Nutritional Methodology

Development of an Assay to Quantify Rumen Ciliate Protozoal Biomass in Cows Using Real-Time PCR[1]

John T. Sylvester,*[†] Sanjay K. R. Karnati,*[†] Zhongtang Yu,[†] Mark Morrison,*[†] and Jeffrey L. Firkins*[†2]

*Ohio State University Interdisciplinary Nutrition Program (OSUN) and [†]Department of Animal Sciences, The Ohio State University, Columbus, OH 43210

ABSTRACT   Currently used microbial markers cannot distinguish protozoal nitrogen (N) from bacterial N, thus limiting research on protozoal quantification in vivo by the lack of a repeatable, accurate marker for protozoal N. We report the development of a real-time PCR assay targeting the gene encoding 18S rDNA to quantify the amount of protozoal biomass in ruminal fluid and duodenal digesta. Protozoal cells were harvested from rumen fluid and concentrated for evaluation of recovery of rDNA in samples from the rumen and the duodenum. The DNA from concentrated cells was extracted with virtually 100% efficiency both before and after column purification. After serial spiking of protozoal cells into duodenal fluid over the entire range of quantification, the recovery was highly linear and constant at 81%. After serially spiking increasing quantities of protozoal rDNA into a constant volume of duodenal samples, nonlinear regression verified constant recovery of background rDNA in duodenal samples regardless of the ratio of target:nontarget rDNA. Recommendations for the procedure, including replication per sample, are described herein.   J. Nutr. 134: 3378–3384, 2004.

KEY WORDS: • rumen protozoa • real-time PCR • 18S rDNA • cattle

Ciliate protozoa are present in the rumens of most dairy cattle in concentrations of 10^5-10^6 cells/mL fluid and can represent up to half of the total microbial N. However, protozoal predation of bacteria and protozoal autolysis promote inefficient metabolism of dietary protein (1) and decrease considerably the protozoal N outflow from the rumen. Based on an elaborate mechanistic model, increasing grain in ruminant diets is predicted not only to increase production of bacterial and protozoal protein but also to substantially increase protozoal-mediated turnover in the rumen (2), thus moderating the supply of amino acids for absorption from the small intestine.

Despite the importance of protozoal ecology and the high sensitivity of models for more measurements of protozoal biomass (3), there is no widely accepted marker to measure the protozoal fraction of microbial protein separate from the bacterial fraction (4,5). With increasing availability of reliable and repeatable kits, molecular-based procedures could help distinguish protozoal from bacterial N, leading to improved modeling and ration formulation efforts.

Advancements in PCR technology have generated several quantification methods targeting the gene (rDNA) encoding the synthesis of ribosomal RNA (rRNA) for the enumeration of microbial species. Competitive PCR was designed to overcome many problems with quantification, and it has been used to enumerate rumen cellulolytic and noncellulolytic bacteria (6). However, for routine usage in molecular-based quantification, real-time PCR offers many advantages (7). Real-time PCR has been used to quantify individual species of rumen bacteria (7–9) and nonrumen protozoa (10) but, to our knowledge, not ruminal protozoa.

The rumen protozoal biomass was semiquantitatively assessed indirectly (Eukarya minus fungal rRNA hybridization signals) (11,12). The latter studies quantified rRNA, which varies considerably per cell over a feeding cycle (13,14) and should compound the error of both rRNA hybridization probes (14). In contrast, rDNA concentration per cell remains relatively stable (15) and should be a more stable marker for protozoal biomass. For rDNA quantification of mixed populations, amplification efficiencies could be different for different species of protozoa, as they are for bacteria (9), or could vary in copy number of rDNA per species, as they do for bacteria (16). Thus, objectives for nutrition studies would require harvesting a reference standard of protozoal cells in the same species distribution and rDNA:N ratio as the entire rumen population. Our previous work showed that protozoa-specific primers can be designed to target diverse types of protozoa from rumen samples (17). The objective of the current research was to develop a real-time PCR assay to quantify protozoal biomass using the 18S rRNA gene, evaluating each step for potential errors and for correction of those errors.

MATERIALS AND METHODS

Animals and sample collection.   Samples were obtained from 2 multiparous Holstein cows fitted with rumen and simple T duodenal

cannulas in a randomized complete block design in which the cows were considered the blocking criterion. The cows were housed in tie stalls and maintained in accordance with The Ohio State University animal care and use guidelines. Both cows were fed each of 2 control diets containing either 21 or 16% forage neutral detergent fiber (NDF; hereafter referred to as high forage and low forage, respectively), in separate periods that were part of a larger study (18). Diets were prepared once daily as a total mixed ration, and cows were fed diets at 110% of their ad libitum consumption at 0600 and 1800 h for 14 d, with the last 5 d of each period used for sample collection. Rumen fluid was collected from 10 sites representing all compartments on d 8 through 11 of each period at 0700, 0900, 1200, 1500, and 1700 h; samples were composited by period to minimize diurnal variation in protozoal counts (19).

Rumen protozoa were harvested using a procedure (**Fig. 1**) adapted from that outlined by Martin et al. (20). Briefly, a mechanical press was created using a 10-cm pipe and a hand press to push fluid through 2 layers of cheesecloth. A 75-mL aliquot was treated with formalin and stored at 4°C until used for protozoal enumeration and DNA extraction. Preliminary experiments verified minimal loss of protozoal rDNA from 1% (wt:v) final concentration of formalin. The dry particulate fraction was washed by hand with 39°C Coleman anaerobic buffer (1) to maximize the recovery of particle-associated protozoa. After flocculation, the floating scum layer was removed via vacuum aspiration and stored for protozoal enumeration. The remaining fluid was treated with formalin (1% wt:v final concentration) to prevent cell lysis. After centrifugation (500 × g, 5 min), pellets were transferred into a filter bag made from nylon filter cloth with a 10-μm

pore size (Sefar America) and dialyzed by gentle agitation of the bag in saline (9 g/L) solution to efflux contaminating bacteria that were not internally or externally associated with protozoa. Protozoal cells were washed until minimal bacterial contamination was observed microscopically (~8 changes in wash buffer). Cells were composited by cow within period, suspended in 100 mL of saline (9 g/L), and stored at 4°C (no further formalin added). Counting and genera differentiation of protozoa were done using the method described by Dehority (21).

