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Comparison of Culture and a Novel 5′ *Taq* Nuclease Assay for Direct Detection of *Campylobacter fetus* subsp. *venerealis* in Clinical Specimens from Cattle

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A *Campylobacter fetus* subsp. *venerealis*-specific 5′ *Taq* nuclease PCR assay using a 3′ minor groove binder-DNA probe (TaqMan MGB) was developed based on a subspecies-specific fragment of unknown identity (S. Hum, K. Quinn, J. Brunner, and S. L. On, Aust. Vet. J. 75:827–831, 1997). The assay specifically detected four *C. fetus* subsp. *venerealis* strains with no observed cross-reaction with *C. fetus* subsp. *fetus*-related *Campylobacter* species or other bovine venereal microflora. The 5′ *Taq* nuclease assay detected approximately one single cell compared to 100 and 10 cells in the conventional PCR assay and 2,500 and 25,000 cells from selective culture from inoculated smegma and mucus, respectively. The respective detection limits following the enrichments from smegma and mucus were 5,000 and 50 cells/inoculum for the conventional PCR compared to 500 and 50 cells/inoculum for the 5′ *Taq* nuclease assay. Field sampling confirmed the sensitivity and the specificity of the 5′ *Taq* nuclease assay by detecting an additional 40 bulls that were not detected by culture. Urine-inoculated samples demonstrated comparable detection of *C. fetus* subsp. *venerealis* by both culture and the 5′ *Taq* nuclease assay; however, urine was found to be less effective than smegma for bull sampling. Three infected bulls were tested repetitively to compare sampling tools, and the bull rasper proved to be the most suitable, as evidenced by the improved ease of specimen collection and the consistent detection of higher levels of *C. fetus* subsp. *venerealis*. The 5′ *Taq* nuclease assay demonstrates a statistically significant association with culture ($\chi^2 = 29.8$; $P < 0.001$) and significant improvements for the detection of *C. fetus* subsp. *venerealis*-infected animals from crude clinical extracts following prolonged transport.

Bovine venereal campylobacteriosis or vibriosis is a major cause of abortion and infertility in cattle and is one of the most important bovine venereal diseases in Australia (6). The disease is caused by *Campylobacter fetus* subsp. *venerealis* and is spread by infected bulls during servicing, by contaminated semen, or between bulls (41). Campylobacteriosis is an Office International des Epizooties list B notifiable disease that is considered to have socioeconomic and/or public health implications and is thus significant in the international trade of animals and animal products. In addition, international semen export guidelines require that bulls be *C. fetus* subsp. *venerealis* negative. Symptoms in female cattle include irregular estrus cycles, infertility through uterine infection, and early embryonic death, while bulls are asymptomatic carriers (6). The symptoms of campylobacteriosis are very similar to those of trichomoniasis (caused by the protozoan *Tritrichomonas fetus*), and these venereal diseases tend to occur in areas with extensive cattle management and natural breeding, such as western North America, Australia, Africa, and Latin America (11). In Australia, it has been estimated that vibriosis causes significant reproductive wastage in infected beef and dairy herds and represents a large economic loss for producers, particularly in the first year of infection, where gross margins can be reduced by as much as 66% (19). When the disease becomes established, gross margins may be 36% below those of noninfected herds (19). Similarly, in Argentina, bovine venereal diseases are considered to be causes of low reproductive efficiency with severe economic losses (5). Several killed bacterial campylobacteriosis vaccines are available, e.g., Vibrovax (Pfizer Animal Health, Australia); Vibrio-Lepto-5 (Boehringer Ingelheim Vetmedica, Inc.); Bioabortogen-H, Biogenesis, and Repropolivac (San Jorge Bago, Argentina); and *Campylobacter (Vibrio) fetus* vaccine (Onderstepoort Biological Products Ltd., South Africa) (9, 10), and such vaccines are considered the most effective means of managing the disease.

The diagnosis of infection is by the direct isolation of the causative agent by selective culture from semen, preputial smegma, or vaginal mucus (20, 31) or through the detection of an immune response in cervico-vaginal mucus by using an enzyme linked immunosorbent assay (ELISA) (23). Several methods for the collection of preputial smegma and vaginal mucus have been investigated in order to improve the reliability of selective culture-based diagnostic procedures or ELISA. These procedures have included preputial washes, scrapes (55), mucus swabs, blotting (23), and the commonly used scrape/aspiration methods with a pipette (26, 41, 55). The use of swabs and blotting has been limited to female cattle. A comparison of three collection methods for preputial smegma (scraping, aspiration, and washing) demonstrated that scraping with a specialized tool that was developed for the collection of preputial smegma for *Tritrichomonas fetus* culture reduced contaminant levels and improved isolation rates compared to those for aspiration and washing (55). Both aspiration and washing require manipulation of a syringe or bulb as well as the

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pipette, requiring at least two people during the collection of diagnostic specimens.

