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Estimation of Ruminal Bacteriophage Numbers by Pulsed-Field Gel Electrophoresis and Laser Densitometry

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To investigate phage activity in the rumen, a method for quantifying phage has been developed. By differential centrifugation and ultrafiltration, phage particles were separated and concentrated from ruminal fluid. Linear double-stranded DNA from this fraction containing predominately tailed phage was isolated and separated by size, using pulsed-field gel electrophoresis (PFGE). Laser densitometry of gel photographs allowed the numbers of phages with DNA in each size region to be calculated and, therefore, the total numbers per milliliter of ruminal fluid to be estimated. Phage numbers were estimated to be between 3×10^9 and 1.6×10^{10} particles ml of ruminal fluid^{-1}. The phage population, as gauged by the appearance of DNA on PFGE gels, had two major components. A broad region of DNA between 30 and 200 kb was always present on PFGE gels. It appears this region comprises DNA from a great many different phages and would include most of the temperate phages. In addition, discrete DNA bands ranging in size from 10 to 850 kb were frequently observed. DNA from one such band, of 12 kb in size, was shown to consist primarily of a single DNA type, suggesting that it originated from a specific phage. It is postulated that the discrete bands are due to epidemics or blooms of phage activity from specific, probably lytic, phages. The method that has been developed will greatly enhance future investigations into the interactions between the ruminal phage population, the ruminal bacterial population, and animal nutrition and growth. It appears the rumen ecosystem contains a dynamic phage population that is maintained at high numbers by a significant and continual lysis of ruminal bacteria.

Over the past five years, knowledge, especially of the molecular biology, of individual phages of a few species of ruminal bacteria has increased (6, 7, 9, 18, 19). This is mainly as a result of their perceived potential in the genetic manipulation of ruminal bacteria (4, 10). However, over the same period, knowledge of the effects of phages on the bacterial populations of the rumen in vivo and the consequent effects on nutrition and animal production has remained largely static.

A large and diverse population of bacteriophages is known to be present in the rumens of sheep and cattle (5, 13, 14). These phages have been known for some considerable time (2) and probably play a major role in the population dynamics of ruminal bacteria. A large and dynamic phage population in the rumen could result in considerable lysis of ruminal bacteria. At times a large proportion of the bacterial pool has been reported to undergo spontaneous lysis (12). Lysis of bacteria in the rumen and subsequent breakdown of the protoplasm results in the reduction of the efficiency of feed conversion in ruminants.

To investigate whether phage activity in the rumen ecosystem accounts for a significant proportion of bacterial lysis, it is first necessary to be able to quantify the phages present. This paper describes a method of quantifying phages that is based on the isolation and analysis of total bacteriophage DNA present in ruminal fluid (RF).

MATERIALS AND METHODS

Source of RF. RF samples (50 to 100 ml) were from a single sheep (*Ovis aries*) fitted with a ruminal cannula, housed indoors in a pen, and fed once daily. The sheep was fed oaten chaff and lucerne chaff (3:1) supplemented with a commercial mineral and vitamin mix. Samples were obtained by aspiration of ruminal contents through nylon stocking.

Phage purification and concentration. Bacteriophages were isolated by a modified method for purifying and concentrating phage for electron microscopy (5). Immediately following collection, the RF sample was heated to 75°C for 20 min to eliminate nuclease activity. The sample was centrifuged twice at 20,000 × *g* for 15 min at 4°C; the supernatant was then filtered through hydrophilic (low-protein-binding) 0.45-μm-pore-size filters (Sterifil aseptic filtering system; Millipore Corp.), and phages were pelleted by centrifugation at 51,000 × *g* for 2 h at 4°C. The supernatants were discarded, and the pellets were resuspended overnight in 50 μl of phage storage buffer (11), after which the centrifuge tubes were filled with TE (10 mM Tris, 1 mM EDTA [pH 8]), and the sample was mixed and again pelleted at 51,000 × *g* for 2 h. Each pellet was resuspended in 100 μl of PSB and pooled in an Eppendorf tube. Material that did not resuspend was removed by centrifugation at 12,000 × *g* for 2 min.

The volume of the supernatant, which contained the phage particles, was measured, and sufficient powdered low-melting-point agarose (Bethesda Research Laboratories) was added to produce a 1.5% (wt/vol) gel. The mixture was heated to 70°C, mixed well, and allowed to cool in 40-μl aliquots prior to DNA extraction.