Duodenal samples (250 mL) were taken on d 11–14 of each period so that every 90-min period in a 24-h period was represented (16 samples total), combined by cow within period, and frozen immediately according to standard practice (22). The effect of multiple freeze/thaw cycles on rDNA recovery was also determined.

To determine whether protozoal rDNA in duodenal samples was degraded during storage, 3 replications of 2 pooled samples of duodenal digesta were used to compare fresh samples to samples frozen at either −20 or −80°C for 3 wk.

DNA extraction and purification. Initially, ruminal and duodenal fluid sample volumes of 0.25, 0.5, or 1.0 mL were used in DNA extraction. After results were obtained from the preceding study, total genomic DNA from 6 replications of each sample containing 0.5 mL of rumen fluid and duodenal fluid and 0.1 mL (~1.0 × 10^6 cells) of concentrated protozoal cells was extracted using the Puregene DNA isolation kit (Gentra Systems). The protocol provided in the kit for the isolation of fixed or paraffin-embedded tissue was used with the following modifications: the reagent volumes were doubled to accommodate sample size, the Proteinase K solution (20 g/L) volume was increased from 3.0 to 50 μL at the initial time and 30 μL after 3 h, and samples were digested for not <12 h at 55°C. When using 0.5 mL of liquid sample (as in the rumen and duodenal samples), the volume of isopropanol was increased to 1.1 mL to maintain the suggested 75% (v:v) isopropanol concentration required to precipitate the DNA.

The extracted DNA previously dissolved in 200 μL of Puregene hydration solution was purified using the column included with the QIAamp Stool Mini Kit (Qiagen) by following steps 10–18 of the kit protocol. The only modifications to the protocol were to eliminate vortex mixing to prevent possible shearing of genomic DNA, and the elution buffer was heated to 50°C. Genomic DNA was stored at 4°C for later use in PCR.

The DNA extraction efficiency was assessed by extracting genomic DNA from 3 replicate extractions of each dilution ranging from 3000 to 2.0 × 10^6 cells (6 dilutions) and then by regressing cell number against the DNA yield determined by absorbance measured at 260 nm.

Genomic DNA was isolated from 3 replications of increasing amounts of protozoal cells ranging from 1000 to 1.0 × 10^6. Aliquots of the extracted DNA were taken from each dilution and stored; the remaining fraction was column-purified. The mean predicted rDNA copies from each dilution before purification were regressed against their purified equivalent.

PCR primers. Two sets of ciliate protozoal-specific PCR primers were designed based on all ruminal protozoan 18S rDNA sequences of GenBank (23) and sequences from Dr. C. J. Newbold, The Institute of Rural Sciences, University of Wales Aberystwyth, Llanbadarn Fawr, Aberystwyth, UK (personal communication). The conventional PCR set (P.SSU-54f, 5′-CAYGTCTAAGTATAAATAAC-TAC-3′; P.SSU-1747r, 5′-CTCTAGGTGATWWGRTTTAC-3′) was used to generate standards for real-time PCR. This set amplifies an approximate 1693-bp fragment (based on *Saccharomyces cerevisiae* numbering) of the 18S rDNA. The second set (316f, 5′-GCTTTCG-WTGGTAGTGTATT-3′; 539r, 5′-CTTGCCCTCYAATCGT-WCT-3′) was designed to amplify an approximate 223-bp fragment of the 18S rDNA located internal to the larger amplicon produced by conventional PCR to be used for real-time PCR.

Real-time PCR standard. The real-time PCR standard was generated by PCR using P.SSU-54f/P.SSU-1747r. PCR was performed with 1 μL of genomic DNA using the Bio-Rad iCycler (Bio-Rad). The amplification conditions were 94°C for 4 min of initial denaturation; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min; and a final extension at 72°C for 6 min. The reaction volume was 100 μL
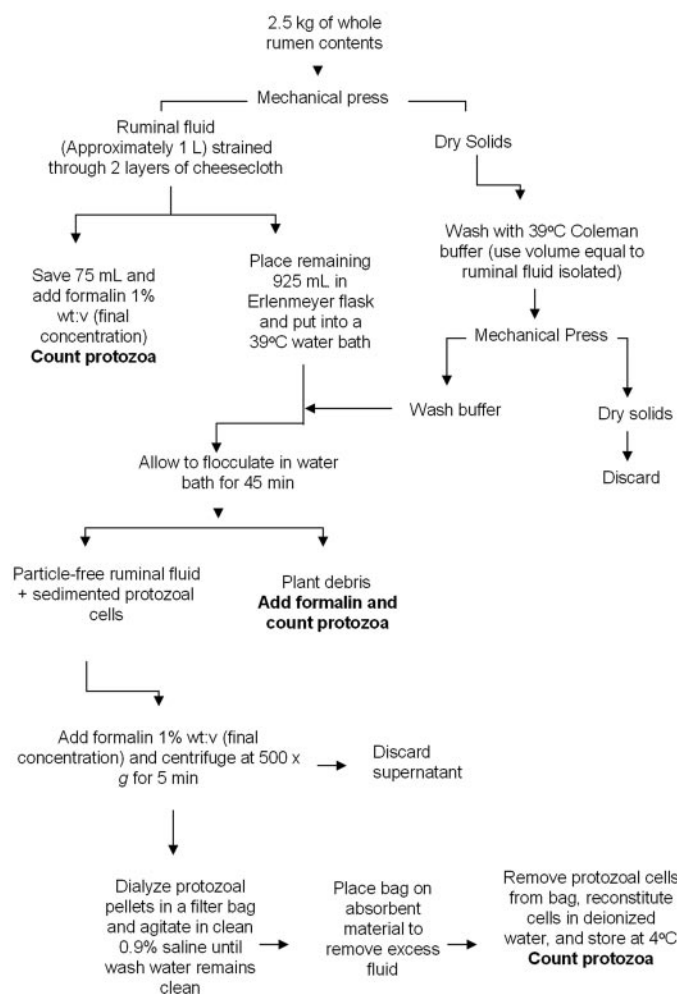

FIGURE 1 Procedure to isolate and concentrate protozoa minimizing nonassociated bacterial contamination and protozoal cell lysis.

containing 100 pmol of each primer, 125 μmol/L of each dNTP mixture, 2 mmol/L magnesium chloride, bovine serum albumin (0.5 g/L), 1X PCR buffer, and 5.0 U of platinum *Taq* DNA polymerase (Invitrogen).