The traditional culture and ELISA diagnostic procedures present with sensitivity and specificity limitations. The ELISA is known to produce false-positive and false-negative results, and a high percentage of *C. fetus* subsp. *venerealis* strains are susceptible to polymyxin B, an antibiotic used in all *Campylobacter* selective media and transport enrichment media (TEM) (20, 26). *Campylobacter* colonies from preputial scrapes and vaginal mucus are visible within 48 h in a microaerobic environment, and the slow-growing *C. fetus* subsp. *venerealis* is readily overgrown by a range of microbes, leading to inaccurate diagnoses (31). These methods are not very sensitive or specific, and discrimination between *C. fetus* subsp. *venerealis* and the morphologically, phenotypically, and genetically similar *C. fetus* subsp. *fetus* is not reliable (57). A direct immunofluorescence test (DIFT) has been developed and applied to the detection of *C. fetus*, and although not widely evaluated, it may present with false-positive results due to nonspecific fluorescence and the inability to differentiate *C. fetus* subspecies (38). *C. fetus* subsp. *fetus* occurs mainly in the intestinal tracts of cattle and sheep and causes only sporadic abortion in these animals (50). Conversely, *C. fetus* subsp. *venerealis* is highly adapted to the genital tract of cattle and cannot survive in the bovine intestine (4). It is thus essential to identify *C. fetus* subspecies in the diagnosis of bovine venereal diseases. Molecular methods such as PCR (22), amplified fragment length polymorphism (57), and pulsed-field gel electrophoresis (17, 44) have been used to discriminate between the two *Campylobacter fetus* subspecies. However, PCR has not been routinely applied for the diagnosis of bovine venereal campylobacteriosis and field studies continue to rely upon either selective culture (20), ELISA (21), or DIFT (38).

Although the isolation and identification of *C. fetus* subsp. *venerealis* appears to be difficult, the adaptation of sensitive molecular methods for direct detection in clinical samples has not been forthcoming. In comparison to conventional PCR techniques, 5′ *Taq* nuclease assays are highly sensitive and specific and the amount of target DNA in the assay can also be accurately quantified (37). The implementation of 5′ *Taq* nuclease assays has improved the detection of a wide range of pathogenic organisms, including *Salmonella enterica* (18), pathogenic *Leptospira* spp. (51), *Campylobacter jejuni* (43), *Actinobacillus pleuropneumoniae* (3), and *Mycobacterium avium* subsp. *paratuberculosis* (28). Minor groove binder (MGB) probes demonstrate higher specificities and sensitivities than non-MGB probes in 5′ *Taq* nuclease assays (30) and thus are highly suited for routine diagnostic applications as demonstrated for the detection of bovine retroviruses (34, 35). This study describes the optimization of sampling, transport, and processing protocols for the diagnosis of bovine venereal campylobacteriosis by using a novel 5′ *Taq* nuclease PCR assay utilizing a 3′ TaqMan MGB probe.

MATERIALS AND METHODS

Bacterial and protozoan culture. Isolates of several *Campylobacter* species were obtained from the Animal Research Institute, Department of Primary Industries and Fisheries (DPI&F), from the American Type Culture Collection, and from the National Collection of Type Cultures (Table 1). *Campylobacter* strains were grown at 37°C in brain-heart infusion broth (Oxoid), 0.2% yeast extract, 0.07% Bacto agar for between 1 and 3 days. *Tritrichomonas fetus* was grown at 37°C in 1.25% neutralized liver digest, 0.5% tryptose, 0.15% Bacto agar, 50% sterile heat-inactivated bovine serum, 0.1% P/S solution (0.75% penicillin and 0.082% streptomycin). *Pseudomonas aeruginosa* and *Proteus vulgaris* were grown at 37°C on blood agar plates for 24 h. *Neospora caninum* tachyzoites were cultured in Vero cells as previously described (14).

TABLE 1. Reference species and isolates used in this study

Species	Strain	Source
C. fetus subsp. *venerealis*	98-109383	Field isolate (DPI&F)
C. fetus subsp. *venerealis*	19438	ATCC
C. fetus subsp. *venerealis*	Biovar venerealis	Pfizer Animal Health, Australia
C. fetus subsp. *venerealis*	Biovar intermedius	Pfizer Animal Health, Australia
C. fetus subsp. *fetus*	98-118432	Field isolate (DPI&F)
C. fetus subsp. *fetus*	15296	ATCC
C. jejuni subsp. *jejuni*	11168	NCTC
Campylobacter hyointestinalis	N3145	Field isolate (DPI&F)
Campylobacter sputorum subsp. *bubulus*	Y4291-1	Field isolate (DPI&F)
Campylobacter coli	11353	NCTC
Tritrichomonas foetus	YVL-W	Field isolate (DPI&F)
T. foetus	30003	ATCC
Tetratrichomonas gallinarum	30097	ATCC
Pentatrichomonas hominis	30000	ATCC
Trichomonas vaginalis	30001	ATCC
Pseudomonas aeruginosa	27853	ATCC
Proteus vulgaris	6380	ATCC
Neospora caninum	50843	ATCC
Leptospira borgpetersenii serovar Hardjobovis	93/94451/3	Field isolate (DPI&F)
Leptospira interrogans serovar Pomona	Pomona	CCRL[a]

[a] CCRL, WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Australia and Western Pacific Region.

Animal sampling. Three techniques were evaluated for the collection of smegma from bulls and the collection of vaginal mucus from female cattle. Collection techniques were evaluated on the basis of ease of use for the veterinarians, lack of adverse impact upon the animals, and suitability of the material gained for assay and culture.

