Development and Validation of a Real-Time PCR Method To Quantify Rumen Protozoa and Examination of Variability between *Entodinium* Populations in Sheep Offered a Hay-Based Diet

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PCR and real-time PCR primers for the 18S rRNA gene of rumen protozoa (*Entodinium* and *Dasytricha* spp.) were designed, and their specificities were tested against a range of rumen microbes and protozoal groups. External standards were prepared from DNA extracts of a rumen matrix containing known numbers and species of protozoa. The efficiency of PCR (ϵ) was calculated following amplification of serial dilutions of each standard and was used to calculate the numbers of protozoa in each sample collected; serial dilutions of DNA were used similarly to calculate PCR efficiency. Species of *Entodinium*, the most prevalent of the rumen protozoa, were enumerated in rumen samples collected from 100 1-year-old merino wethers by microscopy and real-time PCR. Both the counts developed by the real-time PCR method and microscopic counts were accurate and repeatable, with a strong correlation between them ($R^2 = 0.8$), particularly when the PCR efficiency was close to optimal (i.e., two copies per cycle). The advantages and disadvantages of each procedure are discussed. *Entodinium* represented on average 98% of the total protozoa, and populations within the same sheep were relatively stable, but greater variation occurred between different sheep (10^0 and 10^6 entodinia per gram of rumen contents). With this inherent variability, it was estimated that, to detect a statistically significant ($P = 0.05$) 20% change in *Entodinium* populations, 52 sheep per treatment group would be required.

Ciliates are the most abundant protozoa found in the rumens of both domesticated and wild ruminants. Rumen ciliates are involved in host metabolism and digestion of plant material (37) and play an important role in the rumen microbial ecosystem by producing hydrogen as a by-product of plant digestion. The hydrogen is then used by methanogenic archaea (i.e., methanogens) to reduce carbon dioxide to methane, a potent greenhouse gas. Removal of protozoa from the rumen (i.e., defaunation) has been shown to reduce methane emission by an average of 13% (14). A more efficient use of nutrients in ciliate-free animals, especially when given poor diets that limit animal production, has also been reported (11). Because of the current interest in methane mitigation (15), it is likely that methods to accurately quantify protozoa in the rumen will become increasingly important. However, estimating population sizes in the rumen is difficult because microbial populations fluctuate dramatically during the day (21) and between animals (19) and because of sample heterogeneity.

Rumen ciliates have complex growth requirements, and most are therefore difficult to culture. Most previous studies have used microscopic counts to enumerate protozoa in rumen samples (7), but these methods may underestimate protozoal populations due to the tendency of some species to lyse or settle during sample collection and processing. Because of their large size (15 to 250 μm) and visible internal structures,

it is easier to identify protozoa than bacteria, for example, by microscopic observation. This has reduced the requirement for the development of molecular analyses for the rumen protozoa. However, there are some drawbacks to using microscopic-counting methods to quantify rumen protozoa, such as cell lysis, sensitivity, and variation in sample consistency. Furthermore, there is evidence that separation of rumen fluid from the solids can be misleading, with regard to both total numbers and generic distribution of rumen protozoa (7, 27). For these reasons, we have developed a real-time PCR assay to quantify *Entodinium* (and *Dasytricha*) populations in total rumen contents.

Real-time PCR is an approach that allows continuous monitoring of PCR product formation, and techniques vary according to the method of fluorescence generation. Real-time PCR has been used to monitor eubacterial populations in the rumen, and a recent study quantified the protozoal biomass in the rumen (31). The work presented here addresses the validity of using real-time PCR in place of microscopic counts to quantify *Entodinium* spp., particularly in relation to the inherent variability in protozoal populations between sheep fed the same diet. PCR efficiency is likely to have a large influence on accuracy, and calculation methods were therefore applied.