Amplification products were electrophoresed on agarose gel, followed by ethidium bromide staining to verify single product formation of the expected MW. Replicate PCR amplification reactions from DNA isolated from each sample type were composited by cow within treatment and purified using the QIAquick PCR purification kit (Qiagen). Purified PCR products were quantified using the PicoGreen dsDNA quantification kit (Molecular Probes) by reading fluorescence on a Tecan SpectraFluor plus microplate spectrofluorometer. The rDNA copies present within each purified PCR standard were calculated using the mass concentration and the mean MW of all theoretical amplicons of all ciliate rDNA sequences (22 sequences representing major genera of common rumen ciliates, 13 available from GenBank (23) and the rest from Dr. C. J. Newbold (personal communication).

Real-time PCR. The quantification of protozoal rDNA copies present in the extracted DNA from each sample was performed using an iCycler iQ real-time PCR detection system (Bio-Rad). Dilutions (1:100) of DNA from all samples were added to amplification reactions (50 μL) containing 50 pmol of each primer, 125 μmol/L concentrations of each deoxynucleoside triphosphate (dNTP mixture), 2 mmol/L magnesium chloride, bovine serum albumin (0.5 g/L), 1X PCR buffer, 2.5 U of platinum *Taq* DNA polymerase (Invitrogen), 1μL of 13X SYBR green (Molecular Probes), and 1 μL of 0.01 mmol/L fluorescein (Bio-Rad). Cycling conditions were 94°C for 4 min; 45 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min; and a final extension of 72°C for 6 min. Fluorescence readings were taken after each extension step, and a final melting analysis was obtained by slow heating with a 0.1°C/s increment from 65 to 95°C, with fluorescence collection at 0.1°C intervals.

The threshold cycle (i.e., the amplification cycle in which product formation exceeds background fluorescence) of each standard dilution was determined during the exponential phase of amplification (24) and regressed against the logarithm (base 10) of known copies of 18S rDNA that were determined using mass concentration and MW as previously described. Standard curves were generated for each respective sample type (i.e., rumen fluid, duodenal digesta, and concentrated protozoa). All postrun data analyses were performed using iCycler software (version 3.0).

To obtain the mean copies of protozoal rDNA present in each sample type, 4 duplicate PCR reactions of 3 replicate DNA extractions were used. To minimize the possibility of plate-to-plate variation, each sample was randomly assigned to the 96-well PCR plates, and a sample with a known concentration of rDNA copies was loaded in 2 wells of each plate as a positive control to monitor for such effects. Along with the positive controls, there were a minimum of 4 no-template negative controls on each plate to screen for possible contamination and dimer formation and to set background fluorescence for plate normalization.

Preliminary data suggested that 3 replications were adequate for real-time PCR for ruminal and concentrated protozoal samples. However, to account for possible increased variation associated with subsampling from a larger volume of duodenal samples, 10 extractions of DNA were performed on this sample and used in real-time PCR (hereafter termed replications) representing each sample type; each replication had 6 separate PCR amplifications (hereafter termed duplications). Outliers were identified and deleted if values exceeded 2 SD from the mean copies of rDNA. A CV then was calculated for the duplication of real-time PCR amplifications within extraction; using the mean of duplications within extraction, a CV then also was calculated among extractions of the same sample.

Evaluation of the real-time PCR procedure. Three replications of each protozoal cell dilution, ranging from 1000 to 1.0×10^6 cells (6 total dilutions), were spiked into 1-mL samples of duodenal digesta. All samples containing the protozoal cells were incubated at room temperature for 2 h, which is the mean retention time in the abomasum (25), before DNA extraction. Then, extraction and PCR procedures were repeated as described previously. Predicted rDNA copies present in spiked protozoal cells were regressed against rDNA

copies recovered from protozoal rDNA from spiked cells plus those initially in the duodenal digesta.

Dilution standards (ranging from 10^2 to 10^6 rDNA copies) generated from duodenal digesta for use during real-time PCR quantification were serially spiked into 1-μL aliquots of extracted genomic DNA containing unknown copies (U) of protozoal rDNA isolated from the same digesta sample. The copies of protozoal rDNA of the standards (X) and the spiked samples (the total copies = X + U) were determined using real-time PCR, as described previously.

Statistical methods. Protozoal generic counts were expressed as percentages of the total counts and were not independently derived (e.g., an increase in one genus would cause a decrease in another); thus, data were analyzed as repeated measures using the Mixed procedure of the Statistical Analysis Systems (SAS) version 8.2 (SAS Institute). The sample was split into total rumen fluid and concentrated protozoal cells taken from the same sample; thus, data were analyzed as a split plot. The original model included effects of cow, treatment, cow (treatment), sample type (split plot), genera (repeated measures), and all possible interactions. Interactions were modeled as random effects except when convergence criteria were not met, in which case they were analyzed as fixed effects. The mean rDNA copies in the duodenal samples subjected to freeze-thaw cycles were analyzed as a split-plot design. The model included the fixed effects of storage temperature, thaw cycle, and the interaction of storage temperature with thaw cycle. Data were analyzed using the Mixed procedure of SAS. The mean DNA extraction yield was calculated from at least 3 replications of each dilution from each cow. The data were analyzed as described previously. The model included the fixed effects of sample type (i.e., rumen, duodenal) and sample volume. Efficiency of DNA extraction and column purification were computed using linear regression with the Proc Reg procedure of SAS. Verification of the real-time PCR procedure was done using nonlinear regression. After quantifying the copies in both the X standards and spiked samples (X + U were measured combined), the single unknown variable, the constant U copies, was solved over the entire range of X using the Proc NLIN procedure of SAS with the following model: log10 (X) = log10 (X + U). Least-squares means were generated for all data. Pooled SEM were reported when replications were equal across means, but individual SEM were reported when means had unequal replications. Mean separation was conducted using Fisher's protected least significant difference test, with significance declared at $\alpha \le 0.05$, unless otherwise specified.