Preputial smegma samples were collected from eight bulls by using sterile pipettes, swabs, or bull raspers. The bulls were restrained in a veterinary crush during the collection procedures. A sterile pipette (10-mm internal diameter, with a beveled edge) was gently scraped along the surface of the penis and internal prepuce near the fornix, with gentle aspiration being applied with an attached bulb or syringe. The collected smegma was rinsed into approximately 5 ml sterile phosphate-buffered saline (PBS) or physiological saline. A sterile McCullough uterine mare swab (Minitube Australia Pty Ltd.) was gently scraped along the surface of the penis and internal prepuce near the fornix. The collected smegma was expressed into approximately 5 ml sterile PBS or physiological saline. A bull rasper (polyethylene, 60 cm long with a 75-mm-long, 8-mm-diameter corrugated scraper head with a 1.5-mm collection bore attached to 6-mm-diameter tubing with a 1.5-mm internal diameter, similar to those produced by Elastecnica, Argentina; based on the original design that was described previously [52]) was gently scraped along the surface of the penis and internal prepuce near the fornix. No aspiration was necessary. The collected smegma was rinsed into approximately 5 ml sterile PBS or physiological saline.

Vaginal mucus samples were collected from eight cows by using artificial insemination pipettes, swabs, or bull raspers during restraint in a veterinary crush. A sterile artificial insemination infusion pipette was inserted so that the anterior end reached the cervix. Gentle suction was applied by using a rubber bulb while moving the pipette gently backwards and forwards. The pipette was removed, and the collected mucus was rinsed into approximately 5 ml sterile physiological saline. A sterile 15-cm swab, held by sterile forceps, was inserted so that the anterior end reached the cervix. The swab was gently moved backwards and forwards while being rotated to saturate the head with mucus. The swab was removed, and the collected mucus was expressed into approximately 5 ml sterile physiological saline. A bull rasper was inserted so that the anterior end reached the cervix. The rasper was moved gently backwards and forwards. No aspiration was necessary. The rasper was removed, and the collected mucus was rinsed into approximately 5 ml sterile physiological saline.

TABLE 2. Primers and fluorescent 3′ MGB-DNA probe used in this study

Primer	Sequence (5′ to 3′)
VenSF	CTTAGCAGTTTGCGATATTGCCATT
VenSR	GCTTTTGAGATAACAATAAGAGCTT
Mg3F	GGTAGCCGCAGCTGCTAAGAT
Mg4R	TAGCTACAATAACCACAACT
CFVF	CCCAGTTATCCCAAGCGATCT
CFVR	CGGTTGGATTATAAATTTTAGCTTGGT
CFVP1	6-FAM-CATGTTATTTAATACCGCAA[a]

[a] The probe was labeled with 6-carboxyfluorescein (6-FAM) phoshoramidite as the 5′ reporter dye and included a nonfluorescent quencher attached to the 3′ MGB moiety.

Urine was investigated to determine its feasibility as an alternative clinical specimen for the diagnosis of venereal *C. fetus* subsp. *venerealis* infection in bulls. Urine from two consecutive voids was collected in a series of sterile collection containers following the subcutaneous administration of a diuretic (Frusemide; Ilium Veterinary Products, Australia). The first container collected was discarded as being the most likely to be heavily contaminated with fecal material, hair, and other debris.

Diagnostic culture. Culture-based diagnosis for *C. fetus* subsp. *venerealis* was conducted by the inoculation of 5 ml modified Weybridge medium (32) with 0.5 ml of preputial smegma in PBS or vaginal mucus in saline, followed by transport at ambient temperatures for up to 48 h. Upon arrival at the laboratory, 100 μl of the inoculated modified Weybridge medium was plated onto *Campylobacter fetus* selective medium (Skirrow's) (49) and incubated at 37°C in a microaerobic environment that was generated by using an anaerobic jar and a Campygen sachet (Oxoid). The presence of *C. fetus* subsp. *venerealis* was indicated by the presence of small (about 0.5 mm in diameter), smooth, translucent colonies arising after 48 to 72 h, followed by microscopic confirmation of *Campylobacter*-like morphology.

PCR template preparation. DNA was extracted from liquid culture, resuspended colonies, preputial smegma, vaginal mucus, and urine samples by using a commercial kit (QIAamp DNA mini kit; QIAGEN) as per the manufacturer's protocol, except for elution of the final product in 50 μl rather than 400 μl.

Crude cell lysates were prepared for the 5′ *Taq* nuclease assay by a heat lysis method. One milliliter of the sample (preputial smegma in PBS; vaginal mucus in saline or urine) was centrifuged for 5 min at 12,000 × *g*, and the supernatant was discarded. The pellet or compressed mucus was resuspended in 500 μl sterile distilled water and heated at 95°C for 10 min. The suspension was centrifuged for 30 s at 2,000 × *g*, and the supernatant was assayed by 5′ *Taq* nuclease assay.

PCR amplification, cloning, and conventional PCR assay. All primers used in this study were synthesized by Proligo Australia Pty Ltd. The 142-bp *C. fetus* subsp. *venerealis*-specific product was amplified in a 15-μl reaction mixture volume by using 500 nM VenSF and VenSR primers (Table 2) (22), 1× PCR buffer with $MgCl_2$ (Roche Diagnostics), 200 μM dNTPs, 1 U *Taq* DNA polymerase (Roche Diagnostics), and 1 ng of target *C. fetus* subsp. *venerealis* DNA. The reactions were cycled in a GeneAmp PCR system model 2700 (Applied Biosystems Inc.) using the following conditions: initial denaturation at 95°C for 10 min, 30 cycles at 95°C for 20 s, annealing at 50°C for 20 s, and extension at 72°C for 2 min, with a final extension at 72°C for 10 min.