MATERIALS AND METHODS

Sample collection. Animal ethics approval was obtained prior to experimentation. One hundred 1-year-old merino wethers were housed in individual pens indoors and offered a pelletized diet consisting of oat hay (63%), wheat (20%), lupins (10%), and molasses (5%), with a supplement of minerals and vitamins (Siromin) (2%). The metabolizable energy requirements for maintenance, live-weight gain, and wool production were calculated using GRAZPLAN (12), and

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the animals were fed once per day in the morning. The changes in *Entodinium* populations in rumen samples collected from subsets of the 100 sheep were also collected after 5 days and 6 weeks. Rumen samples were collected by aspiration using a suction pump and a 1.0-cm-diameter stomach tube with a brass filter (2.0-mm pore size) inserted down the esophagus. Samples were mixed with an equal volume of 2% formalin in phosphate-buffered saline for microscopic counts, and another aliquot was weighed in a sterile container, immediately frozen, and freeze-dried before DNA extraction. Sample appearances and consistencies were similar, with dry matter between 2 and 6% of the total wet weight.

Microscopic counts of protozoa. Protozoa were counted by light microscopy as previously described (3, 7) at $\times 100$ magnification with a Sedgewick-Rafter (1.16- μ l volume) counting chamber (J. A. Whitlock & Co., Eastwood, Australia). Each sample was added to the counting chamber and covered with a coverslip, and the protozoa were allowed to settle. This step was performed quickly so that the protozoa were randomly distributed and settled uniformly. The dilution of the samples was adjusted, if necessary, so that between 10 and 20 cells were visible per field. Counts of vestibuliferids (*Isotricha* and *Dasytricha* spp.), *Entodinium* spp., and total protozoa (e.g., *Ophryoscolex* and *Polyplastron*) were recorded. Twenty fields of view were counted, and the highest and lowest counts were discarded.

Primer specificity and protozoal-separation techniques. The new protozoal primers were designed for the predominant species of rumen protozoa by sequence comparisons using ClustalW alignments and protozoal sequences available in GenBank. To test the specificities of the newly designed protozoal primers (Table 1), DNA was extracted from methanogenic archaea (*Methanobrevibacter ruminantium* M1, *Methanobrevibacter smithii* PS, and *Methanosarcina mazei* C16), bacteria (*Streptococcus bovis* ATCC 15352, *Fibrobacter succinogenes* ATCC 19169, and *Prevotella ruminicola* ATCC 19189), a plant (clover; *Trifolium repens*), and the free-living ciliate *Tetrahymena corlissi*. In addition, four different genera of rumen protozoa were also tested, *Entodinium*, *Polyplastron*, *Dasytricha*, and *Isotricha*. Rumen ciliates were separated by size exclusion using progressively smaller filters based on the methods described by Williams and Coleman (37): rumen contents (liquid and solid) were strained through Nitex mesh (300 μ M) and washed once in salt solution [per liter, pH 6.8 to 7.0, 5.0 g NaCl, 1.0 g KH_2PO_4 , 0.2 g K_2HPO_4 , 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g $\text{Ca}(\text{CH}_3\text{COO})_2$, 0.8 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 1.8 g $\text{Na}(\text{CH}_3\text{COO})$, 1.0 g NaHCO_3]. The strained rumen fluid and washes were combined and placed in a prewarmed (39°C) separating funnel and allowed to separate at 39°C for 30 to 60 min. Following incubation, three layers were visible: the top layer (plant debris) was removed by suction and discarded; the middle layer, containing small *Entodinium*-like species and the highly motile *Dasytricha* and *Isotricha*; and the lower layer, containing larger rumen protozoa. The middle layer was spun at $1,000 \times g$ for 10 min, while the pellet was resuspended in the salt solution and combined with protozoa from the bottom layer. The cells were then separated by successive washings with the salts solution through progressively smaller-diameter filters: 300, 250, 100, 80, 45, 30, 20, and 10 μ m. DNA was extracted as outlined below from preparations containing *Entodinium* spp., *Dasytricha ruminantium*, and *Isotricha prostoma* and preparations of larger rumen protozoa, including *Polyplastron multivesiculatum* and *Ophryoscolex caudatus* ($>160 \mu$ m). For microscopic identification, the cells were fixed in 1% isotonic formalin.