RESULTS

Sample collection and storage. The protozoal isolation method **(Fig. 1)** allows the solid fraction to be washed while maintaining a temperature conducive to protozoal survival because the mechanical press decreased processing time from 45 to 15 min compared with hand-squeezing. The protozoal generic distribution in rumen fluid or in the respective concentrated cells showed no evidence ($P > 0.8$) of selective protozoal loss during concentration **(Table 1)**. Recovery of protozoal cells after scum removal from ruminal fluid (Fig. 1) averaged 96% (data not shown), and this step removed nearly all of the contaminating plant matter (based on visual appraisal under a microscope).

Freezing the samples at either -80 or -20°C did not decrease ($P > 0.98$) the mean rDNA copies, nor was there an interaction ($P > 0.37$) between storage temperature and the number of thaw cycles. After the samples had been frozen and thawed twice, the mean copies were significantly reduced ($P < 0.01$) by 51% (data not shown).

DNA extraction and purification. The amount of genomic DNA isolated from concentrated protozoal cells increased linearly (slope of 1.00) with increasing numbers of cells (data not shown). Similarly, the regression of rDNA copies present before purification against copies present after purification gave a slope of 0.98. In both cases, r^2 were >0.98 and the slopes and intercepts were not different ($P > 0.26$)

TABLE 1

Generic distribution of ciliate protozoa in ruminal fluid (RF) after concentrating (Conc) protozoal cells from 2 cows fed low- or high-forage neutral detergent fiber diets

	Cow 660				Cow 647			
	Low-forage		High-forage		Low-forage		High-forage	
	RF	Conc	RF	Conc	RF	Conc	RF	Conc
Total counts × 10⁸/L	8.35	236	9.82	336	1.72	42.5	2.25	49.3
Genera,[1,2] % of total								
Entodinium	98.1	93.3	98.8	98.4	78.7	77.3	88.3	82.2
Diplodiniinae[3]	0.48	0.39	0.49	0.84	ND[4]	ND	ND	ND
Epidinium	ND	ND	ND	ND	15.1	17.4	9.31	15.7
Isotricha	0.29	0.47	0.49	0.29	2.32	0.29	2.31	0.21
Dasytricha	1.15	0.86	0.33	0.43	3.87	0.25	ND	0.18

[1] The main effect of method was not significant, $P = 0.8$; pooled SEM = 0.26.
[2] The main effect of genera was $P < 0.05$, with *Entodinium* the only genus different ($P < 0.05$) from all of the others; pooled SEM for total counts were: RF = 0.34, Conc = 2.81.
[3] Subfamily, containing the genera *Diplodinium, Eudiplodinium, Ostracodinium, Metadinium, Enopoplastron,* and *Polyplastron.*
[4] ND, not detectable.

from 1 and 0, respectively. On the basis of these data, we have not applied any correction factors resulting from this step.

The volume of ruminal fluid and duodenal digesta used in DNA extraction did affect ($P < 0.05$) the mean total measured copies of protozoal rDNA (**Table 2**). Using 0.5 mL of each sample for DNA extraction consistently produced higher numerical yields of rDNA and was chosen as the optimal volume, regardless of sample type, for subsequent extractions.

Quantification using real-time PCR. Protozoal rDNA was consistently amplified as a single band from all sample types using conventional PCR. The average amplicon length generated by conventional PCR had a mean MW of 9.4×10^5 g/mol or 6.38×10^8 copies/ng of PCR product. Typically, ten $100-\mu L$ reactions were pooled per sample to ensure that adequate product was available for purification, quantification, and use as a standard.

A typical amplification output representing a dilution series used to generate a duodenal sample standard curve is shown in **Figure 2**. When dilutions containing template concentrations $>10^7$ copies/reaction were used, the real-time PCR curve collapsed due to excessive template. Consequently, when default values were used (i.e., cycles 3–10) for PCR plate normalization, high template concentrations exceeded the capabilities of the machine. If subsequent dilutions are used in

standard curve formation, quantification is inaccurate (see the dashed line in Fig. 2B). Melt curve analyses still consistently showed that amplification of all samples of standards (includ-
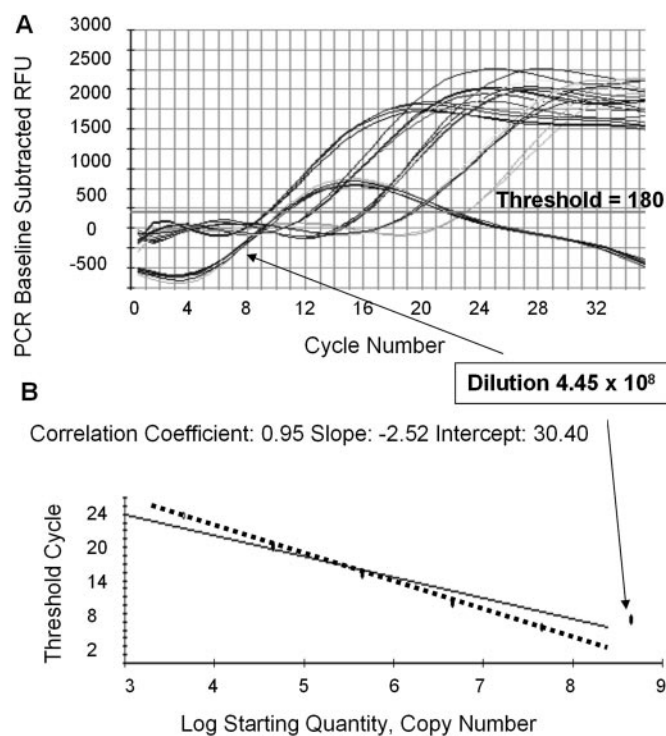

FIGURE 2 The effect of amplification plots (*A*) and standard curves (*B*) from duodenal digesta using default background correction values. (*A*) Product formation exceeds background fluorescence (set at 180 relative fluorescent units, RFU) at advancing cycles coinciding with greater dilution of standards. When excess standard was used (4.45 × 10⁸ dilution), the default background fluorescence normalization procedure caused the appearance that product formation increased and then decreased. (*B*) The effect of generating the standard curve of threshold cycle vs. logarithm (base 10) of standards when all standard dilutions are used: 4.45 × 10³ to 4.45 × 10⁸ copies. The dashed line represents the standard curve when the 4.45 × 10⁸ standard is deleted: $Y = 35.06 - 3.39X$; $r^2 = 0.99$.

