1$ hour or $> 1$ hour).

The potential improvement in sensitivity with repeated sampling and testing by qRT-PCR assay was analyzed by identifying groups of 3 sequential samples for each Cfv-infected bull on the basis of the date of sample collection. The resulting sets of 3 samples from successive weekly samples were used to calculate the cumulative sensitivity for 1, 2, or 3 consecutive samples. Potential differences in cumulative sensitivity between the numbers of consecutive samplings were evaluated by use of generalized linear mixed models with a binomial distribution and logit link function. Within-bull clustering was accounted for with a random intercept.

Variables evaluated as potential risk factors in each model were retained if they were either significant ($P < 0.05$) or acted as important confounders. Risk factors were considered to be confounders if removing or adding the factor from the model changed the regression coefficient.
for another risk factor of interest by > 10%. Variables were considered significantly different at \( P \leq 0.05 \).

**Results**

**qRT-PCR assay cut point value**—An ROC curve was generated from all the qRT-PCR assay data generated in the study to determine the optimal cut point for the assay. The cut point at which sensitivity and specificity were maximized was determined as 2.5% greater than the background value (Figure 1) and corresponded to a sensitivity and specificity of 85%. The minimum cut point value that resulted in a sensitivity > 90% was 0.9%. However, the specificity at this value was only 70.8%. Conversely, the minimum cut point value that resulted in a specificity > 95% was 17%, but the sensitivity at this cut point decreased to 61.9%.

**Specificity**—The group of 300 virgin bulls were tested to generate a collection of samples anticipated to be Cfv negative. A positive result from these samples was considered a false-negative result. Crude specificity estimates from these samples were 85% (95% CI, 80.5% to 88.6%; 253/300 negative test results) for qRT-PCR assay and 100% (95% CI, 98.7% to 100%; 300/300 negative test results) for bacteriologic culture in SKA or CSA after transport in TEM and were significantly (\( P = 0.01 \)) different. There was no significant (\( P = 0.45 \)) effect of order of sampling, and thus sampling order was removed from the model.

**Sensitivity**—A group of Cfv-positive bulls was sampled repeatedly, once a week, to generate a collection of samples anticipated to be Cfv positive. A negative result from these samples was considered a false-negative result. Crude sensitivity estimates from these samples for the different tests evaluated were 85.4% (95% CI, 80.6 to 89.2%; 222/260 positive test results) for qRT-PCR assay, 82.3% (95% CI, 77.2 to 86.5%; 214/260 positive test results) for direct culture on BA, 72.1% (95% CI, 66.2 to 77.4%; 176/244 positive test results) for DFAT, 32.7% (95% CI, 27.2 to 38.7%; 82/251 positive test results) for direct culture on SKA, 30% (95% CI, 23.4 to 37.9%; 48/160 positive test results) for TEM and BA, and 38.1% (95% CI, 31 to 45.9%; 61/160 positive test results) for TEM and SKA.

Median (range) ambient outdoor temperature for the samples collected was 5.1°C (27.5° to –34°C). The effect of ambient outdoor temperature on sensitivity varied by test type (\( P < 0.001 \); Figure 2; Table 1). Overall, the qRT-PCR assay and direct bacteriologic culture on BA had superior sensitivity, compared with all other tests. When outdoor temperature was > 5°C, the sensitivities of the qRT-PCR assay (94.2% and 95.8%, for 10° and 15°C, respectively) were significantly higher than those of direct bacteriologic culture on BA (87.0% and 86.9%, for 10° and 15°C, respectively). Conversely, when the outdoor temperature was colder (<5°C), direct bacteriologic culture on BA had higher sensitivity than did qRT-PCR assay (87.0% vs 85.3%, respectively). The sensitivity of the qRT-PCR assay increased as ambient outdoor temperature increased (range, 85.5 to 95.8%), whereas the sensitivity of direct bacteriologic culture on BA remained almost constant (range, 86.9% to 87.0%).

The sensitivity of DFAT (range, 71.3% to 80.3%) was superior to that of bacteriologic culture using TEM (BA range, 21.0% to 25.3%; SKA range, 29.3% to 43.4%) and direct bacteriologic culture on SKA (range, 11.8% to 39.6%); however, it was less than that of the qRT-PCR assay (range, 85.5% to 95.8%) and direct bacteriologic culture on BA (range, 86.9% to 87.0%). An increase in the sensitivity of DFAT with increasing outdoor temperature was observed similar to that described for the qRT-PCR assay (Table 1). Both direct bacteriologic culture and the use of TEM with SKA resulted in a decrease in sensitivity as outdoor temperature increased. Conversely, the use of TEM with BA resulted in higher sensitivity with increasing outdoor temperatures.

