Short Communication

Ovine rumen papillae biopsy via oral endoscopy; a rapid and repeatable method for serial sampling

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Abstract

AIMS: To explore and validate the utility of rumen endoscopy for collection of rumen papillae for gene expression measurement.

METHODS: Four adult Coopworth ewes were fasted for either 4 or 24 hours. Animals were sedated, placed in a dorsally recumbent position at 45 degrees with the head upright, and an endoscope inserted via a tube inserted into the mouth. Biopsies of rumen papillae were taken from the ventral surface of the rumen atrium under visual guidance. Two biopsies were collected from one of the animals that had been fasted for 4 hours, and three from one of the animals that had been fasted for 24 hours. Video of the rumen atrium and reticulum was also collected. The animals recovered uneventfully. Biopsies were subsequently used for extraction and sequencing of mRNA.

RESULTS: The ventral surface of the rumen atrium was accessible after 4 hours off pasture, but a larger region was accessible after 24 hours of fasting. Sedation allowed access for endoscope use for around 5 to 10 minutes after which increased saliva flow was noted. Rumen papillae biopsies were easily collected, with samples from a variety of sites collected in the ~10 minute time window. High quality RNA was obtained for stranded mRNA sequencing. Of the resulting reads, 69–70% mapped uniquely to version 3.1 of the ovine genome, and 48–49% to a known gene. The rumen mRNA profiles were consistent with a previously reported study.

CONCLUSIONS: This method for obtaining rumenal tissue was found to be rapid and resulted in no apparent short or long term effects on the animal. High quality RNA was successfully extracted and amplified from the rumen papillae biopsies, indicating that this technique could be used for future gene expression studies. The use of rumen endoscopy could be extended to collection of a variety of rumen and reticulum anatomical measurements and deposition and retrieval of small sensors from the rumen. Rumen endoscopy offers an attractive and cost effective approach to repeated rumen biopsies compared with serial slaughter or use of cannulated animals.

KEY WORDS: *Rumen, papillae, sheep, endoscopy, biopsy, gene expression*

Introduction

In recent years there has been increasing interest in understanding ruminant digestion, driven by the objective of genetically mitigating emissions of methane by ruminants (Clark 2013), either through direct selection on methane-yield (Pinares-Patiño *et al.*. 2013), or improved ruminant feed efficiency (Basarab *et al.* 2013). Improvement of these traits through genomic selection is currently being investigated (Pickering *et al.* 2015), and as a consequence the number of animals that have rumen samples being collected for microbial analysis has increased rapidly from 10–20 to thousands per annum. With the recent publication of repeatable and detailed differences in rumen contents between animals with differing methane-yield phenotypes (Kittelmann *et al.* 2014; Shi *et al.* 2014) interest has extended to the rapid sampling of rumen contents. Separately, the recent publication of the sheep genome and transcriptome demonstrated that high expression of genes encoding keratin cross-linking proteins was associated with adaptation to a ruminant lifestyle (Jiang *et al.* 2014).

Historically, rumen gene expression studies have involved either slaughter of animals (e.g. Naeem *et al.* 2012), or rumen cannulation, also referred to as fistulation (e.g. Steele *et al.* 2011). Both these methods are expensive, time consuming and not suitable for animals that are to be retained for breeding. Moreover, slaughter of the animal is not feasible for time-course studies as serial sampling of the same animals is impossible. Serial sampling can be undertaken using rumen cannulation, which allows long-term, minimally invasive access to the rumen (Hecker 1969), however, this process is not convenient for studies requiring large numbers of animals.

While cannulation is currently the conventional method for studies that require sequential sampling of rumen contents, endoscopic biopsies are a viable alternative. In domestic ruminants endoscopy via a ruminal cannula has been used for evaluation of the rumen (McBride *et al.* 1983), and for sampling to

RIN RNA integrity number

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determine gene expression (Suominen *et al.* 1998; Taylor-Edwards *et al.* 2010; Steele *et al.* 2015). An alternative method of obtaining tissue for gene expression studies is via oral endoscopy. In calves, oral endoscopy has been evaluated as a tool to describe the visible structures of the rumen. This technique was found to be unsatisfactory in comparison to use of a ruminal cannula; however, the animals were not sedated during the procedure. As a result endoscopic examination of the rumen was only successful in 4/9 calves examined (Franz *et al.* 2006).