TABLE 2

Mean protozoal rDNA copies from ruminal fluid and duodenal digesta samples from 2 cows determined using increasing sample volumes for DNA extraction[1,2]

	Cow 660		Cow 647	
Sample volume, *mL*	Rumen fluid	Duodenal digesta	Rumen fluid	Duodenal digesta
1.0	8.0 ± 2.0ᵃ	2.0 ± 0.8	7.0 ± 2.2	5.2 ± 1.0ᵃ
0.5	18.0 ± 1.6ᵇ	3.4 ± 0.6	8.8 ± 2.4	8.0 ± 0.6ᵇ
0.25	8.6 ± 2.0ᵃ	2.8 ± 1.4	4.6 ± 2.4	6.6 ± 0.6ᵃᵇ

[1] Values are mean rDNA copies × 10¹¹/L ± SEM. Means in a column with different superscript letters differ, $P < 0.05$.
[2] Determined from 3 replicate DNA extractions of each dilution.

ing those with excessive template) plus those from the samples produced 1 sharp peak (data not shown), confirming single product formation.

Evaluation of the real-time PCR assay. Replication and duplication CV averaged 21.2 and 18.6% across sample types, respectively (data not shown). From these data, we determined that 4 PCR duplications of 3 replicated DNA extractions were needed for optimal representation of all sample types, and all subsequent results used are based on $n = 12$ unless outliers (averaging 1 of 12) were detected.

The rDNA copies present in each protozoal dilution were determined using real-time PCR and transformed to logarithms (base 10) to normalize data. When protozoal cell dilutions were spiked into a constant amount of duodenal digesta, the regression of log rDNA copies against the log of copies present in cell dilutions gave a slope of 0.81 (**Fig 3**).

The determination of unknown copies was constant over the entire range of standards (**Fig. 4**), and the best-fit curve from the nonlinear regression had a CV of 1.5%. Because the model fit a log function based on the actual number of copies, r^2 over such a large range with increasing distance between points has limited meaning. The unknown rDNA copies predicted in this sample using nonlinear regression was 6.5×10^5, compared with the 7.2×10^5 copies determined by real-time PCR (using the standard curve in Fig. 2 and correcting for 81% recovery through the column; Fig. 3) when determined in the same sample (data not shown).

DISCUSSION

To our knowledge, direct quantification of rumen ciliate biomass using molecular techniques has not been reported. Moreover, the biomass of mixed populations of microbial cells in any system has not been quantified using real-time PCR because of the difficulty in the collection of a reference microbial sample to standardize the amount of copies to the biomass represented by that sample. For our objectives to be met, 2 criteria had to be resolved. First, the copies of PCR-amplified rDNA had to be reconstituted back to the original sample (i.e., recovery determined at each step). Second, the
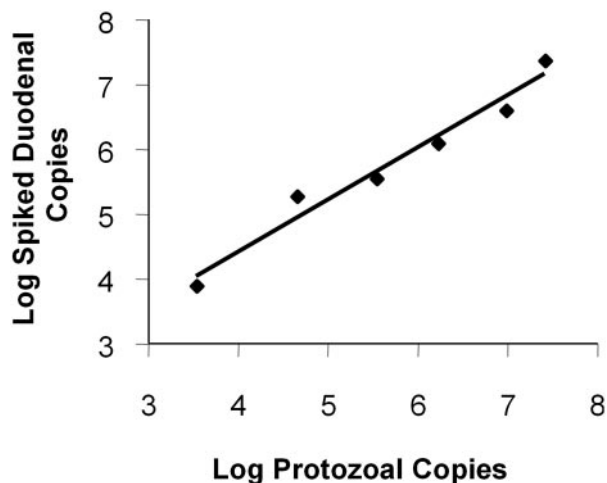

FIGURE 4 Logarithm-transformed copies of purified PCR product from DNA extracted from a duodenal sample as a standard for real-time PCR (X axis). The Y-axis depicts the logarithm (base 10) of copies after the same dilution of standard was added to 1 μL DNA extracted from the same duodenal sample. The best-fit curve from nonlinear regression had a CV of 1.5%.

rDNA copies:N ratio had to be assessed relative to a protozoal reference standard that was sufficiently representative of the rumen population and was free enough from plant and bacterial matter so the rDNA:N ratio was accurate.

Sample collection and storage. Rumen protozoal populations can exhibit daily diurnal variation (26); thus, the validity of results of all protozoal experiments depends on the accuracy of rumen sampling (27,28). We assume that increasing sample volume will help ensure that the sample more accurately represents the entire protozoal population. Our protozoal isolation procedure (Fig. 1) allows for larger samples to be taken (2.5 kg), using less time than other methods based on gravity filtration (1,20) while still preserving protozoal diversity (Table 1).

Isolating protozoa using 1 or 2 layers of cheesecloth does not bias the protozoal sample as can more layers (29). Although excess exposure to oxygen can induce lysis (26), short-term exposure of rumen fluid to oxygen appears not to be harmful to protozoa (30). Formalin is introduced early in the procedure to fix the cells, preventing possible lysis (5) that can occur during centrifugation and filtration. Formalin can react with protein (31) and amino acids (32), although washing to remove excess formalin, which occurs later in the procedure, should reduce these effects (31).

Sylvester et al. (33) demonstrated, using denaturing gradient gel electrophoresis, that a concentrated protozoal sample from the rumen provided an appropriate standard for estimation of flow of protozoal N to the duodenum. Although species in the family Isotrichidae were reported to pass more slowly from the rumen than the entodiniomorphid protozoa (34), our results (33) did not detect any differential passage of major groups of protozoa. Fluid-associated bacteria can have significantly higher N concentrations than protozoa (5); thus, the accuracy of this assay should be improved by the filtration method, which reduces bacterial contamination of N and RNA to <5% (w:w) (Sylvester et al., unpublished results).