An experiment was performed to determine the effect that frozen storage of RF samples had on phage yield. A 150-ml sample of RF was heated and then divided into two 75-ml aliquots. One aliquot was processed in the usual manner; the other was frozen at −20°C overnight, thawed, and then processed.

A further experiment was performed to determine whether hydrophobic filters reduced the recovery of phage DNA. A 100-ml sample of RF was obtained, heated, and centrifuged

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twice at 20,000 × *g*. The sample was divided into 50-ml aliquots; one was filtered with hydrophilic filters, and the other was filtered with hydrophobic filters (pore size, 0.45 μm; Millipore). After filtration, the aliquots were processed, separately, as described above.

The isolation and manipulation of phage DNA. DNA was released from phage particles and prepared for pulsed-field gel electrophoresis (PFGE) by the procedures used for viruses of *Chlorella* spp. (15). Agarose blocks containing phage DNA were stored as recommended by Sambrook et al. (16).

On occasion, agarose blocks containing DNA were excised from pulsed-field agarose gels and the DNA was digested in situ with restriction endonucleases. The agarose block was equilibrated with buffer for 1 h prior to digestion. Digestion was under the conditions specified by the manufacturer (Pharmacia). When high-molecular-weight DNA from PFGE gels was to be used to produce DNA probes for use in DNA-DNA hybridization, the DNA was restricted with endonucleases in situ prior to being recovered with Geneclean (Geneclean kit; BIO 101 Inc.).

Methods for Southern blotting and hybridization to detect homologous DNA sequences have been reported previously (7). The Southern blotting procedure was modified to facilitate the migration of large DNA molecules to the membrane by increasing the time the gel was soaked in 0.25 M HCl to two half-hour periods (3).

Agarose gel electrophoresis. Conventional electrophoretic techniques were generally as described by Maniatis et al. (11). The sizes of DNA fragments were determined by comparison with the *Hin*dIII digest of phage lambda DNA (Pharmacia LKB Biotechnology).

PFGE was performed with a Pulsaphor plus system (Pharmacia). Gels of 1% (wt/vol) agarose were run with a Tris-borate-EDTA buffer, as detailed by the manufacturer. Electrophoresis conditions comprised an initial switch time of 50 s for 14 h and then 90 s for 20 h at 150 V. These conditions were those specified for use with the lambda ladder (concatamerized phage lambda DNA; Promega) DNA size markers. This size marker and the *Hin*dIII digest of phage lambda DNA were used to determine DNA sizes of the phage DNA samples.

Laser densitometry. The relative amount of DNA in each gel band was determined from image density on photographic negatives of ethidium bromide-stained agarose gels (both conventional and PFGE) with a laser densitometer (ULTROSCAN XL; Pharmacia).

Determination of total phage numbers. The total DNA content of a sample was determined by electrophoresing a small volume of gelled sample and linearized (*Eco*RI-digested) pBR322 plasmid of known DNA concentration together on an agarose gel. Laser densitometric analysis showed the relative amounts of DNA present due to the plasmid DNA compared with the remaining DNA present on the gel. Hence, the DNA concentration of the sample was calculated.

PFGE separated the phage DNA sample by size, and laser densitometric analysis divided the sample into a number of regions of differing DNA size and gave the relative amount of DNA present in each region. As the total DNA concentration was known, the amount present in each region was calculated and converted to a value of total kilobase pairs (kb). By overlaying the densitometric scan of the size markers from the gel, the average size (in kilobase pairs) of the DNA molecules in each region was estimated. This allowed calculation of the number of linear double-stranded DNA molecules present in each region. As the ratio of double-stranded DNA molecules to phage particles (tailed phages) is one to one, this also gave the number of phage represented by each region. Addition of numbers represented in each region showed the total phage present in that volume of gelled sample. Hence, the concentration of phage present in the original RF sample was calculated.