18S clone libraries. In addition to rumen fractions of different-size protozoa, 18S rRNA gene sequences from single species of rumen protozoa were generated by PCR and cloned into *Escherichia coli*. The PCR products were generated using the protozoal primers 82F and Medlin B (22) or Oph-151F and Medlin B (Table 1). Cloning was also used to confirm the specificities of the primer pairs against DNA from the complex rumen community. PCRs, in a final volume of 50 μ l, were carried out in a PTC-100 Thermal Cycler (MJ Research Inc., CA) and contained 20 mM Tris HCl (pH 8.4); 50 mM KCl; 2.5 mM MgCl_2 ; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 0.5 μ M of each primer; and 0.25 μ l of Platinum *Taq* DNA polymerase (5 U/ μ l; Invitrogen). PCR amplification was initiated by denaturation at 94°C for 10 min and was followed by six cycles of denaturation (94°C; 45 s), annealing (55°C; 45 s), and extension (72°C; 90 s) and then 35 cycles of denaturation (94°C; 30 s), annealing (55°C; 30 s), and extension (72°C; 60 s). A final extension was carried out at 72°C for 8 min. The PCR products were purified using a QIAGEN PCR purification kit (QIAGEN Inc. CA) according to the manufacturer's guidelines. Purified PCR products were ligated into the pCR2.1-TOPO vector (Invitrogen, CA) and transformed into chemically competent *E. coli* TOP10 cells according to the manufacturer's instructions (Invitrogen), and recombinants (white colonies) were selected at random.

Plasmid DNA was extracted using a GenElute Plasmid miniprep kit (Sigma) and digested by EcoRI endonuclease at 37°C for 90 min, and the insert size was checked by agarose gel electrophoresis. The clones were separated into groups

TABLE 1. PCR primers selected for identification and quantitation of protozoa in the rumen

Forward primer	Sequence (5'-3')	Position within 18S gene ^a	Reverse primer	Sequence (5'-3')	Position within 18S gene ^a	Annealing temp (°C) ^b	Specificity	Application
Oph-151F	GAG CTA ATA CAT GCT AAG GC	145-165	Medlin B ^c	TGA TCC TTC TGC AGG TTC ACC TAC	1690-1667	56	<i>Entodinium</i> spp., <i>Epithium</i> spp., <i>Ophryoscolex</i>	PCR
Euk-82F	AAA CTG CGA ATG GCT C	81-96	Medlin B ^c	TGA TCC TTC TGC AGG TTC ACC TAC	1690-1667	56	All eukaryotes	PCR
Oph-151F	GAG CTA ATA CAT GCT AAG GC	145-165	Ento-472R	CCC TCA CTA CAA TCG AGA TTT AAG G	453-428	55	<i>Entodinium</i>	Real-time PCR
Iso-Das-151F	CTA GAG CTA ATA CAT GCC	145-163	Das-472R	CTA CAA TCA CAA TTA AAT TGC	449-428	55	<i>Dasytricha ruminantium</i>	Real-time PCR

^a *Tetrahymena thermophila* numbering.

^b Annealing temperatures for PCRs containing 2.5 mM MgCl_2 .

^c Medlin et al. (22).

by restriction enzyme digestion with RsaI (25), and representatives from each group were sequenced using protozoal sequencing primers (up to threefold coverage). The nucleotide sequences of the inserts were determined on a capillary sequencer using a BigDye terminator v. 3.0 cycle-sequencing kit (Perkin-Elmer). Sequences were assembled using Seqman in the DNA-STAR suite of programs (Lasergene, CA). Putative identification of each sequence was carried out using the Basic Local Alignment search tool (1), and potential chimeras were identified using the Chimera Check program on the Ribosomal Database Project website (4; <http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU>). Clones were identified as 97% identical to *Eudiplodinium maggii*, 96 to 99% identical to *Entodinium caudatum*, and 99% identical to *Dasytricha ruminantium*.

DNA extraction. DNA was extracted from approximately 50 mg freeze-dried rumen sample material by physical disruption and phenol-chloroform extractions using the method of Stahl et al. (29) with the modifications of Skillman et al. (28).