The amount of material used in DNA extraction should affect DNA yield if reagents or columns are saturated. Because

FIGURE 3 Logarithm-transformed (base 10) copies of protozoal rDNA determined from protozoal cell dilutions ranging from 1000 to 10^6 cells (X-axis). The Y-axis depicts the logarithm of copies after the same protozoal cell dilutions were spiked into 1 mL of duodenal digesta from the same original sample. The regression gave the following equation: $Y = 0.81 \pm 0.08 X + 1.2 \pm 0.45$, $r^2 = 0.97$.
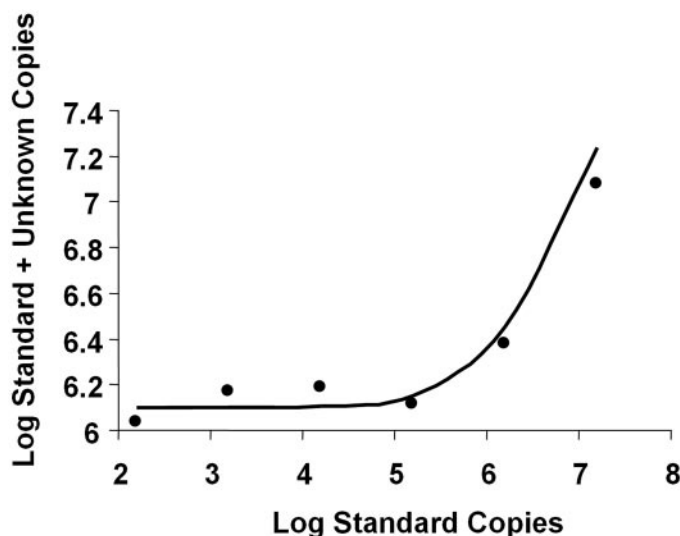
genomic DNA from the rumen is a variable mixture originating from bacterial, protozoal, fungal, plant, and even animal (sloughed epithelial cells) sources, the volume of material extracted must be evaluated experimentally in future studies. The use of 0.5 mL seems to optimize extraction recovery while still maintaining the needed volume to reduce sampling error.

Protozoal genomic DNA could not be recovered from formalin-treated duodenal digesta (data not shown). Formalin cross-links genomic DNA with histone proteins if cell membranes are lysed (35). Samples were therefore frozen immediately to prevent possible DNA degradation. Because multiple samples must be taken in nutrition experiments, our finding that freezing duodenal digesta did not decrease rDNA recovery ($P > 0.37$) at either temperature is critical to the method's future potential. Duodenal digesta subjected to 2 freeze-thaw cycles decreased recovery by almost half, but 1 freezing (a standard practice) actually appeared to increase recovery. Loss from thawing samples has not been well studied, but recovery seems dependent on sample volume and whether the freezing-thawing was fast or slow (36). The lower storage temperature did not increase the DNA stability (no storage temperature × freeze cycle interaction, $P > 0.37$), although storage at lower temperatures for extended periods warrants further assessment. Although chemical preservatives were not added to duodenal samples, DNA did not appear to be destabilized by the low pH of ruminant duodenal digesta (37,38). In fact, this low pH of 2–3 appears to protect protozoal rDNA from the high DNA degradation rate reported in ruminal fluid (39) unless duodenal samples are stored unfrozen for relatively long periods.

DNA extraction and purification. As shown in Figure 2, research is required to carefully scale the range for optimal accuracy. Some standard curves from published literature seem to show tailing at the highest concentration. Using spike and recovery assays from 3 replications of 2 experiments using 2 different cows, we consistently recovered ~80% of rDNA from duodenal digesta, as shown for 1 experiment (Fig. 3). Our results seem to contradict the much lower yield of rDNA from cellulolytic bacteria in the abomasum or duodenum relative to the rumen or omasum (40), although we note several differences in methodology between our procedure and theirs.

The purity of DNA extracted from heterogeneous material is critical for PCR analysis (41). Complex environmental samples such as feces and digesta contain many compounds that can be coextracted with DNA (42). Our high recovery of rDNA after sample purification corroborates data from McOrist et al. (41), who demonstrated that the QIAamp kit was the most effective of 4 methods tested for extracting bacterial DNA from fecal samples. On the basis of preliminary spike and recovery experiments using both herring sperm and protozoal DNA, we also found that the quantitative DNA recovery was not consistent (data not shown) with methods other than the one chosen.

Verification of real-time PCR. Because primer sets were modified from those reported by Karnati et al. (17), preliminary studies were done to verify primer specificity and effectiveness for duodenal samples. Using cloning ($n = 15$) and sequencing procedures described previously (17), the specificity of the 2 sets of primers was confirmed. Primer specificity is important because SYBR green detection is nonspecific (24). Single bands from PCR amplification and sharp peaks from melt curve analyses consistently verified single product formation, even for template concentrations above 10^8 copies.

After serially spiking known amounts of standard rDNA, the constant recovery of target rDNA in duodenal samples and good fit (Fig. 4) support the removal of potential inhibitors and an apparently consistent PCR efficiency with varying ratios of competing nontarget DNA relative to the amount of target DNA. Other replications of this validation experiment showed similar reliability; data calculated as shown in Figure 4 were within 5–15% of the copies calculated using the standard curves and with comparable or lower CV (data not shown). This nonlinear procedure overcomes the problem of using linear spiking methodology, when the unknown copies in the solution are not known (8). Those authors discussed a lag effect of the first few cycles needed to overcome inhibitors, resulting in standard curves from real-time PCR that were parallel (similar slope but different intercept) for pure cultures of bacteria or the pure cultures spiked into rumen fluid. Our DNA purification procedures appeared to remove potential inhibitors that likely were present in their rumen samples.