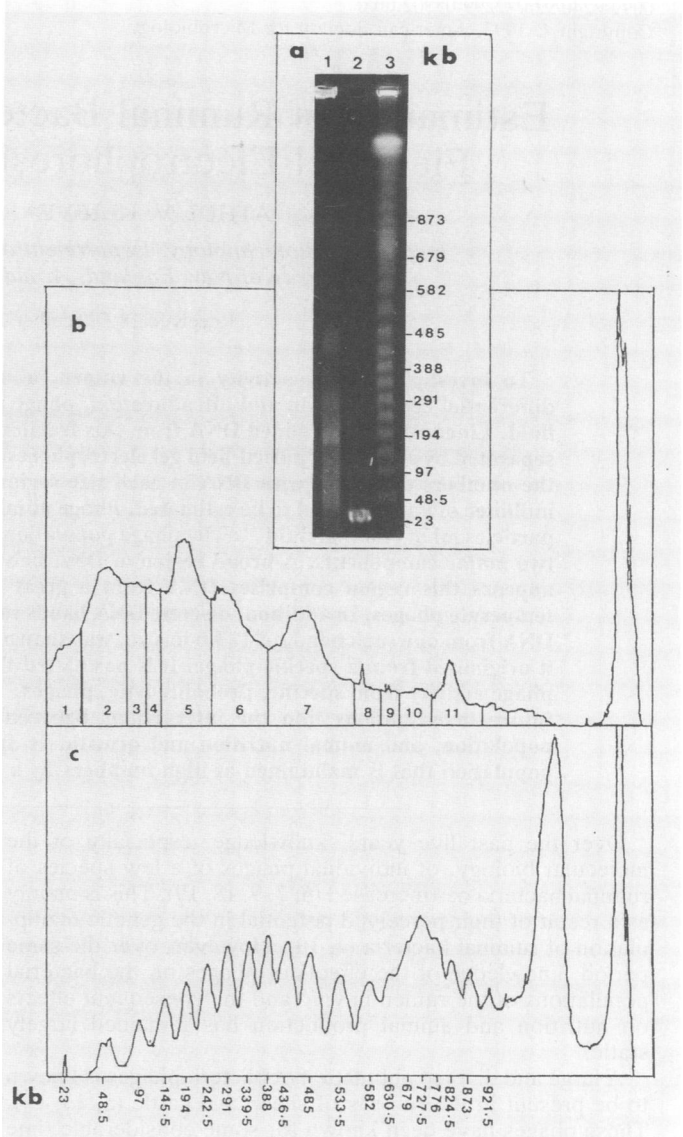

FIG. 1. (a) PFGE of ruminal phage DNA (lane 1), the *Hin*dIII digest of phage lambda DNA (lane 2), and the lambda ladder DNA size marker (lane 3). Laser densitometric scans of ruminal phage DNA (b) and the lambda ladder (c). Regions of differing DNA size from the laser densitometer scan of ruminal phage DNA (b) and the size in kilobase pairs (kb) as determined from the DNA size markers (c) are shown.

RESULTS

Estimation of bacteriophage numbers. An example of a typical PFGE gel, the densitometric scans of lanes containing ruminal phage DNA and the lambda ladder DNA size marker, are shown in Fig. 1. By comparison with a DNA standard, the ruminal phage sample, shown in Fig. 1, was

TABLE 1. Quantification of phage numbers from a laser densitometric scan[a]

Region of scan	Relative area (%)	Amt of DNA represented (ng)	Total DNA present (kb)	Avg DNA size (kb)	No. of DNA molecules present/μl of gelled sample	Phage particles/ml of RF[b]
1	6	6.8×10^2	6.2×10^{11}	23	2.7×10^{10}	4.7×10^9
2	14	1.6×10^3	1.4×10^{12}	49	2.9×10^{10}	5.1×10^9
3	6.1	7.0×10^2	6.3×10^{11}	97	6.5×10^9	1.1×10^9
4	4.4	5.0×10^2	4.5×10^{11}	1.2×10^2	3.7×10^9	6.5×10^8
5	17	1.9×10^3	1.7×10^{12}	1.9×10^2	8.8×10^9	1.5×10^9
6	14	1.6×10^3	1.4×10^{12}	3.3×10^2	4.3×10^9	7.5×10^8
7	15	1.7×10^3	1.5×10^{12}	4.9×10^2	3.1×10^9	5.4×10^8
8	2.3	2.6×10^2	2.4×10^{11}	5.8×10^2	4.1×10^8	7.2×10^7
9	1.3	1.5×10^2	1.3×10^{11}	6.3×10^2	2.1×10^8	3.7×10^7
10	2.3	2.6×10^2	2.4×10^{11}	7.3×10^2	3.3×10^8	5.8×10^7
11	3.8	4.4×10^2	4.0×10^{11}	8.2×10^2	4.9×10^8	8.6×10^7

