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RESEARCH ARTICLE

Metagenomic DNA isolation from sheep feces and PCR detection of several rumen bacteria

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Koyun dışkısından metagenomik DNA izolasyonu ve bazı rumen bakterilerinin varlığının PZR ile tespit edilmesi

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Öz

Amaç: Bu çalışmanın amacı taze ve kuru koyun dışkısından metagenomik DNA'nın izolasyonunu yapmak ve spesifik primerler kullanarak çeşitli rumen bakterilerini tespit etmektir.

Gereç ve Yöntem: Metagenomik DNA izolasyonu ticari I-Genomic Dışkı DNA izolasyon kiti kullanılarak gerçekleştirildi. *Anaerovibrio lipolytica, Fibrobacter succinogenes, Prevotella bryantii, Prevotella ruminicola, Ruminobacter amylophilus, Ruminococcus albus, Ruminococcus flavefaciens, Streptococcus bovis, Selenomonas ruminantium* ve *Succinovibrio dextrinosolvens* spesifik primerler ile metagenomik DNA kullanılarak polimeraz zincir reaksiyonu yardımıyla tarandı. *R. amylophilus, R. albus* ve *S. dextrinosolvens* yokluğunu doğrulamak için 16S rRNA bölgesinin SphI ile reaksiyonu gerçekleştirildi.

Bulgular: Dışkı örnekleri hızlıca kurutuldu ve yaş ağırlığının %53.72'si kaybettirildi. Taze ve kuru örneklerdeki DNA izolasyonlarından sonra DNA konsantrasyonları ve saflığı sırasıyla 25.60-59.50 ng/µL ve 1.72-1.90 arasında değiştiği belirlendi. Dışkıdaki inhibitörlerin PZR üzerinde etkisinin olmadığı görüldü. *A. lipolytica, F. succinogenes, P. bryantii, P. ruminicola, R. flavefaciens, S. bovis* ve *S. ruminantium* spesifik primerler ile tespit edildi, fakat PCR ile *R. amylophilus, R. albus* ve *S. dextrinosolvens* varlığına rastlanılmadı. 16S rRNA bölgesinin SphI ile kesimi bu sonucu doğruladı.

Öneriler: Bu çalışma, doğal şartlarda kurumanın dışkı örneklerinden metagenomik DNA izolasyonu üzerine etkilerini tanımlamıştır. Ayrıca, izole edilen DNA kullanılarak çeşitli rumen bakterilerinin tespiti gerçekleştirilmiştir. Sonuç olarak kurumuş dışkıdan izole edilen metagenomik DNA'nın bakteri populasyonlarının belirlenmesinde kullanılabileceği ifade edilebilir.

Anahtar kelimeler: Ruminant, rumen bakterisi, metagenomik DNA, PZR

Abstract

Aim: The aim of this study was to isolate metagenomic DNA from fresh and dry sheep feces and to detect several rumen bacteria using the specific primers.

Materials and Methods: The metagenomic DNA isolation was performed by using commercial I-Genomic Stool DNA Isolation Kit. *Anaerovibrio lipolytica, Fibrobacter succinogenes, Prevotella bryantii, Prevotella ruminicola, Ruminobacter amylophilus, Ruminococcus albus, Ruminococcus flavefaciens, Streptococcus bovis, Selenomonas ruminantium* and *Succinovibrio dextrinosolvens* were screened using metagenomic DNA with polymerase chain reaction and spesific primers. Reaction of 16S rRNA region with SphI was carried out to confirm the absence of *R. amylophilus, R. albus* and *S. dextrinosolvens.*

Results: Fecal samples dried rapidly and lost its 53.72% of fresh mass. After the DNA isolations from fresh and dried samples, DNA concentrations and purity were varied between 25.60-59.50 ng/µL and 1.72-1.90, respectively. It was observed that fecal inhibitors had no effect on PCR. *A. lipolytica, F. succinogenes, P. bryantii, P. ruminicola, R. flavefaciens, S. bovis* and *S. ruminantium* were detected with specific primers however PCR did not reveal the presence of *R. amylophilus, R. albus* and *S. dextrinosolvens*. SphI digestion of 16S rDNA regions has confirmed this result.

Conclusion: In this study, effect of drying in natural conditions on metagenomic DNA isolation from fecal samples was determined. Furthermore, PCR detection of several rumen bacteria was performed by using isolated DNA. In conclusion, it may be stated that the metagenomic DNA isolated from dried fecal samples could be an effective tool for the detection of bacterial populations.