Real-time PCR conditions. The primer pair selected to quantify *Entodinium* spp., Oph-151F and Ento-472R, or *Dasytricha* only, Iso-Das-151F and Das-472R (Table 1), was included in 25- μ l real-time PCR mixtures containing 10 μ l SYBR Green mix (IQ SybrGreen Supermix; Bio-Rad), 7 μ l distilled H₂O, 1 μ l forward primer (10 μ M concentration), 1 μ l reverse primer (10 μ M concentration), and 1 μ l DNA. Real-time PCR amplification was conducted on an Icyler (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Real-time PCR amplification was initiated by denaturation at 95°C for 10 min and was followed by 45 cycles of denaturation at 95°C for 15 s and annealing at 55°C for 30 s, and then by extension at 72°C for 30 s. Fluorescence was acquired during extension using an excitation wavelength of 470 nm and emission detection at 530 nm. A final melting-curve analysis was carried out by continuously monitoring fluorescence between 55°C and 95°C with 0.5°C increments every 10 s. Threshold cycles were calculated automatically by the Bio-Rad Icyler software (version 3.5), although a standardized amount of protozoal clone DNA (*Entodinium caudatum*) was included in each run to monitor and correct any between-run variability. The sensitivities of the primers designed in this study were checked against equal concentrations of DNAs from different species, and it was found that amplification occurred at the same threshold cycle for each target species (data not shown).

External standards for real-time PCR. For quantitation by real-time PCR, external standards were prepared. A simulated sample of rumen contents consisting of clarified (0.22 μ m filtered) rumen fluid (100 ml) was mixed with 20 ground feed pellets consisting of oat hay (63%), wheat (20%), lupins (10%), molasses (5%), and Siromin (2%); 5×10^8 methanogens (*M. ruminantium* M1); and 9×10^9 bacteria (*S. bovis* ATCC 15352). Ten-milliliter aliquots were dispensed, and rumen protozoa (11% *Dasytricha*, 5% *Isotricha*, 0.6% *Polyplastron*, and 83.4% *Entodinium*) enumerated by microscopy were added to a final cell density between 1×10^4 and 5×10^4 cells g⁻¹ wet weight. The samples were then freeze-dried, and DNA was extracted from approximately 50-mg portions as outlined above according to the method of Skillman et al. (28).

Real-time PCR quantitation. As the levels of PCR inhibitors may vary in each rumen sample, it was crucial to estimate DNA "quality" or PCR efficiency, as this can have a large influence on protozoal quantitation (6, 13, 17, 34). DNA from the prepared standards containing enumerated protozoa was diluted (10- to 1,000-fold) in distilled H₂O and amplified by real-time PCR as outlined above. A linear regression of the threshold cycle (C_t) (or the PCR cycle number where fluorescence is first detected) for each dilution versus the log dilution allowed calculation of PCR efficiency (ϵ) according to the following equation: $\epsilon = 10^{-1/\text{slope}}$. Real-time PCRs (24) can be described by the formula $N = N_0 \times \epsilon^n$, where N_0 is the number of protozoa/amount of DNA present initially (before the PCR), ϵ is the efficiency of the PCR, and n is the number of cycles. The amount of DNA required to reach the threshold of fluorescence detection is the same for all PCRs. Therefore, the amount of DNA present in any PCR at its C_t is the same, a constant, N . This enables the PCR efficiency and number of protozoa in each standard (N_0) to be used to calculate the constant (N), which can be used in subsequent enumerations of unknown samples.

The numbers of *Entodinium* sp. (or *Dasytricha*) cells in the sheep rumen samples (N_0) were similarly obtained by real-time PCR of up to seven serial dilutions of DNA, calculation of PCR efficiency, and substitution of the constant (N) calculated from the standards into the following equation: $N_0 = N/\epsilon^n$. Wet and dry weights of samples collected and DNA extraction procedures were taken into account to convert the calculated numbers of protozoa (N_0) in the samples measured by real-time PCR to numbers of protozoa per g (wet weight) rumen contents.

Statistics. Statistical analyses were carried out in Genstat (version 7.1.0.198; Lawes Agricultural Trust). Paired t tests were carried out to determine the statistical significance of differences between and among animals. Power plant

(5) calculations were carried out to determine the number of animals required to detect a statistically significant ($P = 0.05$) treatment effect (2).

Nucleotide sequence accession numbers. Small-subunit rRNA gene sequences of the ruminal clones from hay-fed sheep have been deposited in the GenBank nucleotide sequence database (<http://www.ncbi.nlm.nih.gov/GenBank/>) under accession numbers DQ118760 to DQ118773.

RESULTS

Protozoal PCR and real-time PCR primer design and specificity. PCR primers for specific genera of rumen ciliates were designed for regions within the 18S gene (Table 1). Two primer pairs that amplified rumen protozoa (Oph-151F and Medlin B; Euk-82F and Medlin B) were also used to amplify rumen ciliate 18S genes from rumen samples to prepare clones of individual ciliate species for specificity testing.