[a] Laser densitometric scan from Fig. 1b.
[b] There are a total of 1.4×10^{10} phage particles present per ml of RF sample.

found to contain 285 ng of DNA per μl of gelled sample or 2 μg of DNA per ml of the original RF sample. The amount of phage DNA electrophoresed on the PFGE gel (Fig. 1a) was 11.4 μg (40 μl of gelled sample). Figure 1b and c show the distribution of the DNA across the DNA size spectrum and the division into regions of differing size. The data generated from these results are shown in Table 1. DNA that didn't migrate into the gel, which accounted for 10 to 15% of the total DNA in all samples examined to date, was subtracted from the total present in the sample and not regarded as phage DNA. The total number of phage from the RF sample in Fig. 1 was 1.4×10^{10} particles per ml. Two further samples had phage populations of 1.6×10^{10} and 3.0×10^9 particles per ml.

The effects of frozen storage and filter type. No difference in the pattern and amount of phage DNA on PFGE gels was found when RF was stored frozen overnight compared with immediate processing of the sample (Fig. 2).

The yield of DNA, when hydrophobic filters were used instead of hydrophilic filters, was reduced by approximately 10 to 20%. However, the effect was generalized and no specific reduction of any phage types appeared to occur, as the overall DNA pattern and relative amount of DNA of different size classes remained constant.

The distribution and integrity of DNA on PFGE gels. Ruminal bacteriophage DNA ranged in size between 10 and 850 kb. Typically a broad region of DNA, from 30 to 200 kb in size, was always present. In addition to this broad region, distinct bands were often seen on PFGE gels, and the sizes of these bands varied considerably between different rumen samples, as shown in Fig. 3. These were the major features observed from PFGE gels. In addition, DNA was observed over much of the 10- to 850-kb size range, the amount varying from sample to sample.

To ascertain that the broad region of DNA between 30 and 200 kb was DNA representing a large, mixed population of discrete DNA molecules and not degraded DNA from larger bands, a block of DNA (50 to 100 kb in size) was excised from a gel digested with restriction endonuclease (to reduce DNA size) and recovered by the Geneclean method. This DNA was labelled as a probe and hybridized to a Southern blot of DNA from the same ruminal phage sample. Hybridization was observed only at the position from which the probe DNA originated, confirming that this DNA represented a population of discrete, intact molecules.

To test whether the discrete bands on gels represented blooms or epidemics of a single phage or were populations of phages with similar sized genomes, the 12-kb DNA band from the ruminal phage sample shown in Fig. 3 (lane 9) was excised from a PFGE gel, digested with *Eco*RI and *Hin*dIII in situ, and electrophoresed on a conventional agarose gel. The DNA was digested to discrete fragments of sizes that added to the intact size of the DNA (Fig. 4). This indicated that the 12-kb DNA was essentially homogenous and was probably from a single phage.

DISCUSSION

A method of estimating ruminal phage numbers by PFGE and laser densitometry has been developed. Initially, large plant material and bacteria are removed from RF by low-speed centrifugation and filtration. Small bacterial spores

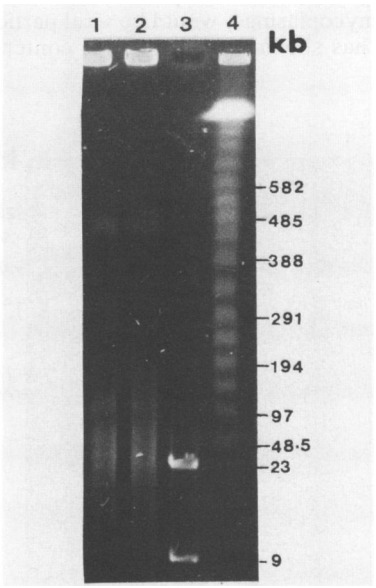

FIG. 2. DNA from a ruminal phage sample, half of which was processed fresh (lane 1) and half of which was processed following overnight storage at −20°C (lane 2). DNA size markers were the *Hin*dIII digest of lambda phage DNA (lane 3) and the lambda ladder (lane 4).