The objective of this study was to explore the viability of oral endoscopy for biopsy of rumen papillae, and evaluate the biopsies obtained via this method for RNA quality and gene expression analysis.

Materials and methods

Animals, sedation and restraint

All work was undertaken by approval of the AgResearch Invermay Animal Ethics Committee (Approval number: 13219).

Four adult female Coopworth sheep weighing 54–73 kg were fasted; two for 24 hours and two for 4 hours prior to sedation. They were each injected I/M with 2 mL atropine (0.65 mg/mL; Phoenix Atropine injection; Phoenix Pharm Distributors Ltd, Auckland, NZ), to reduce salivation and to stabilise the heart rate, followed approximately 10 minutes later by slow I/V xylazine (Xylase 20, Bayer Animal Health, Auckland, NZ) as detailed in Table 1. Each sheep was then placed in dorsal recumbency and lightly restrained in a modified laparoscopy cradle so that it could be positioned at 45 degrees for the procedure. This allowed the papillae in the anterio-ventral region of the rumen to be visualised in the gas dome of the rumen. The procedure was completed between 10 and 24 minutes after initial administration of xylazine (Table 1) when the animal was removed from the cradle and returned to its pen. At this stage they could walk and stand, although initially mildly ataxic.

Endoscopy

Following sedation and restraint in the modified laparoscopy cradle, a tube was introduced into the sheep's mouth and pharynx to protect the sheep and endoscope, and held by hand. The tube consisted of a PVC pipe (20 mm wide and 200 mm long), surrounded by a rubber dairy teat liner (DeLaval liner 24M; DeLaval Ltd, Hamilton, NZ) cut to fit over the pipe. A standard Olympus colonoscope (Olympus Flexible Video Evis CF-140L, Olympus, Auckland, NZ) with a diameter of 12.5 mm and a length of 170 cm connected to a light source and processor (Olympus Evis Exera II CV-180 and CLV-180) was introduced through the pipe and advanced through the oesophagus and into the rumen with visual guidance (Olympus OEV191H monitor). Following visual identification of the correct location of the papillae in the anterio-ventral region of the rumen, biopsies were taken using single-use biopsy forceps (Olympus Disposable Biopsy Forceps FB-230U) introduced through the instrumentation channel. Two rumen papillae biopsies were collected from one of the animals that had been fasted for 4 hours, and three biopsies from one of the animals that had been fasted for 24 hours. A video of the procedure and collection of biopsies is available (Supplementary Information 1[1] and https://youtu.be/7CI0m9KPtUo). Between sheep, the outside of the instrument was cleaned with detergent (Medivators Intercept Detergent, CR Kennedy (NZ) Ltd., Auckland, NZ), the working channel was cleaned with a Pull-thru (CR Kennedy (NZ) Ltd.) to remove any remaining tissue and flushed with detergent. Rumen papillae biopsies were snap-frozen in liquid nitrogen then stored at –80 °C.

RNA extraction and sequencing

Total RNA was isolated from ~10 mg of tissue using an RNeasy mini kit and an on-column DNase digestion (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA integrity was assessed using an Agilent RNA 6000 Nano Assay on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and total RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

The MiSeq Reagent Nano Kit v2 (Illumina, San Diego, CA, USA) was utilised for this proof-of-concept experiment. This kit is only capable of producing a maximum of one million reads, therefore, the two samples with the highest RNA integrity number (RIN; with a scale of 1–10, where 1 is completely degraded and 10 intact; Schroeder *et al.* 2006) were chosen for sequencing. Illumina TruSeq Stranded mRNA libraries (Illumina) were prepared according to the manufacturer's instructions. Libraries were visualised using an Agilent DNA High Sensitivity assay on an Agilent 2100 Bioanalyzer, and quantified using the Qubit dsDNA HS assay (Invitrogen, Life Technologies, Carlsbad CA, USA) according to the manufacturer's instructions. Qubit concentrations were used to pool the two indexed cDNA libraries containing the specific Illumina TruSeq adapters. The denatured cDNA libraries were pooled together with 1% PhiX control

Table 1. Details of sheep using to assess oral endoscopy for biopsy of the rumen, with the dose of atropine and xylazine given to establish sedation, and the duration of the procedure.