PCR assays were conducted under the same conditions using both the VenSF-and-VenSR primer pair and MG3F-and-MG4R primer pair (Table 2), which amplify a 960-bp *C. fetus*-specific product (22). Two microliters of QIAGEN kit-purified genomic DNA (gDNA) prepared from smegma, mucus, and urine extracts was added as template for conventional PCR assays. The presence of *C. fetus* subsp. *venerealis* is indicated by the presence of both the 960-bp *C. fetus*-specific amplicon and the 142-bp *C. fetus* subsp. *venerealis*-specific amplicon.

Amplification products were separated in 2% TBE (89 mM Tris borate, 2 mM EDTA [pH 8]) agarose gels by using size markers (Marker XIV; Roche Molecular Biochemicals, Germany) and were visualized under UV illumination by ethidium bromide staining.

Sequencing. The 142-bp *C. fetus* subsp. *venerealis*-specific amplicon from strain 98-118432 was ligated into a cloning vector (pCR2.1, TOPO-TA cloning kit; Invitrogen Corporation) as described in the manufacturer's protocol. Plasmids with inserts were sequenced using the T7 and M13 reverse primers and the BigDye Terminator mix (Applied Biosystems, Inc.), following the manufacturer's protocols. Sequences were analyzed by the Griffith University DNA Sequencing Facility (School of Biomolecular and Biomedical Science, Griffith University, Nathan, QLD 4111, Australia) on an ABI 377 DNA sequencer.

5′ *Taq* nuclease assay. Primer and probe combinations for 5′ *Taq* nuclease assay using fluorescent 3′ MGB-DNA probes (synthesized by Applied Biosystems, Inc.) were designed for *C. fetus* subsp. *venerealis* by using Primer Express, version 2 (Applied Biosystems, Inc.), and BLASTn searches (http://www.ncbi.nlm.nih.gov/BLAST/) were conducted to confirm sequence specificity. Sequences of primers and the probe are provided in Table 2. The 5′ *Taq* nuclease assay for *C. fetus* subsp. *venerealis* was conducted in a 25-μl volume by using either Platinum Quantitative PCR SuperMix-UDG (Invitrogen Life Technologies) or RealMasterMix probe mix (Eppendorf) with 900-nM CFVF and CFVR primers, 170 nM CFVP1 fluorescent 3′ MGB-DNA probe, and 5 μl of either heat-lysed cells or kit-purified DNA templates in a Corbett Rotor-Gene RG-3000 (Corbett Research, Australia). The thermal profile was 50°C for 2 min, 95°C for 2 min, and 45 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 20 s, and extension at 72°C for 20 s. The acquisition of fluorescence occurred at the end of each extension step. A positive result was indicated by the fluorescence (normalized to a no-template control) passing a threshold of 0.1. Assay conditions were optimized by using serial dilutions of *C. fetus* subsp. *venerealis*-purified DNA. Quantitative estimates of target cells/ml were calculated by using a standard curve generated with either kit DNA extracts or crude cell extracts prepared from serial dilutions of known numbers of *C. fetus* subsp. *venerealis* isolates. All 5′ *Taq* nuclease assays were prepared in duplicate, with cycle threshold or quantitative cell estimates averaged for each sample. Inconsistently positive samples (one of two repeats) were repeated.

Sensitivity evaluation and effects of transport. *C. fetus* subsp. *venerealis* strain 98-109383 cells from 2-day-old fresh cultures were treated 1:1 with methanol to reduce cell motility and were counted by using a hemocytometer. Serial log dilutions of the 2-day-old *C. fetus* subsp. *venerealis* cultures were prepared at 10^5 cells/ml and were diluted to 1 cell/ml. These dilutions were inoculated into smegma, mucus, and urine that were obtained from healthy animals which had previously tested negative for both *C. fetus* subsp. *venerealis* (by selective culture and 5′ *Taq* nuclease assay) and *T. fetus* (microscopic examination of InPouchTF cultures) (Biomed Diagnostics). The viability of the cells that were used for these evaluations was determined by spreading 100 μl of each dilution onto prepoured Columbia sheep blood agar plates (Oxoid), and colonies were counted following 2 days of incubation at 37°C in a microaerobic environment (as described above). Genomic DNA was extracted from the spiked specimens by using a commercial DNA extraction kit (QIAamp DNA mini kit; QIAGEN), which was assayed by both conventional PCR and 5′ *Taq* nuclease assays. Inoculated specimens were also prepared for 5′ *Taq* nuclease assay by heat lysis. Modified Weybridge TEM were inoculated with these laboratory-spiked specimens, as was done for diagnostic culture. DNA was also extracted from aliquots of inoculated TEM by using a commercial kit (QIAamp; QIAGEN) and assayed by both 5′ *Taq* nuclease assay and conventional PCR assay. Selective media were inoculated from the TEM as was done for diagnostic culture. Estimates of cell equivalents/assay or cells/inoculum were calculated from the enumerated spiked specimens by determining equivalent cell numbers contained in the final volume used either as template for PCR or as inoculum in cultures.

5′ *Taq* nuclease assays were scored positive if the fluorescence (normalized to a no-template control) passed a threshold of 0.1. A positive conventional PCR assay required the detection of both the 960-bp, species-specific and the subspecies-specific, 142-bp amplicons. Results from cultured samples were scored positive for the presence of *Campylobacter*-like colonies, followed by microscopic confirmation of *Campylobacter*-like morphology and motility.