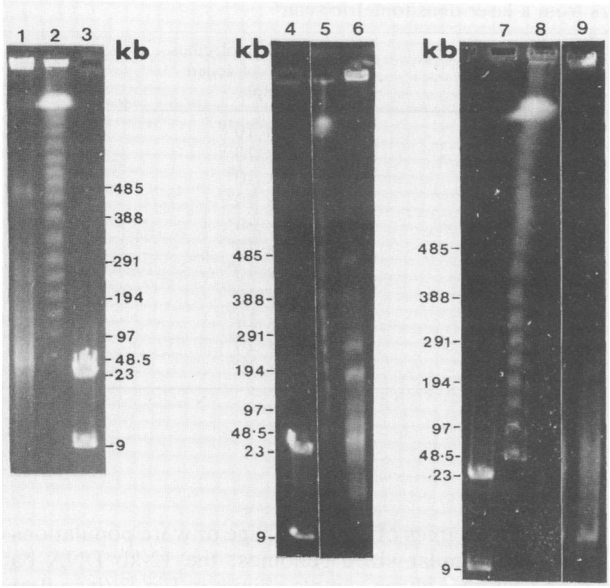

FIG. 3. Ruminal phage DNA from RF samples taken from the same animal on separate occasions (lanes 1, 6, and 9). DNA size markers were the *Hin*dIII digest of phage lambda DNA (lanes 3, 4, and 7) and the lambda ladder (lanes 2, 5, and 8).

and mycoplasmas could theoretically pass through the filters and might be sedimented with phage particles. However, we have never been able to culture organisms from the filtrate, although this does not in itself preclude the possibility that these particles are present. Any DNA released from bacteria prior to or during the filtration process would be heavily sheared (chromosomal DNA) or too small (plasmid DNA) to sediment with the phage particles. Hence, following centrifugation of filtered rumen fluid, the only nucleic acid-containing particles present, with the possible exception of bacterial spores and mycoplasmas, would be viral particles. Electron microscopy has shown that in ruminal contents these viral

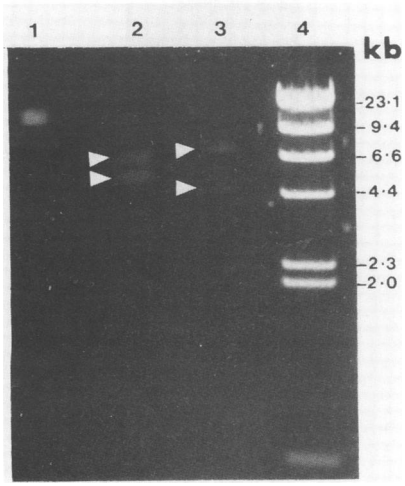

FIG. 4. DNA from a 12-kb band excised from a PFGE gel (lane 1) and digested with either *Hin*dIII (lane 2) or *Eco*RI (lane 3). The major products of digestion are shown with white arrowheads. The *Hin*dIII digest of phage lambda DNA (lane 4) was used as a size marker.

particles are usually tailed bacteriophages (5, 14). As tailed bacteriophages represent 95% of all known phages (1), it would be expected that the overwhelming viral pathogens of ruminal bacteria would be tailed phages. Virtually all tailed phage particles contain a single molecule of linear double-stranded DNA. Hence, the DNA from a population of largely double-stranded DNA phages would be separated according to the length of the DNA on an agarose gel. RNA and single-stranded DNA-containing phages appear not to have been detected by the method used, as genomes smaller than 10 kb (which would be expected for these types of phage) were not observed. Although these phages may exist in the rumen, and require investigation, it is thought that from the general considerations and electron microscopic studies cited above that they would be a minor component of the viral ecosystem.

Up to 15% of the DNA from the ruminal phage DNA samples did not migrate into the gel and was discounted as not being phage DNA (at least not linear double-stranded DNA from tailed phages). This DNA is possibly circular DNA from bacterial spores and mycoplasmas. However, it could possibly be circular DNA from phages or phage DNA that was not released from particles by the treatment used. DNA excised from the gel did not hybridize to any sample DNA in the well, nor did any DNA remain in the well when DNA was excised from the gel and rerun on another PFGE gel.

The estimate of phage numbers is probably less than the numbers present, because losses occur in sample preparation. In addition, the method can only measure particles present at one point in time in the free rumen liquor. Phage attached to, or inside, bacterial cells are not included. The numbers present are at least as high as the highest previous estimate, by electron microscopy, of $>10^9$ particles per ml of RF (14).