			Atropine[a]		Initial xylazine[b]		
Sheep	Fasting (hours)	Live weight (kg)	Dose (mL)	Dose rate (mg/kg)	Dose (mL)	Dose rate (mg/kg)	Duration (minutes)
1	24	54	2.0	0.024	0.45	0.17	24
2	24	57	2.0	0.023	0.7	0.25	15
3	4	73	2.0	0.018	0.9	0.25	12
4	4	62	2.0	0.021	0.75	0.24	10

[a] Atropine sulphate, 0.65 mg/mL
[b] Xylazine HCl, 20 mg/mL
[c] A further 0.2 mL xylazine was administered 5 minutes after initial treatment, due to lack of sedation (total dose rate 0.24 mg/kg)

[1] http://dx.doi.org/10.1080/00480169.2015.1121845

(Illumina), which acted as a quality control for cluster generation, sequencing, and alignment. Two pM of the combined sample library and PhiX control was loaded on an Illumina MiSeq (Illumina) for 300 cycles, resulting in paired-end reads of 150 base pairs. The Illumina adapters were removed from the sequences, along with bases with a median Phred scaled quality score below 20, using Trim Galore (version 0.3.7, Babraham Bioinformatics, Cambridge, UK). Trimmed reads were mapped to version 3.1 of the ovine reference genome (Jiang *et al.* 2014) using STAR (Dobin *et al.* 2013), with the supplied Ensembl *Ovis aries* transcriptome annotation (release 78). Only uniquely mapped reads with a maximum of two mismatches to the reference genome were retained for further analysis. The mapped reads were used to estimate raw read counts per gene using HTSeq (version 0.6.1p1, Anders *et al.* 2015) with the union overlap resolution mode.

Results

Biopsy using oral endoscopy

After the endoscope was passed into the rumen of the sheep the ventral surface of the rumen atrium was accessible after 4 hours off pasture, but a larger region was accessible after 24 hours of fasting. We were able to visualise the papillae in the anterioventral region of the rumen easily, but the remainder of the rumen was filled with debris in sheep fasted for either 4 hours or 24 hours. Some improvement was achieved by insufflating air into the lumen of the rumen. Sedation allowed access for endoscope use for around 5–10 minutes after which increased saliva flow was noted. Two to three biopsies were taken from each sheep under visual guidance (Figure 1). The sheep tolerated the procedure without noticeable discomfort and recovered uneventfully. There was minimal bleeding following the sampling.

RNA sequencing

Sample RIN for the five samples ranged from 6.3–8.2. The two samples with the highest RIN (≥8) were chosen for sequencing. They were both from the first sheep biopsied. Results for the two samples are presented in Table 2. Of the paired-end cDNA reads, 69–70% mapped to a unique region of the ovine genome, and 48–49% aligned to a known feature. The full list of read counts per gene is available in Supplementary Information 2[2]. The mRNA sequencing data have been deposited in the NCBI Gene Expression Omnibus Database, with the accession number GSE68791.

Discussion

Rumen biopsy using oral endoscopy was found to be rapid, able to undertaken in animals after 4 hours of fasting, and resulted in no apparent short or long term effects on the animal. Cannulation is currently the conventional method for studies that require sampling of rumen contents; however it is impracticable to repeat on a large scale. The alternative process of collecting samples post-slaughter cannot be used to collect samples serially. Perhaps most relevant to genetic studies, cannulated animals are very unlikely to be retained as part of an ongoing breeding flock or herd. Oral endoscopy, however, is a quick method to obtain rumen tissue biopsies that has no, or minimal, impact

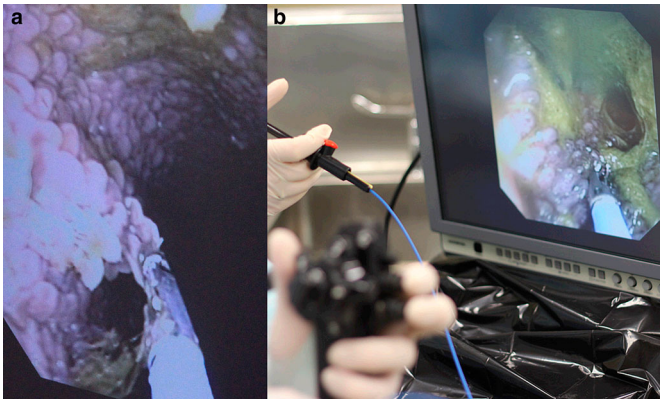

Figure 1. Photographs of (a) guided biopsy of papillae in the anterioventral region of the rumen of a sheep using oral endoscopy, and (b) the endoscopy procedure showing the hand held steering mechanism for the endoscope in the foreground, the hand piece to operate the biopsy forceps above this and the monitor showing the rumen with the papillae in the background.

on the animal and can be done serially over time; while also imposing various treatments. A PVC pipe was used to protect not only the scope from the teeth of the sheep but also the sensitive oral surface from the scope.