Sample transport was simulated by storing the inoculated TEM and animal samples at ambient temperatures for smegma and mucus and at 4°C for urine for up to 5 days. Samples for 5′ *Taq* nuclease assays, conventional PCR assays, and selective culture were processed as described above at time zero and after 2 and 5 days of storage. To examine changes in cell numbers under these storage conditions, quantified cell estimates were compared by using 5′ *Taq* nuclease assay results of the 10^4 cells/ml-spiked samples.

Field sampling evaluation. Preputial smegma ($n = 249$) and cervico-vaginal mucus ($n = 120$) specimens were collected from 369 animals originating from 38 properties throughout northeastern Australia (Queensland) by using the bull rasper and the collection protocols described above. Specimens were assayed for *C. fetus* subsp. *venerealis* by both diagnostic culture and 5′ *Taq* nuclease assay. Urine was collected (as described above) from 16 bulls whose samples were positive by smegma 5′ *Taq* nuclease assay and processed for 5′ *Taq* nuclease assay testing. A chi-square test was used to compare the distribution of positive and negative results for the two methods of testing, culture and 5′ *Taq* nuclease assay. The proportions that were detected as positive by the two methods were also compared by using a normal approximation. Mucus samples from female cattle were also tested by using the *C. fetus* subsp. *venerealis* ELISA as described previously (23).

TABLE 3. Colony counts of dilution series prepared from fresh 2-day cultures of *C. fetus* subsp. *venerealis* subsequently used for sensitivity and transport inoculation experiments

Cells/inoculum[a]	Avg colony count	SD
2×10^3	>1,000[b]	NA[c]
2×10^2	210	28.28427
2×10^1	22	4.242641
2×10^0	2	1.414214
2×10^{-1}	0	0

[a] Cells/inoculum are based on an initial direct count using a hemocytometer followed by serial dilution. Inoculum volume was 100 μl/plate.
[b] The plate contained too many colonies to accurately count, with many colonies in physical contact.
[c] NA, not applicable.

RESULTS

Assay specificity and sensitivity. The genomic sequence of the species-specific amplicon from *C. fetus* subsp. *venerealis* strain 98-118432 is described in GenBank under accession no. AY903214. 5′ *Taq* nuclease primer and probe sequences are described in Table 2. BLASTn searches identified sequence identity between the primers, the probe, and the *C. fetus* subsp. *venerealis* plasmid ParA-like protein gene (GenBank accession no. AY750964). A single base difference was observed between the ParA-like protein gene sequence and the species-specific *C. fetus* subsp. *venerealis* amplicon that is described here.

By using serial dilutions of *C. fetus* subsp. *venerealis* genomic DNA (gDNA), optimal amplification and fluorescence were obtained using a three-step thermal profile (described in Materials and Methods) as opposed to the probe manufacturer's two-step preferred protocol (results not shown). 5′ *Taq* nuclease assay of genomic DNA from a range of related organisms and bovine venereal microflora (Table 1) did not produce any nonspecific amplification. Notably, the morphologically, phenotypically, and genetically similar *C. fetus* subsp. *fetus* did not produce a positive assay result in the *C. fetus* subsp. *venerealis* assay.

The viability counts of *C. fetus* subsp. *venerealis* dilutions correlated well with the hemocytometer counts as indicated in Table 3. The 5′ *Taq* nuclease assay ably detected approximately a single cell equivalent per assay from heat-lysed spiked preputial smegma and vaginal mucus preparations and 100 cell equivalents per assay from urine (Table 4). Culture-based detection was less sensitive (Table 4), and the *C. fetus* subsp. *venerealis* selective media suffered from significant levels of overgrowth by non-*Campylobacter*-like organisms, particularly from vaginal mucus. Conventional PCR assays generally improved upon the sensitivity of culture-based diagnosis (Table 4), with the greatest improvements being observed when vaginal mucus specimens were assayed. The 5′ *Taq* nuclease assay provided at least a 10-fold increase in sensitivity compared to those of the other methods that were evaluated and an improvement of 250-fold or higher compared to that for selective culture (Table 4).

Sample processing. A number of the samples collected from healthy animals contained visible contamination with feces, semen, and blood. Despite repeated attempts, the conventional PCR did not yield detectable products from crude cell extracts, and kit-purified gDNA had to be used as a template for these assays. The sensitivity limits and quantitative estimates that were observed by using the 5′ *Taq* nuclease assay did not change in the presence of potentially inhibitory substances in crude extracts; in fact, the sensitivity of detection improved slightly compared to that of gDNA extracts (Tables 4 and 5). Smegma specimens that were prepared for conventional PCR assay by a commercial DNA

TABLE 4. Detection limits for *Campylobacter fetus* subsp. *venerealis* from stored samples determined by selective culture, conventional PCR assay, and 5′ *Taq* nuclease assay[a]

Sample type	Storage time (days)[b]	Sensitivity limit[c]					
		Selective culture	Assay postenrichment culture[d]		Conventional PCR	5′ *Taq* nuclease assay	
			5′ *Taq* nuclease assay	Conventional PCR	QIAamp	QIAamp	Heat lysis
Smegma	0	>25,000	500	5,000[e]	>1,000	10	1
	2	25,000	500	5,000[e]	100	10	1
	5	2,500	5,000	>5,000	100	1	10
Mucus	0	>25,000	50	50	100	10	10[e]
	2	>25,000	500	500[e]	10	10[e]	1
	5	>25,000	500	5,000	10	10	1
Urine	0	500	ND[f]	ND	ND	10	100[e]
	2	>25,000	ND	ND	ND	10	1
	5	>25,000	ND	ND	ND	10	1