When RF samples were split into two and each half was processed separately, the results on PFGE gels were visually identical, suggesting the method gave reproducible results. This reproducibility within RF samples has been demonstrated repeatedly. The wide variability between samples taken on different days (Fig. 3) is indicative of a dynamic and continuously changing phage population. However, when RF was sampled from a sheep at 2-h intervals (to be published separately), the PFGE DNA banding patterns changed slowly, and between some 2-h intervals no visible changes had occurred. It appears that samples taken from an animal at the same time, or very shortly thereafter, give reproducible DNA banding patterns, while longer times between samples show much greater differences.

There was no difference between the DNA banding patterns of RF samples that had either been processed immediately or frozen at −20°C prior to processing. This should allow for future experiments to be planned so that RF samples can be stored after collection to be processed at a later date.

The total amount of DNA recovered following passage of phage through hydrophobic filters was 10 to 20% less than when the same sample was passed through hydrophilic filters. This reduction was concluded to be due to binding of phage particles but was less than expected from previously reported data (17).

Two major features were observed from the migration of DNA through PFGE gels. Firstly, a broad region of DNA (30 to 200 kb in size) was always present. Since a sample of the entire phage population of the rumen is being examined at one time, it was predicted that a broad band of DNA over

this size range would occur. Ackermann and Du Bow (1) showed that the majority of tailed phages have DNA between 10 and 120 MDa (approximately 15 to 180 kb) in molecular mass. The rumen possesses a diverse population of tailed phages, with more than 40 morphologically distinct types being reported in one study (14) and 25 reported in another (5). Each of these phage types could represent tens or hundreds of genetically distinct phages, each of which could possess DNA of differing size. This broad region of DNA often represents 50% or more of the total DNA in the sample. Altering the electrophoresis conditions to spread this DNA over a greater area of the gel did not resolve this region into individual bands, although bright bands frequently occur within the region, suggesting that the numbers of different phages contributing DNA to this region is large. All the temperate phages isolated from ruminal bacteria that have had their chromosomal DNA sizes determined have DNA between 35 and 50 kb (4a, 9, 19). Also, the head diameter of most of the other phages that have been isolated from RF and 60 to 70% of the types observed with electron microscopy (5) indicate that these would probably contain DNA in the size range 15 to 180 kb.

In addition to the broad region of phage DNA, distinct bands of a wide variety of sizes were also frequently observed (Fig. 3). Some bands occurred within the broad region, but many were considerably smaller or larger, with the extremes of phage sizes observed being from such bands (10 and 850 kb, respectively). It is postulated that these bands represent epidemics or blooms of phage activity and probably arise from lytic phage activity. This is due to the fact that many known temperate phages (and almost all isolated from ruminal bacteria) are of the B1 morphotype, with DNA size commonly between 30 and 70 kb. Also, as the animal had been fed the same ration daily over an extended period, it would be unlikely that a new inducing agent would enter the system in sufficient quantity to cause widespread lysis of a lysogenic population of bacteria. In support of this hypothesis, one such band has been shown, by endonuclease digestion, to consist of DNA from a single phage genotype (Fig. 4). It is possible that this single phage has appeared in the RF in such high numbers as the result of the massive lysis of a limited population of bacteria.

We intend to use this method of examining the ruminal phage population to investigate the link between ruminal phage and bacterial lysis in the rumen. A cause of reduced efficiency of feed utilization is the nonspecific lysis of bacteria within the rumen and subsequent breakdown of the protoplasm. At times a large proportion of the bacterial pool can be affected (12). It is most likely that phages are responsible for a significant proportion of this lysis. We also intend to investigate natural mechanisms of induction of temperate phages in the rumen, as a large proportion (up to 25%) of ruminal bacteria possess inducible temperate phages (8). Such knowledge may allow the manipulation of the phage population in the rumen and the subsequent affects on animal nutrition and growth to be measured.

In conclusion, we have presented an objective means of quantifying the phage population of the rumen. The method also gives an indication of the composition of the phage population at a given point in time. From the limited evidence available, it appears that the rumen ecosystem contains a large and dynamic phage population which may be present because of a significant and continual lysis of ruminal bacteria.

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