Primer combinations amplifying smaller PCR products (100 to 700 bp) were chosen to obtain optimal amplification and fluorescence detection on the Icyler platform. Problems of primer complementarity, hairpin formation, and occurrence of primer dimers were avoided entirely following analyses with Primer Check (DNA Club package; Xiangfong Chen, Cornell University, Ithaca, NY). Of the 14 real-time PCR primers, 11 primer combinations were tested on all microbial groups for specific quantitation of rumen protozoa (data not shown); the primer pair Oph-151F and Ento-472R amplified DNA from *E. caudatum*, but not *E. maggii*. This primer pair was therefore used to specifically quantify *Entodinium* species by real-time PCR.

Gel electrophoresis of the real-time PCR products confirmed specific amplification of a 180-bp target, and melting-curve analyses showed that the melting temperatures of amplified products were consistent. There was no evidence of primer dimers (nonspecific, smaller PCR artifacts) from thermal-melting properties. The sensitivity of the primer pair for *Entodinium* spp. was also assessed over a range of protozoan DNA concentrations between 1 pg and 100 ng. Amplification was successful over the entire range (5 orders of magnitude), and different protozoal targets in equal concentrations amplified at the same threshold cycle (data not shown).

A forward primer (Iso-Das-151F) specific for *Isotricha* and *Dasytricha* was designed in the same region as Oph-151F but was shortened by 2 bp to ensure primer specificity with a conserved nucleotide at the 3' terminus (Table 1). Similarly, a *D. ruminantium*-specific reverse primer (Das-472R) was designed in the same region as Ento-472R but contained five different bases with two substitutions at the extending end of the primer (Table 1). When tested, the primer pair amplified only *D. ruminantium* from a rumen sample and allowed specific amplification and quantitation of *Dasytricha* by real-time PCR.

Detection and quantitation of *Entodinium* and *Dasytricha* spp. by real-time PCR. External standards for real-time PCR were prepared as described above from a simulated rumen matrix. For each standard, a linear regression derived from the C_t of each DNA dilution versus the log dilution (Fig. 1) enabled the PCR efficiency and a constant, N , to be calculated. The calculated constants (*Entodinium* $N = 3.36 \times 10^{10}$; *Dasytricha* $N = 2.13 \times 10^{11}$) were used for subsequent calculations of protozoal numbers in the rumen samples from sheep.

Each sheep sample collected was quantified by real-time PCR of five serial dilutions of the DNA, and the PCR effi-

FIG. 1. Example of real-time PCR amplification of serially diluted rumen DNA to calculate ϵ from the slope of the linear-regression plot (threshold cycle versus log dilution) according to the equation $\epsilon = 10^{-1/\text{slope}}$. In this example, the slope is -3.82 , and the PCR efficiency is 1.82 .

ciency was again calculated for each sample by linear regression of the threshold cycle versus log dilution (Fig. 1), with $>90\%$ of samples ranging from 1.75 to 2.05 with a mean of 1.94 . For each dilution, protozoal numbers per gram (wet weight) of rumen contents were calculated from the protozoal standards (Table 2) divided by the PCR efficiency to the power of the threshold cycle. The mean ($n = 5$) was corrected for

freeze-drying and DNA extraction procedures by calculating the equivalent wet weight of material the DNA was extracted from to determine the number of protozoa per gram (wet weight) rumen contents.

In general, the numbers of *Entodinium* cells calculated using real-time PCR (mean, 4.86×10^5) were higher than microscopic estimations (mean, 1.74×10^5). Samples collected from

TABLE 2. Real-time PCR of protozoal standards prepared in a rumen matrix using *Entodinium* (Oph-151F and Ento-472R) and *Dasytricha* (Iso-Das-151F and Das-472R)