[a] See Table 3 for viability counts of each dilution.
[b] Smegma and mucus samples for 5′ *Taq* nuclease or conventional PCR assay were stored at ambient temperatures in either PBS or physiological saline. Urine samples were stored at 4°C. Samples for culture were stored in modified TEM at ambient temperatures.
[c] Sensitivity limits for both conventional PCR and 5′ *Taq* nuclease assay are given in cell equivalents/assay. Selective culture sensitivity limits are indicated as the minimum number of cells/inoculum in TEM required to produce *Campylobacter*-like colonies on subsequently inoculated selective media. Standard deviations on viable cell counts for each dilution are presented in Table 3.
[d] DNA was extracted from TEM for 5′ *Taq* nuclease and conventional PCR assay using a commercial kit (QIAamp).
[e] Positive assay results were possible for the log dilutions below this concentration but were not reliably obtained.
[f] ND, not done.

TABLE 5. Comparison of quantitative estimates for *Campylobacter fetus* subsp. *venerealis* by 5′ *Taq* nuclease assay of stored samples inoculated with 10^4 cells

Sample type	Storage time (days)[a]	Avg no. of cells/ml		
		Assay postenrichment culture[b,c]	5′ *Taq* nuclease assay	
			QIAamp	Heat lysis
Smegma	0	15,510	379	1,259
	2	3,380	94	1,836
	5	0	47	183
Mucus	0	7,905	996	5,282
	2	5,948	253	1,838
	5	2,764	206	810
Urine	0	ND[d]	976	693
	2	ND	414	426
	5	ND	393	776

[a] Smegma and mucus samples for 5′ *Taq* nuclease or conventional PCR assay were stored at ambient temperatures in either PBS or physiological saline. Urine samples were stored at 4°C. Samples for culture were stored in modified TEM at ambient temperatures.
[b] DNA was extracted from TEM for 5′ *Taq* nuclease assay using a commercial kit (QIAamp).
[c] Quantitative 5′ *Taq* nuclease assay estimates are given in cells/ml.
[d] ND, not done.

purification kit appeared to be inhibited relative to vaginal mucus specimens prepared the same way (Table 4).

Sample storage/transport. Selective culture of vaginal mucus samples for *C. fetus* subsp. *venerealis* led to significant levels of overgrowth by other venereal microflora, limiting identification of the slow-growing *Campylobacter* colonies following prolonged storage. Urine that had been stored at 4°C for 2 or more days also proved to have significant levels of contaminating organisms that were capable of growing on the *Campylobacter* selective media. Both the 5′ *Taq* nuclease and conventional PCR assays generally improved upon the detection sensitivity of selective culture following enrichment in TEM. But detection limits for both PCR-based techniques rose over the course of 5 days while detection limits for selective culture either improved or were unaltered (Table 4).

The direct detection of *C. fetus* subsp. *venerealis* from clinical specimens that were not stored in culture medium exhibited higher sensitivity by both conventional PCR and 5′ *Taq* nuclease assay compared with that of PCR detection postenrichment culture. In fact, estimates of *C. fetus* subsp. *venerealis* numbers in the TEM obtained from 5′ *Taq* nuclease assay quantitation were shown to drop steadily over the course of storage (Table 5). The sensitivity of the conventional PCR improved slightly when specimens were tested at days 2 and 5 (Table 4). A similar trend was generally observed for the 5′ *Taq* nuclease assay results following sample storage. Overall, 5′ *Taq* nuclease assay detection of crude cell lysates prepared from uncultured clinical material provided the highest level of *C. fetus* subsp. *venerealis* detection despite prolonged storage or transport at ambient temperatures (for mucus and smegma). Thus, this protocol was applied for detection of *C. fetus* subsp. *venerealis* for all subsequent animal testing.

Sample collection. The three techniques that were assessed for the collection of genital mucus specimens from cattle caused minimal adverse impact and no obvious discomfort upon the animals. The 10-mm pipette and artificial insemination infusion pipette both required the application of suction via a bulb or syringe, often requiring two operators in order to obtain a suitable sample. The bull rasper was less cumbersome to use and could be effectively manipulated with one hand. Three of the eight bulls tested were identified as having natural *C. fetus* subsp. *venerealis* infections, based upon at least one positive result with selective culture. Selective culture and 5′ *Taq* nuclease assay results for these three animals are presented in Table 6. Specimens that were collected by using the bull rasper obtained positive selective culture results for all three bulls, while those that were collected by using either a pipette or swab provided selective culture positive results from only one of the three bulls. The 5′ *Taq* nuclease assay provided positive results for all three bulls by using each collection technique, and estimates of cells/ml in the original specimens were highest in the specimens that were collected with the bull rasper (Table 6).

TABLE 6. Diagnostic assay results from successive testing of naturally infected bulls via different specimen collection tools

Bull	Collection tool	Selective culture[a]	Quantitative 5′ *Taq* nuclease assay[b]
1	Pipette	−	1,430
	Rasper	+	2,258
	Swab	−	85
2	Pipette	+	72
	Rasper	+	1,501
	Swab	−	148
3	Pipette	−	2,876
	Rasper	+	8,118
	Swab	+	2,910

[a] +, positive culture result; −, negative culture result.
[b] Quantitative 5′ *Taq* nuclease assay estimates are given in cells/ml, which is an average value calculated from duplicate results.