No other measured factors were associated with the sensitivity of the tests including order of sampling (\( P = 0.92 \)), type of infection (natural vs experimental; \( P = 0.35 \)), breed (\( P = 0.75 \)), age of the bull (\( P = 0.34 \)), or sample transport time (≤ 1 hour vs > 1 hour; \( P = 0.25 \)). Differences among bulls were responsible for 12.8% of the unexplained variation, and differences among samples within bull were responsible for 35.2% of the unexplained variation.

**Performance of the qRT-PCR assay on carrier bulls and with repeated testing**—Only 2 of the 13 bulls had positive results of qRT-PCR assay in every sample collected. The percentage of samples with positive results and the mean quantity of organisms per milliliter of sample for each bull were determined. The percent-

### Table 1—Adjusted sensitivity (% [95% CI]) estimates for bacteriologic culture, the DFAT, and a qRT-PCR assay for detection of Cfv infection in bulls in preputial samples collected at various ambient temperatures.

<table>
<thead>
<tr>
<th>Test</th>
<th>Ambient temperature (°C)</th>
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<tr>
<td></td>
<td>-5</td>
</tr>
<tr>
<td>Direct bacteriologic culture</td>
<td>260</td>
</tr>
<tr>
<td>BA (Filter)</td>
<td>251</td>
</tr>
<tr>
<td>Direct bacteriologic culture + TEM</td>
<td>160</td>
</tr>
<tr>
<td>BA (Filter)</td>
<td>160</td>
</tr>
<tr>
<td>Skirrow</td>
<td>244</td>
</tr>
<tr>
<td>DFAT</td>
<td>260</td>
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</tbody>
</table>

*Values with different superscript letters in a column are significantly (\( P < 0.05 \)) different.

Filter = 0.85-μm mixed cellulose ester membrane filter overlaid on the BA plates. Skirrow = Skirrow agar plates.
age of samples with positive results obtained by qRT-PCR assay for each bull ranged from 46% to 100%. The median (range) quantity of organisms per milliliter of sample, determined from the qRT-PCR assay, was $7.0 \times 10^7$ (0 to $2.5 \times 10^8$), and the range of $C_v$ values obtained in the qRT-PCR assay was 21.1 to 35.6.

The use of repeated testing at weekly intervals significantly ($P < 0.001$) improved the sensitivity of the qRT-PCR assay. Cumulative sensitivity was 85.4% (95% CI, 81.0% to 89.5%; 222/260 positive test results) for a single sample, 95.5% (95% CI, 92.2% to 97.5%; 236/247 positive test results) for 2 samples, and 98.7% (95% CI, 96.3% to 99.6%; 231/234 positive test results) for 3 samples. The increase was significant for 2 sequential samplings, and no further improvement was seen when 3 sequential samples were examined ($P = 0.051$).

**Discussion**

The objectives of this study were to determine the clinical sensitivity and specificity of a published qRT-PCR assay for the detection of Cfv from preputial samples, compare its performance with established bacteriologic culture techniques and DAFT, and investigate the effect of ambient outdoor temperature at the time of sample collection on the sensitivity of different diagnostic tests. Once a cut point of 2.5% RFU signal greater than the known negative background was determined empirically, the qRT-PCR assay had both a sensitivity and specificity of 85%. The optimal cut point for an assay can be adjusted depending on the intended goal of the testing. The choice of cut point should be made on the basis of the pretest probability in the population of interest as well as the consequences of false-positive and false-negative results. For example, screening the bull population in a commercial cow-calf operation in which Cfv is suspected could require a cut point that maximizes sensitivity because the pretest probability is high and the cost of false-negative results can be high. Conversely, a test used for a purebred bull from a semen collection center should initially optimize specificity to avoid unnecessary culling of a valuable animal. Ultimately, the objective of the testing strategy and the pretest probability of the infection should dictate the selected cut point.

The sensitivity of 85% for the qRT-PCR assay was comparable to that of the direct bacteriologic culture with BA medium and similar to the sensitivity reported in a study based on a conventional multiplex PCR assay that used the same primers. The only other real-time PCR assay described for Cfv detection directly from preputial samples is the 5′ Taq nuclease assay described by McMillen et al. Although the analytic sensitivities of the qRT-PCR assay of the present study and the McMillen assay are comparable, there is presently limited information available on clinical sensitivity and specificity of the McMillen assay. One potential limitation of the Taq nuclease assay is that the primers used by McMillen et al. targeting the parA gene (different from the VenSF-VenSR primer set used in the qRT-PCR assay) have mismatches with certain Cfv sequence types and also cross-react with a strain of *Campylobacter hyointestinalis*. The specificity estimate obtained for the qRT-PCR assay was also 85%; 15% of virgin bull samples generated a positive result by use of the assay. For the purpose of this study, virgin bulls were assumed to be negative for Cfv because of the lack of exposure to breeding as reported by the owners. Although it is possible that some of these bulls had been exposed to breeding without notice by the owner, it is unlikely that this could explain such a high percentage of positive results. Bull-to-bull transmission has also been suggested in the literature as a potential means of transmission because mounting behavior is often observed, particularly in younger bulls. However, to the best of our knowledge, there have been no scientific data to support this assumption, and the high susceptibility of the organism to environmental conditions makes this possibility less likely. More investigation into possible sources of cross-reacting signal is needed to understand this specificity result better.