Standard	No. of cells added (per g [wet wt])	Wet wt (g) extracted	N_0 entodinia (per g [wet wt])	C_t	PCR efficiency	N (constant) ^a
<i>Entodinium</i>						
A	0	NA ^b	NA	37.4	NA	NA
B	9.00×10^3	1.39	1.25×10^4	23.1	1.87	1.71×10^{10}
C	1.72×10^4	1.37	2.36×10^4	23.1	1.75	7.07×10^{10}
D	2.47×10^4	1.35	3.33×10^4	21.1	2.03	7.59×10^{10}
E	3.15×10^4	1.01	3.18×10^4	18.2	2.17	4.19×10^{10}
F	3.78×10^4	0.94	3.55×10^4	19.6	2.05	4.87×10^{10}
G	4.36×10^4	1.14	4.97×10^4	21.6	1.78	1.12×10^{10}
<i>Dasytricha</i>						
A	0	NA	NA	39	NA	NA
B	1.22×10^3	1.39	8.76×10^2	28.2	1.87	4.06×10^4
C	2.32×10^3	1.37	1.70×10^3	26	1.75	4.84×10^9
D	3.33×10^3	1.35	2.47×10^3	26.8	2.05	7.55×10^{11}
E	4.26×10^3	1.01	4.22×10^3	24.8	2.08	3.29×10^{11}
F	5.11×10^3	0.94	5.44×10^3	25	1.98	1.33×10^{11}
G	5.90×10^3	1.14	5.18×10^3	24.3	1.84	1.61×10^{10}

^a Means: 3.36×10^{10} for *Entodinium* and 2.13×10^{11} for *Dasytricha*.

^b NA, not applicable.

FIG. 2. Correlation between *Entodinium* populations in the sheep rumen measured by microscopic counts and real-time PCR. The data are presented as log numbers of *Entodinium* cells per gram (wet weight) rumen contents. The real-time PCR primers used were Oph-151F and Ento-472R. Standard errors ranged from 0.8 to 11%.

100 sheep were compared and showed reasonable correlation ($R^2 = 0.8$) (Fig. 2). Correlation between the two methods was lower if the PCR efficiency deviated below 1.7 or above 2.15 (data not shown).

Standards and samples from 38 sheep were also tested using *Dasytricha* real-time PCR primers. Of these 38 samples, five contained vestibuliferids (*Dasytricha* and/or *Isotricha*) at $>1,000$ cells per gram (wet weight). The threshold cycle for the 33 samples that showed no evidence of *Dasytricha* under microscopic examination was >34 cycles. Of the five samples that contained *Dasytricha*, three were detected by the primers and two were not (Table 3), perhaps reflecting the lower cell density of *Dasytricha* in these samples.

Variation in rumen *Entodinium* populations. Further samples were collected from 78 animals 5 days later, and *Entodinium* populations were again measured by microscopic counts. Populations varied greatly, from 1.02×10^3 to 2.58×10^6 per gram (wet weight) (mean, 3.1×10^5). Variation was greater between individuals (coefficient of variation, 416%) than in the same individual sampled on separate occasions (coefficient of variation, 124%). Twenty sheep were also sam-

pled 6 weeks later, and again, the variation in *Entodinium* populations was greater between different sheep than within the same sheep sampled 6 weeks apart (coefficients of variation, 339% versus 163%).

As some samples collected (2 h postfeeding) contained a large proportion of lysed protozoal cells, 16 sheep were sampled before and after feeding to examine the effect of feeding on *Entodinium* populations (Table 4). The pH after feeding was much lower (mean, pH 5.66) than before feeding (mean, pH 6.95), and *Entodinium* numbers had fallen by 50%. In all but one sample collected after feeding, protozoal cells were lysed or cell debris was evident, whereas before feeding, all samples were cleaner with protozoa intact.

DISCUSSION

Techniques based on the amplification of 16S rRNA genes for comparing bacterial communities are now widely used in microbial ecology (13, 30), including studies of the rumen (10, 18, 26, 32), but calibration of these techniques with traditional culture methods has been conspicuously absent (9). We chose to enumerate *Entodinium* spp., as they are the most abundant

TABLE 3. Detection of *Dasytricha* by real-time PCR in 5 of 38 rumen samples tested

C_t	PCR efficiency	No. of <i>Dasytricha</i> cells calculated from standards	Wet wt extracted	No. of <i>Dasytricha</i> cells (per g [wet wt])	<i>Dasytricha</i> microscopic count per g (wet wt)
28.7	1.92	1.58×10^3	1.16	1.36×10^3	3.69×10^3
30.0	1.91	7.90×10^2	0.52	1.52×10^3	1.87×10^3
27.7	1.98	1.29×10^3	0.92	1.40×10^3	1.84×10^3
>35.0	NA ^a	NA	NA	NA	9.43×10^2
>35.0	NA	NA	NA	NA	9.26×10^2

^a NA, not applicable.