On the basis of a statistical power analysis, Dionisi et al. (43) determined that 3–5 PCR runs (i.e., duplication) were necessary to detect a 2-fold difference ($P < 0.05$) in bacterial 16S rDNA in sludge with 80% power. They based their conclusion of lower variability associated with DNA extraction (i.e., replication) partly on a low CV for threshold cycle of unknown samples. In our study, threshold cycle data were discrete (i.e., discontinuous), thus not following a normal distribution or allowing an accurate CV to be determined. Using rRNA hybridization to quantify cellulolytic bacteria in the rumen, Krause et al. (44) also noted a required n of 4–6, even though the CV was slightly higher than ours. We chose not to do a power analysis because it was based on a priori assumptions associated with expected differences and variation of means for which no expectations can be determined. On the basis of our algorithm of random, progressive removal of data from our study, however, we determined that 3 separate extractions with 4 real-time PCR amplifications per extraction provided a mean that was sufficiently insensitive to random loss or exclusion of copy data. The variation between replicate DNA extractions is most likely due to the combination of the difficulty in taking representative 0.5-mL aliquots from environmental samples and the variation in real-time PCR amplification resulting from pipetting error associated with small template volumes (1 μL). Multiple 96-well PCR plates reduce the cost and time for replication; although we noted no consistent effects of plate-to-plate variation, we still recommend at least 2 wells containing a sample with known copies as a positive control.

We developed a method to quantify protozoal rDNA in duodenal digesta and rumen fluid. Using the mean rDNA copies generated from the isolated protozoal cells, protozoal N flow can now be determined independently from bacterial N. Preliminary data measuring protozoal N pools in the rumen for these 2 cows showed similar treatment responses compared with a nonmarker-based approach (Sylvester et al., unpublished results). When cows were fed the low forage NDF diet, protozoal pool size tended to decrease ($P < 0.08$), and the duodenal flow of protozoal N was reduced ($P < 0.05$).

For commentary on this article, see the article by Bergen in this issue (45).

ACKNOWLEDGMENTS

LITERATURE CITED

1. Williams, A. G. & Coleman, G. S. (1992) The Rumen Protozoa. Springer-Verlag, New York, NY.

2. Dijkstra, J., France, J. & Tamminga, S. (1998) Quantification of the recycling of microbial nitrogen in the rumen using a mechanistic model of rumen fermentation processes. J. Agric. Sci. 130: 81–94.

3. Bannink, A. & De Visser, H. (1997) Comparison of mechanistic rumen models on mathematical formulation of extramicrobial and microbial processes. J. Dairy Sci. 80: 1296–1314.

4. Firkins, J. L., Allen, M. S., Oldick, B. S. & St-Pierre, N. R. (1998) Modeling ruminal digestibility of carbohydrates and microbial protein flow to the duodenum. J. Dairy Sci. 81: 3350–3369.

5. Broderick, G. A. & Merchen, N. R. (1992) Markers for quantifying microbial protein synthesis in the rumen. J. Dairy Sci. 75: 2618–2632.

6. Reilly, K., Carruthers, V. R. & Attwood, G. T. (2002) Design and use of 16S ribosomal DNA-directed primers in competitive PCRs to enumerate proteolytic bacteria in the rumen. Microb. Ecol. 43: 259–270.

7. Ouwerkerk, D., Klieve, A. V. & Forster, R. J. (2002) Enumeration of *Megasphaera elsdenii* in rumen contents by real-time Taq nuclease assay. J. Appl. Microbiol. 92: 753–758.

8. Klieve, A. V., Hennessey, D., Ouwerkerk, D., Forster, R. J., Mackie, R. I. & Attwood, G. T. (2003) Establishing populations of *Megasphaera elsdenii* YE34 and *Butyrivibrio fibrisolvens* YE44 in the rumen of cattle fed high grain diets. J. Appl. Microbiol. 95: 621–630.

9. Tajima, K., Aminov, R. I., Nagamine, H., Matsui, H., Nakamura, M. & Benno, Y. (2001) Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. Appl. Environ. Microbiol. 67: 2766–2774.

10. Petroni, G., Rosati, G., Vannini, C., Modeo, L., Dini, F. & Verni, F. (2003) In situ identification by fluorescently labeled oligonucleotide probes of morphologically similar, closely related ciliate species. Microb. Ecol. 45:156–162.

11. Faichney, G. J., Poncet, C., Lassalas, B., Jouany, J. P., Millet, L., Dore, J. & Brownlee, A. G. (1997) Effects of concentrates in a hay diet on the contribution of anaerobic fungi, protozoa and bacteria to nitrogen in rumen and duodenal digesta in sheep. Anim. Feed Sci. Technol. 64: 193–213.

12. Krause, D. O., Smith, W.J.M., Ryan, F.M.E., Mackie, R. I. & McSweeney, C. S. (2000) Use of 16S-rRNA based techniques to investigate the ecological succession of microbial populations in the immature lamb rumen: tracking of a specific strain of inoculated *Ruminococcus* and interactions with other microbial populations *in vivo*. Microb. Ecol. 38: 365–376.

13. Zoetendal, E. G., Collier, C. T., Koike, S., Mackie, R. I. & Gaskins, H. R. (2004) Molecular ecological analysis of the gastrointestinal microbiota: a review. J. Nutr. 134: 465–472.

14. Raskin, L., Capman, W. C., Sharp, R., Poulsen, L. K. & Stahl, D. A. (1997) Molecular ecology of gastrointestinal ecosystems. In: Gastrointestinal Microbiology, Vol. 2. Gastrointestinal Microbes and Host Interactions (Mackie, R. I., White, B. A. & Isaacson, R. E., eds.), pp. 243–298. Chapman and Hall, New York, NY.

15. Blomberg, P., Randolph, C., Yao, C.-H. & Yao, M.-C. (1997) Regulatory sequences for the amplification and replication of the ribosomal DNA minichromosome in *Tetrahymena thermophila*. Mol. Cell. Biol. 17: 7237–7247.

16. Crosby, L. D. & Criddle, C. S. (2003) Understanding bias in microbial community analysis techniques due to *rrn* operon copy number heterogeneity. BioTechniques 34: 790–802.