Field sampling evaluation. Results of the diagnostic culture and 5′ *Taq* nuclease assays for bull testing (smegma) are presented in Table 7. All mucus samples were negative by both culture and 5′ *Taq* nuclease assay; however, 14 were positive by ELISA. In two herds with four ELISA-positive results, bulls associated with these herds were identified as positive by the 5′ *Taq* nuclease assay. However, bulls associated with properties of herds for the remaining 10 ELISA positives were not tested. Of the 249 bull smegma samples, 13 were positive by culture, with 9 of these correlating to samples that were positive by the 5′ *Taq* nuclease assay. The four culture-positive specimens which were 5′ *Taq* nuclease assay negative were confirmed as false culture positives following 16S rRNA gene sequencing

TABLE 7. Comparison of diagnostic culture and 5′ *Taq* nuclease assay results from smegma specimens collected from northern Australian properties

Culture result	No. of 5′ *Taq* nuclease assay results that were:		Total
	Positive	Negative	
Positive	9	4[a]	13
Negative	30	206	236
Total	39	210	249

[a] Confirmed that the four 5′ *Taq* nuclease assay negative-culture positive isolates were nonspecific positives.

and conventional subspecies-specific PCR. A further 30 bulls were negative by culture yet were positive by 5′ *Taq* nuclease. Of the 16 urine samples collected from 16 smegma-positive bulls, only 2 urine samples tested positive with the 5′ *Taq* nuclease assay (results not shown). Chi-square analysis of the data indicated a strong association between results for the 5′ *Taq* nuclease assay and selective culture ($\chi^2 = 29.8; P < 0.001$). Comparison of the proportions that were positive for the 5′ *Taq* nuclease assay (16%) and selective culture (5%) indicated the 5′ *Taq* nuclease assay is detecting significantly more infected animals ($P < 0.001$).

DISCUSSION

Real-time PCR-based techniques, such as 5′ *Taq* nuclease assays, have been applied to the clinical diagnosis of a wide range of pathogens from various sources, including *C. jejuni* from human stools (24, 43) and *Trichomonas vaginalis* from female genital secretions (27). These assays provide improvements in sensitivity and specificity relative to selective culture and direct microscopic examination, and diagnoses can be obtained in significantly less time. Real-time PCR techniques are less labor intensive than conventional PCR-based assays, as there is no need for electrophoretic analysis, and the use of specific probes offers improved assay sensitivity and specificity (58). These assays can also provide quantitative measures of target organisms, providing useful tools to clinicians and diagnosticians (13, 37, 53). The assay described here is the first real-time probe-based PCR assay that was developed for the specific detection and quantification of *C. fetus* subsp. *venerealis* to improve the identification of campylobacteriosis in bull carriers.

The assay designed here was based on a subspecies-specific PCR target that was shown to previously identify *C. fetus* subsp. *venerealis* successfully, differentiating among 99 strains of *C. fetus* subspecies (22). Subsequently, this PCR has demonstrated suitability for subspecies-specific diagnostic and research identification of *C. fetus* subsp. *venerealis* (42, 56, 57) and was therefore considered a suitable target for the development of the 5′ *Taq* nuclease assay that is described here. The sensitivity of the 5′ *Taq* nuclease assay was higher than that of the conventional PCR assay, as demonstrated during testing of laboratory-spiked clinical specimens or DNA prepared from similarly inoculated transport medium. In addition, the conventional PCR did not consistently amplify product from crude extracts, requiring pure DNA as a template from both clinical specimens and culture medium. The specificity of this target fragment for the identification of *C. fetus* subsp. *venerealis* was further confirmed by specific detection by 5′ *Taq* nuclease assay of all strains of this subspecies that were tested in this study while also providing considerable improvement on the conventional PCR assay based on this same target.

The 5′ *Taq* nuclease assay provides several significant improvements over conventional culture diagnostic methods. Approximately one single target cell was sufficient for a positive result from smegma or cervico-vaginal mucus, whereas a culture-based diagnosis to isolate *Campylobacter*-like colonies required a minimum of 1,000 cells. A method which can withstand prolonged transport conditions is critical for the sampling of animals from extensively grazed cattle regions. Prolonged transport results in poor culture isolation of *C. fetus* subsp. *venerealis*, and it is recommended that for successful culture, samples be transported for up to only 48 h prior to subculture onto selective medium (7). The slow growth and fastidious nutritional requirements of *C. fetus* subsp. *venerealis* allow rapid overgrowth by more vigorously multiplying contaminating organisms. The organism also maintains limited viability under normal levels of atmospheric oxygen, limiting its survival during transport (8). In addition, antimicrobial susceptibility differs between isolates of *C. fetus* subsp. *venerealis*, with a majority of isolates showing susceptibility to polymyxin B, which is used in most *Campylobacter fetus* selective media (26). The decline in quantitative 5′ *Taq* nuclease assay estimates of *C. fetus* subsp. *venerealis* numbers during 5 days of simulated transport in modified Weybridge media illustrates the impact of these factors on the subsequent isolation of the pathogen. Therefore, these factors reduce the effectiveness of culture-based diagnosis, leading to false-negative results for infected animals as confirmed by our field investigation in this study.