TABLE 4. Effects of feeding on pH and *Entodinium* populations

Sheep no.	Total no. of cells		pH	
	Prefeeding	Postfeeding	Prefeeding	Postfeeding
1	$8.50\text{E} \times 10^5$	$6.36\text{E} \times 10^5$	6.78	5.53
2	$2.80\text{E} \times 10^4$	$5.64\text{E} \times 10^3$	7.01	5.55
3	$8.74\text{E} \times 10^2$	$2.06\text{E} \times 10^3$	7.29	5.54
4	$1.15\text{E} \times 10^4$	$1.53\text{E} \times 10^4$	7.02	5.39
5	$3.41\text{E} \times 10^5$	$1.47\text{E} \times 10^5$	7.16	5.33
6	$9.15\text{E} \times 10^5$	$3.13\text{E} \times 10^5$	7.15	6.31
7	$1.01\text{E} \times 10^6$	$4.62\text{E} \times 10^5$	6.84	5.59
8	$9.41\text{E} \times 10^4$	$1.87\text{E} \times 10^4$	6.84	5.18
9	$3.26\text{E} \times 10^5$	$1.54\text{E} \times 10^5$	6.67	6.24
10	$5.42\text{E} \times 10^5$	$3.19\text{E} \times 10^5$	7.04	5.54
11	$4.10\text{E} \times 10^5$	$2.87\text{E} \times 10^5$	6.71	5.75
12	$3.17\text{E} \times 10^5$	$1.36\text{E} \times 10^5$	7.03	6.42
13	$1.38\text{E} \times 10^5$	$7.15\text{E} \times 10^5$	6.89	6.06
14	$1.17\text{E} \times 10^5$	$6.49\text{E} \times 10^5$	6.88	5.49
15	$5.26\text{E} \times 10^4$	$3.38\text{E} \times 10^4$	7.01	5.35
16	$5.47\text{E} \times 10^4$	$2.71\text{E} \times 10^4$	6.94	5.32
Mean	$4.68\text{E} \times 10^5$	$2.45\text{E} \times 10^5$	6.95	5.66

ciliate protozoa in the rumen and also have the largest number of species (37). In this study, based on microscopy, 98% ($n = 100$) of all rumen protozoa were *Entodinium* spp. We have demonstrated a real-time PCR method that is reproducible and correlated ($R^2 = 0.8$) with a conventional cell-counting technique. However, there are advantages and disadvantages to each method. Both techniques (cell counts and real-time PCR) can be used to quantify total rumen protozoa and different genera of rumen protozoa based on morphology (microscopy) or primer choice (real-time PCR). Microscopic methods are faster and more cost-effective, but during microscopy, lysed or ingested protozoa are not counted. This may reflect protozoal numbers in the rumen more accurately than real-time PCR, unless the sampling procedures adversely affect the protozoa. It is likely that the temperature changes (39°C to ambient) associated with sampling may cause lysis of the fragile protozoal cell membrane and may therefore underestimate protozoal populations by microscopic counts. By contrast, the rapid freezing of samples for DNA extraction is likely to preserve DNA from lysed protozoal cells. Although real-time PCR is more expensive and time-consuming than microscopic counts, it is more sensitive, detecting 1 to 10 million protozoal cells. One of the major advantages of real-time PCR is that the bacterial and total microbial populations can be measured concurrently. This is likely to be particularly important when dealing with heterogeneous rumen samples.

Tichopad et al. (35) demonstrated that primer selection is crucial to the accuracy of real-time PCR and that reactions with higher amplification efficiency proceed with lower variability and are better suited to measurement purposes. The primer Ento-472R was sequence specific to *Entodinium* and had >5 base mismatches with all other rumen protozoa. The forward primer (Oph-151F) matched the 18S gene sequences of *Entodinium* spp., *Epidinium* spp., and *Ophyroscolex caudatus* and would be expected to detect these three protozoal genera. However, Oph-151F had three nucleotide mismatches between the 3' end of the primer and *E. maggii*, four mismatches with *Diplodinium* and *Polyplastron*, and five mismatches with *Da-*

sytricha. As the predicted specificities of primers may differ from the practical outcome, it is important to test primer specificity against target and nontarget groups. The real-time PCR primers designed during this study specifically amplified the 18S rRNA genes of specific genera of rumen protozoa, including *Entodinium* species (Oph-151F and Ento-472R) and *D. ruminantium* (Iso-Das-151F and Das-472R).