While the integrity of the RNA obtained from the rumen biopsies varied, high quality RNA was able to be extracted for successful amplification. The quality of RNA may vary extensively between extractions (Schroeder *et al.* 2006), however steps can be taken to minimise degradation. These steps include reducing the time between sampling and immersing the tissue in liquid nitrogen, the addition of an RNA stabilisation reagent, and ensuring that the sample does not thaw before RNA extraction.

With optimal clustering, the kit that was used for this study is capable of producing one million reads, however our output was approximately 436,000 reads, under half of what was expected. This was the result of under-clustering of the flow cell due to the concentration of the library being under-estimated, and could be remedied by loading 6 pM of library onto the MiSeq instead of 2 pM. The gene expression profile was similar to previously reported rumen transcriptomes (Jiang *et al.* 2014). The two most highly expressed genes in both samples were the mitochondrial genes cytochrome c oxidase subunit I and cytochrome B.

The successful extraction and amplification of high quality RNA from the rumen biopsies indicates that this technique could be used for future gene expression studies. The amount of tissue could be increased if multiple biopsies were collected, or larger forceps used. Additionally, the use of an endoscope allows serial sampling of the same animal. Differences in the morphology (Bain *et al.* 2014), microbial community structure (Kittelmann *et al.* 2014) and microbiome (Shi *et al.* 2014) of the rumen have all been linked to methane-yield phenotypes in sheep. While these studies have primarily focussed on either the microbes or the host, in future the technique reported here could provide the opportunity to understand the interaction between the host transcriptome and the microbiome through serial sampling of both rumen papillae and rumen contents.

Although not investigated in the present study, rumen endoscopy could easily be extended to the collection of a variety of rumen and reticulum anatomical measurements and perhaps deposition

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Table 2. Results for mRNA sequences obtained from two biopsy samples from a sheep rumen, acquired using endoscopy, showing the metrics generated by bioinformatics packages for alignment to the ovine genome (Jiang *et al*. 2014). The percentage of processed reads is given in brackets.

Bioinformatics metric	Sample A	Sample B
Trim Galore[a]		
Number of processed reads	212,245	223,537
Number of reads post-trimming	212,138	223,342
STAR[b]		
Number of uniquely mapped reads	148,700 (70%)	154,032 (69%)
Number of reads mapped to >1 locus	47,148 (22%)	52,475 (23%)
HTSeq[c]		
Number of reads aligned uniquely to known feature	103,999 (49%)	106,750 (48%)
Number of reads that did not map to a known feature	43,977 (21%)	46604 (21%)
Number of reads that could have been assigned to more than one feature (ambiguous)	724 (0.34%)	678 (0.30%)

[a] Babraham Bioinfomatics, Cambridge, UK.
[b] Dobin *et al*. (2013).
[c] Anders *et al*. (2015).

and retrieval of small sensors from the rumen. There is precedence for this in humans, where pH and pressure sensors have been introduced via the nasogastric route for 24-hour measurements (Ghosh *et al*. 2011). A probe might also be clipped to the stomach/oesophageal wall for continuous wireless measurement (Chang *et al*. 2009).

In summary, biopsy via rumen endoscopy was found to be rapid, able to be undertaken in animals after 4 hours of fasting, and resulted in no apparent short or long term effects on the animal. High quality RNA was successfully extracted and amplified from the rumen papillae biopsies, indicating that this is a viable alternative for sample collection in future host gene expression studies. The use of rumen endoscopy could also easily be extended to collection of a variety of rumen and reticulum anatomical and pH measurements. Rumen endoscopy offers an attractive and cost effective approach to repeated rumen biopsies compared to serial slaughter or use of cannulated animals.

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