ELISA-based diagnosis of campylobacteriosis has several significant limitations as a diagnostic tool. It is an indirect diagnostic method, detecting, rather than the organism itself, immunoglobulin A antibodies that are specific for the organism. This immune response can persist for up to 10 months after infection, long after the infection has been eliminated (23). As such, it is not an indicator of current infection status, but rather of exposure within the previous 10 months. The ELISA is unsuitable for use in diagnosis of bulls, due to a lack of sufficient titers of antibody in preputial fluids (59). All results for female cattle tested in this study were negative by culture and 5′ *Taq* nuclease assays despite the demonstrated high sensitivity of 5′ *Taq* nuclease assay in spiked mucus samples. We did, however, identify previously infected females by using the ELISA, demonstrating the effectiveness of the ELISA to detect previous exposure. It is feasible that the seasonal timing of our sample collections did not coincide with current or recent infection of the female cattle that were sampled for this study. We did not have access to the DIFT used in Argentina to determine whether this assay is suitable as an alternative confirmatory diagnostic tool (38).

The direct PCR detection of pathogens in clinical specimens without culture enrichment is increasingly being applied for disease diagnosis (1, 12, 16, 39, 46, 47, 54, 60). Although PCR methods have been developed to differentiate the *C. fetus* subspecies following enrichment culture (22, 44), very few studies describe the direct amplification from clinical specimens (15). Preputial and cervico-vaginal mucus specimens may contain a range of contaminating materials, including blood, urine, feces, pus, and semen. These potentially inhibitory materials can limit the effectiveness of PCR as a reliable diagnostic tool unless adequate DNA purification steps are undertaken (2, 25). The 5′ *Taq* nuclease assay described here suffers only minor inhibition in the presence of urine following crude cell lysis and no significant loss of sensitivity or specificity in the presence of smegma or mucus, including specimens contaminated with the blood, feces, or semen as observed during this study. Urine is commonly used for the diagnosis of human venereal diseases in males but is less suitable for similar diagnoses in females (33, 48). Laboratory-spiked urine specimens were suitable for 5′ *Taq* nuclease assay, but the suitability of

urine as a clinical specimen proved less satisfactory than smegma in our study. Preputial smegma is recommended as the most reliable clinical sample for the specific diagnosis of *C. fetus* subsp. *venerealis* from bulls. Mucus samples can also be tested by using the protocols described here, but success is dependent upon recent colonization of the bacteria in infected female cattle as described above.

Heat lysis techniques have been successfully applied for the isolation of template DNA from diagnostic specimens and thus offer considerable time and labor savings for the routine application of DNA-based diagnostics (29, 36, 40, 45, 61). Although crude sample processing does not remove all potential PCR-inhibitory substances, 5′ *Taq* nuclease assays appear to be more robust, enabling successful amplification of target material as demonstrated in this study. This also simplifies the requirements for transport from the field to the laboratory, without the need for complex transport enrichment media. Although, with prolonged storage of some samples, the sensitivity of detection following heat lysis diminished slightly, it was determined that amplification following this crude preparation method proved more sensitive than that of 5′ *Taq* nuclease assay by using kit-purified templates. Further transport studies examining the statistical differences of each processing method and *C. fetus* subsp. *venerealis* viability are required to confirm the preliminary outcomes that were identified in this study. Nevertheless, the heat lysis processing of clinical samples, followed by 5′ *Taq* nuclease assay, provided the most sensitive and practical protocol for the reliable detection of *C. fetus* subsp. *venerealis* for future routine application in diagnostic laboratories.

Previous studies have demonstrated that the bull rasper could be an effective tool for the collection of venereal samples for diagnosis (55). By comparing quantitative 5′ *Taq* nuclease assay results, we were able to confirm that specimens collected from infected bulls by using the bull rasper yielded higher estimates of *C. fetus* subsp. *venerealis* cells than did other collection tools. This was also confirmed by a higher success rate of positive culture from some specimens. In addition, the bull rasper led to marked improvements in the ease of specimen collection from both male and female cattle. Ease of use for the veterinarian, combined with improved specimen quality and no notable adverse impact upon the animal, makes the bull rasper a superior tool for the collection of genital specimens from cattle for the diagnosis of campylobacteriosis by either selective culture or 5′ *Taq* nuclease assay. A bull rasper may be an effective tool for the collection of diagnostic specimens for other venereally localized organisms, such as *Tritrichomonas fetus*.

In summary, the 5′ *Taq* nuclease assay described here is a reliable, sensitive, and specific detection method for *C. fetus* subsp. *venerealis* in bovine venereal diagnostic specimens, providing reliable detection of as few as approximately one cell equivalent per assay, and is able to readily discriminate between the target organism and the phenotypically and genotypically similar *C. fetus* subsp. *fetus*. Specimen collection from male and female cattle by using a bull rasper has been found to be simple and efficient, and specimens that are suspended in physiological saline have proven to be stable during transport at ambient temperatures. Diagnostic specimens can be processed by a simple and rapid heat lysis technique rather than DNA extraction, with no loss of sensitivity. Significant improvements in sensitivity and specificity over those obtained with selective culture-based and conventional PCR-based techniques was observed, with bull testing proving to be the most reliable specimen for screening herds for this pathogen. The assay should be suitable for routine use within diagnostic laboratories with continued use of "gold standard" culture methods. A multicenter evaluation of the specimen collection, transport, processing, and assay procedures should prove valuable. As the detection of *C. fetus* subsp. *venerealis* is significant for trade restrictions, it will be crucial to develop standardized and robust internal positive and negative control protocols, to develop an alternative sensitive assay to confirm positive results, and to obtain sequential samples from animals to confirm test results.

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ADDENDUM IN PROOF

The data summarized in Table 7 were subjected to reanalysis after the paper was submitted. Some samples were removed from the analysis, with slight consequent changes to the statistical results. The changes are reflected in the text and do not affect the interpretation of the results or any subsequent conclusions.

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