The model used to determine sensitivity and specificity in the present study had several advantages and disadvantages. Commonly, sensitivity and specificity are determined by comparison with the gold standard. However, in the case of Cfv, it has been well established that the best method available, organism isolation by bacteriologic culture, lacks sensitivity under field conditions. We attempted to use the true infection status of the bulls as a gold standard; although this would be ideal, it was based on certain assumptions. For estimation of sensitivity, we determined the status of a Cfv-positive carrier on the basis of bacteriologic culture of Cfv. For a bull to be considered a Cfv carrier, Cfv had to be cultured from the prepuce at the beginning and end of the testing period. For example, if a bull was determined to be culture positive on week 1, that bull was sampled every week thereafter. Subsequently, if week 20 was the last sampling point of that bull, and that sample was culture positive, all 20 samples from week 1 to week 20 were considered to be Cfv positive. As a result, the sensitivity of a particular test would be determined as the proportion of those 20 samples that tested positive for that given test.

The potential pitfall of this approach is the assumption that every sample had Cfv in it to be detected. With no knowledge about how the numbers of Cfv fluctuate in a carrier bull over time, it is possible that some samples obtained between culture-confirmed positive samples could have had undetectable numbers of Cfv by any testing strategy. This would artificially lower the sensitivity of all the testing methods. In addition, this approach eliminates the incubation period and the period around clearing of the infection. Given that bacteriologic culture is not 100% sensitive, these 2 periods were excluded in this model, which could lead to an overestimation of the sensitivity of diagnostic tests. However, one would expect that differences among tests would remain the same, although precise estimates would differ in their value. Despite this, we believe that this was the best available method to determine test sensitivity in the absence of a 100% sensitive gold standard. This type of sensitivity evaluation has been successfully used with other organisms.

Conversely, for specificity, it was assumed that virgin bulls < 2 years of age were Cfv negative. Our current understanding of Cfv suggests that its trans-
mission is exclusively venereal, making young virgin animals a suitable negative sample group. One area of little knowledge is how the preputial environment is different between mature and virgin animals, leading to different types of organisms that may be present to generate false-positive test results. A test designed to be specific for the prepuce of a mature animal may have cross-reactive issues because of prepuce differences in a juvenile animal. Although little work has been done to characterize the prepuce microbiome, known inhabitants of the preputial cavity in bulls include streptococci, micrococci, Bacillus spp, corynebacteria, coliforms, Bacteroides spp, and Pseudomonas spp. In addition, the mounting behavior of young bulls when housed together leads to frequent contamination of the prepuce with leces, which could further complicate the reliable identification of Cfv. Further research is needed in regards to the prepuce microbiome and its relationship with age and Cfv infection to better understand specificity issues related to an animal’s age.

The sensitivity and specificity of the qRT-PCR assay differed from the other techniques used. The specificity was significantly lower than for direct bacteriologic culture in SKA or CSA after transport in TEM; all specificity was significantly lower than for direct bacteriologic culture results in this study is that sample handling and transport time were ideal. Transport times were < 2 hours, and samples were kept at an almost constant temperature. Such conditions are hard to attain in most field situations. Diagnostic laboratories are often located at great distances from where the samples are collected, which requires shipment of the samples by courier services and transport times > 24 hours. Given these challenges, bacteriologic culture results from the field often have lower sensitivities than expected under ideal conditions.

Another technique investigated was DFAT. This test is appealing because it does not require samples to be collected in specialized media and has a good sensitivity. Previous reports2,23 have indicated a sensitivity > 90% for this test, whereas the sensitivity in the present study was lower. A potential explanation might be based on the degree of experience of the operator because samples are determined to have positive results not only by positive fluorescence, but also by examination of the sample for organisms with the correct morphological characteristics. In addition, this technique has reported cross-reactivity with certain strains of Cff of serotype A.9 Overall, the qRT-PCR assay used in the present study has the advantage that no special media or transport conditions are required and the assay is somewhat fast and has better sensitivity than most other tests available.