17. Karnati, S.K.R., Yu, Z., Sylvester, J. T., Dehority, B. A., Morrison, M. & Firkins, J. L. (2003) Technical note: specific PCR amplification of protozoal 18S rDNA sequences from DNA extracted from ruminal samples of cows. J. Anim. Sci. 81: 812–815.

18. Lima, M.L.M., Firkins, J. L., Sylvester, J. T., Karnati, S.K.R. & Mattos, W. (2003) Physical effectiveness of whole cottonseed as affected by lint and particle size. J. Dairy Sci. 86 (suppl. 1): 64 (abs.).

19. Dehority, B. A. & Tirabasso, P. A. (1989) Factors affecting the migration and sequestration of rumen protozoa in the family Isotrichidae. J. Gen. Microbiol. 135: 539–548.

20. Martin, C., Williams, A. G. & Michalet-Doreau, B. (1994) Isolation and characteristics of the protozoal and bacterial fractions from bovine ruminal contents. J. Anim. Sci. 72: 2962–2968.

21. Dehority, B. A. (1993) Laboratory Manual for Classification and Morphology of Rumen Ciliate Protozoa. CRC Press, Boca Raton, FL.

22. Oldick, B. S. & Firkins, J. L. (2000) Effects of degree of fat saturation on fiber digestion and microbial protein synthesis when diets are fed twelve times daily. J. Anim. Sci. 78: 2412–2420.

23. Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J. & Wheeler, D. L. (2004) GenBank: update. Nucleic Acids Res. 32: D23–D26.

24. Malewski, T., Malewska, A. & Rutkowski, R. (2004) RT-PCR technique and its applications. State of the art. J. Anim. Feed Sci. 12: 403–416.

25. Ellis, W. C., Matis, J. H., Hill, T. M. & Murphy, M. R. (1994) Methodology for estimating digestion and passage kinetics of forages. In: Forage Quality, Evaluation, and Utilization (Fahey, G. C., Jr., Collins, M., Mertens, D. R. & Moser, L. E., eds.), pp. 682–756. American Society of Agronomy, Madison, WI.

26. Dehority, B. A. (2003) Rumen Microbiology. Nottingham University Press, Nottingham, UK.

27. Coleman, G. S. (1979) The role of rumen protozoa in the metabolism of ruminants given tropical feeds. Trop. Anim. Prod. 4: 199–212.

28. Martin, C., Devillard, E. & Michalet-Doreau, B. (1999) Influence of sampling site on concentrations and carbohydrate-degrading enzyme activities of protozoa and bacteria in the rumen. J. Anim. Sci. 77: 979–987.

29. Dehority, B. A. (1984) Evaluation of subsampling and fixation procedures used for counting rumen protozoa. Appl. Environ. Microbiol. 48: 182–185.

30. Coleman, G. S. (1985) The cellulase content of 15 species of entodiniomorphid protozoa, mixed bacteria and plant debris isolated from the ovine rumen. J. Agric. Sci. 104: 349–360.

31. Karlsen, F., Kalantari, M., Chitemerere, M., Johansson, B. & Hagmar, B. (1994) Modifications of human and viral deoxyribonucleic acid by formaldehyde fixation. Lab. Investig. 71: 604–611.

32. Towne, G., Nagaraja, T. G., Brandt, R. T., Jr. & Kemp, K. E. (1990) Dynamics of ruminal ciliated protozoa in feedlot cattle. Appl. Environ. Microbiol. 56: 3174–3178.

33. Sylvester, J. T., Karnati, S.K.R., Yu, Z., Newbold, C. J., Morrison, M. & Firkins, J. L. (2004) Determining if protozoal cells isolated from ruminal fluid represent those passing to the duodenum for a quantitative assay of protozoal N. Reprod. Nutr. Dev. 44 (suppl. 1): S30 (abs.).

34. Leng, R. A. & Nolan, J. V. (1984) Nitrogen metabolism in the rumen. J. Dairy Sci. 67: 1072–1089.

35. Davis, S. K. & Bardeen, C. J. (2003) Cross-linking of histone proteins to DNA by UV illumination of chromatin stained with Hoechst 33342. Photochem. Photobiol. 77: 675–679.

36. Bellete, B., Flori, P., Hafid, J., Raberin, H. & Sung, R.T.M. (2003) Influence of the quantity of nonspecific DNA and repeated freezing of samples on the quantification of DNA by the light cycler. J. Microbiol. Methods 55: 213–219.

37. Chargaff, E. & Davidson, J. N. (1955) The Nucleic Acids. Chemistry and Biology. Vol. 1. Academic Press, New York, NY.

38. McAllan, A. B. & Smith, R. D. (1969) Nucleic acid metabolism in the ruminant. Determination of nucleic acids in digesta. Br. J. Nutr. 23: 671–682.

39. Morrison, M. (1996) Do ruminal bacteria exchange genetic material. J. Dairy Sci. 79: 1476–1486.

40. Koike, S. & Kobayashi, Y. (2001) Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. FEMS Microbiol. Lett. 204: 361–366.

41. McOrist, A. L., Jackson, M. & Bird, A. R. (2002) A comparison of five methods for extraction of bacterial DNA from human faecal samples. J. Microbiol. Methods 50: 131–139.

42. Guthrie, J. N., Moriarty, D.J.W. & Blackall, L. L. (2000) DNA extraction from coral reef sediment bacteria for the polymerase chain reaction. J. Microbiol. Methods 43: 73–80.

43. Dionisi, H. M., Harms, G., Layton, A. C., Gregory, I. R., Parker, J., Hawkins, S. A., Robinson, K. G. & Sayler, G. S. (2003) Power analysis for real-time PCR quantification of genes in activated sludge and analysis of the variability introduced by DNA extraction. Appl. Environ. Microbiol. 69: 6597–6604.

44. Krause, D. O., Dalrymple, B. P., Smith, W. J., Mackie, R. I. & McSweeney, C. S. (1999) 16S rDNA sequencing of *Ruminococcus albus* and *Ruminococcus flavefaciens*: design of a signature probe and its application in adult sheep. Microbiology 145: 1797–1807.

45. Bergen, W. G. (2004) Quantitative determination of rumen ciliate protozoal biomass with real-time PCR. J. Nutr. 134: 3223–3224.