The *Dasytricha* primer pair successfully detected three of five samples containing the vestibuliferids *Dasytricha* and/or *Isotricha* with no false positives. The failure to detect two of the samples may have reflected lower cell density (9×10^2 versus 4×10^3), a prevalence of *Isotricha*, or poor PCR efficiency in these samples.

The sensitivity of PCR assays to different target sequences may vary, and this has important implications for subsequent quantitation. If a primer pair amplifies one target more effectively than another, then the proportion of each species would have to be known for accurate quantitation. The sensitivities of the protozoal primers (Oph-151F and Ento-472R) designed in this study were checked against equal concentrations of DNAs from different target species, and it was found that amplification occurred at the same threshold cycle for each species (data not shown).

Dionisi et al. (8) examined the effects of DNA extraction methods and real-time PCR procedures on assay variability. They concluded that increasing the number of real-time PCR assays performed with a single DNA extract may have as large an effect on statistical power as increasing the number of DNA extractions and real-time PCR assays performed with each of their activated sludge samples. In a recent study to quantify rumen ciliate biomass (31), the copy number of 18S rRNA genes was measured in bovine rumen samples, and although the PCR efficiency of a spiked control was measured, the individual PCR efficiency for each sample was not. According to the equation $N = N_0 \times \epsilon^n$ (24), PCR efficiency has a large influence on calculated populations, particularly for rumen samples, which often contain PCR inhibitors coextracted with DNA (20). Accurate estimation of PCR efficiency (i.e., by serial dilutions) should improve assay reliability to a greater extent than repeated measures or DNA extractions.

The quality of DNA or ability to perform PCR on DNA extracted from rumen samples is often poor (19), due in part to high levels of PCR inhibitors, such as humic acids. Therefore, it is important to estimate DNA quality or PCR efficiency for each rumen sample for accurate quantitation (23, 33, 34, 35). The optimal PCR efficiency is 2, where the copies of DNA double each cycle. Deviations below 2 may occur when the PCR is inhibited by compounds coextracted with the DNA. Occasionally (i.e., in 5% of rumen samples), PCR efficiency rose above 2 (>2 copies generated per cycle). Where possible, the range of dilutions chosen should be as close to optimal PCR conditions as possible ($\epsilon = 2$), with the R^2 value of the linear regression as close to 1.0 as possible. A computing method based on the kinetics of real-time PCR has recently been shown to be more accurate than serial dilutions for amplification efficiency estimation (33). However, this mathematical method is based on the logarithmic portion of the real-time PCR and is most suitable for optimal PCR runs with high and constant plateau fluorescence. In practice, we found that the

size of the plateau varied between samples and was not always constant and flat.

Statistical comparisons of protozoal populations have shown that the variation between different sheep is greater than variation in the same sheep sampled 5 days or 6 weeks apart. As the protozoal populations between different sheep varied considerably over 6 orders of magnitude, the number of animals required to detect a statistically significant ($P = 0.05$) treatment effect at 80% power was calculated to be 52 animals. Preliminary data have indicated that intersheep variability was reduced once it was standardized to the total microbial population, suggesting that real-time PCR may provide better comparisons between individual sheep.

The reduced number of protozoa measured after feeding in this study may be the result of dilution, with food and saliva entering the rumen, or may reflect protozoal-cell lysis at the lower pH. This may increase the amount of DNA present in samples collected postfeeding and contribute to the overestimation of protozoal populations by real-time PCR as opposed to microscopic counts. In hindsight, it may be better to sample before feeding rather than the more usual practice (16, 36) of sampling postfeeding. The new protozoal primers and methods described provide a rapid and specific quantitative assay for different groups of rumen protozoa. It is likely that the use of robust methods to assess changes in protozoal populations will become increasingly important to assess potential treatments to modify or manipulate microbial populations in the rumen.

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