The relationship between ambient outdoor temperature at the time of sample collection and sensitivity of the tests evaluated in the present study provides novel information that could aid in the design of testing strategies that would maximize detection of carrier bulls. To the authors’ knowledge, this is the first report that identifies this type of relationship. The improvement in sensitivity observed for the qRT-PCR assay and DFAT when temperatures increase could be explained by differences in the population of Cfv organisms in the preputial cavity. Variations in the number of organisms recovered among examinations and among bulls have been described.7 It is possible that ambient outdoor temperatures could affect the level of infection in bulls by changes in the temperature in the preputial cavity, resulting in higher numbers of Cfv in warmer temperatures. As a result, the likelihood of identifying carrier bulls would increase when it is warmer outdoors. However, accurate information regarding numbers of organisms in the preputial cavity is lacking because current sampling methods do not generate consistent sample volumes. Thus, further research is needed to properly evaluate the number of organisms present and whether fluctuations in their numbers are associated with ambient outdoor temperatures.

The relationship between bacteriologic culture results and ambient outdoor temperature revealed some interesting differences among tests. When SKA media were used directly or with TEM, sensitivity decreased as ambient outdoor temperature increased. Fungal overgrowth in selective agar for Cfv has been described and can result in significant reductions in recovery rates.8,22 Overgrowth by fungi could potentially explain why sensitivity decreases at progressively warmer temperatures because the warmer temperatures provide better fungal growth conditions. Seasonal variations in the occurrence of fungal contamination have been suggested.23

The influence of outdoor temperature when using the passive filtration system in nonselective agar differed depending on whether bacteriologic culture was performed directly or after transport in TEM. The relationship with passive filtration in nonselective agar when using TEM is similar to that described for qRT-PCR assay and DFAT and might be a reflection of differing numbers of organisms in the prepuce. However, when bacteriologic culture was done directly on nonselective agar, there was almost no influence of temperature. This could be attributable to the short transport time and appropriate control of contaminants, maximizing the opportunity for isolation. Although some organisms are commonly isolated from preputial samples, such as Campylobacter sputorum biovar buhulus,50 Proteus spp,4,60 and Pseudomonas aeruginosa,22 little is known about the microbial population of the preputial cavity and how it could influence the establishment and persistence of Cfv.

Given the proportion of known Cfv-positive bulls that tested positive at every sampling and the variability in Cfv numbers among samplings, we believe that a single test is likely not sufficient to ensure identification of all carrier bulls. Variations in the numbers of Cfv isolated have been reported,7 and others in the field
also discourage reliance on a single test. The examination of 3 to 6 consecutive samples collected at weekly intervals by bacteriologic culture or DFAT has been the most common recommendation. The cumulative sensitivity of the qRT-PCR assay over 2 consecutive preputial samples was significantly higher than the sensitivity of a single sample (95.5% vs 85%, respectively). Although no significant improvement was found when 3 examinations were performed, compared with 2, the use of 3 samples would allow identification of almost 100% of infected bulls (98.7%). The use of repeated sampling has been a common practice in the diagnosis of Trichomonas fetus infection, and both practitioners and producers are already familiar with this strategy. Moreover, the use of direct preputial samples collected in PBS solution would allow for the evaluation of the 2 main venereal diseases from the same sample using the qRT-PCR assay used in the present study and a real-time PCR assay for T. fetus. Finally, there is an ongoing debate in the Cfv community as to the appropriateness of using the parA gene (via the VenSF-VenSR primer set) as a Cfv-specific target. A recent report indicated a positive reaction with a C. hyointestinalis strain in New Zealand with the primers VenSF-VenSR used in the present study. Despite its originally assigned name as a plasmid partitioning gene, the parA gene has been found in several strains to be located in a chromosomal genomic island. It is possible this genomic island is a mobile genetic element and could have been acquired by a strain of C. hyointestinalis by horizontal gene transfer. In addition, a Cff strain recently isolated from veal calves contains at least part of the genomic island believed to be exclusive to Cfv, which included a truncated portion of the parA gene. Whether use of the qRT-PCR assay used in the present study would result in misidentification of the new Cff strain as Cfv is not known, and further research is needed to evaluate this possibility. Both Cff and C. hyointestinalis are commonly isolated from feces of cattle and could potentially contaminate the prepuce in young bulls, in which mounting and sodomizing is a common behavior. If non-Cfv organisms have obtained the parA gene, this could explain the cross-reactivity that resulted in the lower specificity of the qRT-PCR assay used in the present study. The potential for misidentification is an important issue because it leads to an increase in false-positive results; for a disease with low prevalence, this is of particular concern. However, a recent study that attempted to develop PCR assays on the basis of new Cfv-specific targets was unable to improve the specificity results obtained with VenSF-VenSR primers, and no studies have yet been conducted with field samples. Therefore, although the parA gene is probably not the ideal testing target, it is the best and most thoroughly described Cfv-specific target found to date. Knowledge of the clinical sensitivity and specificity of this qRT-PCR assay and how it compares with other testing methods provides further information regarding Cfv management and control.

References


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