Keywords: Ruminant, rumen bacteria, metagenomic DNA, PCR

Introduction

Ruminants carry a very diverse and intense microbial population, which performs the biological conversion of feed in the rumen (Bekele et al 2010). These complex microbial communities are bacteria, archaea, fungi, protozoa and bacteriophage (Kobayashi 2006). Rumen bacteria are constituted the largest population of microbial flora and performed an important part of the biological degradation of vegetable fibers (Koike and Kobayashi 2009).

Rumen bacteria are a complex population according to their morphological and physiological characters (Krause and Russel 1996) and almost all of them are obligate anaerobes (Kamra 2005). Rumen bacteria could be isolated from both rumen (Kuhnert et al 2010) and stool (Ziemer 2014), and studied in pure cultures using anaerobic culture techniques. These studies have broadened of our knowledge of rumen microbial ecosystem.

Although many studies carried out until today, small proportion of bacteria could be isolated from rumen (Kobayashi 2006). However, acceleration of the metagenomics approach in the last 15 years has increased the information about rumen microbiome exponentially (Singh et al 2014). Rumen contents (Duan et al 2009) or feces of ruminants (Durso et al 2010) are the main source for rumen metagenomics studies. Besides the easy sampling, stool samples are important genetic and ecological resources for wild animals (Zhang et al 2006). Important information about microbial populations can be obtained from stool samples by metagenomic studies. Furthermore, it is also possible to isolate yet undiscovered genes. However, effects of drying in natural conditions are not clear for stool metagenome. In this study, the fresh feces and air-dried feces of a sheep were compared in terms of the metagenomic DNA quantity and specific primers belong to several rumen microorganisms.

Materials and Methods

Stool samples

Stool samples were taken from a 3-year-old female sheep after 14 days of forage based feeding. Fecal samples were divided into 9 groups. The first group was stored immediately at -20°C as fresh sample for further studies and the other eight groups allowed for dry under the sun and on the soil. A sample group was then stored at -20°C at 1 week interval for 8 weeks to use in subsequent studies. The degree of drying was monitored by weighing the fecal samples daily.

Table 1. Specific primers, sequences and amplicon sizes used in this study.

Microorganisms	Primer Sequence (5'-3')	Amplicon Size (bp)	Reference
Anaerovibrio lipolytica	F: TGGGTGTTAGAAATGGATTC R: CTCTCCTGCACTCAAGAATT	597	Tajima et al 2001
Fibrobacter succinegenes	F: GGTATGGGATGAGCTTGC R: GCCTGCCCCTGAACTATC	445	Tajima et al 2001, Koike and Kobayashi 2001
Prevotella bryantii	F: ACTGCAGCGCGAACTGTCAGA R: ACCTTACGGTGGCAGTGTCTC	540	Tajima et al 2001
Prevotella ruminicola	F: GGTTATCTTGAGTGAGTT R: CTGATGGCAACTAAAGAA	485	Tajima et al 2001
Ruminobacter amylophilus	F: CAACCAGTCGCATTCAGA R: CACTACTCATGGCAACAT	642	Tajima et al 2001
Ruminococcus albus	F: CCCTAAACAGTCTTAGTTCG R: CCTCCTTGCGGTTAGAACA	175	Koike and Kobayashi 2001
Ruminococcus flavefaciens	F: GGACGATAATGACGGTACTT R: GCAATCYGAACTGGGACAAT	835	Tajima et al 2001
Streptococcus bovis	F: CTAATACCGCATAACAGCAT R: AGAAACTTCCTATCTCTAGG	869	Tajima et al 2001
Succinovibrio dextrinosolvens	F: TGGGAAGCTACCTGATAGAG R: CCTTCAGAGAGGTTCTCACT	854	Tajima et al 2001
Selenomonas ruminantium	F: TGCTAATACCGAATGTTG R: TCCTGCACTCAAGAAAGA	513	Tajima et al 2001

Metagenomic DNA isolation

Two fecal pellets were used from each group. The outer surface of the fecal pellet was removed with a sterile scalpel, and 200 mg of interior stool sample was used for DNA isolation. Metagenomic DNA isolation was performed by using Stool DNA Isolation Kit (I-Genomic, South Korea) according to manufacturer's protocol. The concentration and purity of the isolated DNA was measured using NanoDrop 2000 spectrophotometry (Thermo Scientific, USA). All DNA isolations were performed in duplicate.

PCR with 16S rDNA and specific primers

A variety of inhibitors can be found in stool metagenomic DNA and can adversely affect PCR process. In order to test this situation, PCR was carried out with the 16S rDNA primers without diluting the metagenomic DNA samples. The 16S ribosomal DNA was amplified from the isolated metagenomic using the 27F: 5'-AGAGTTTGATYMTGGCTCAG-3' (Edwards et al 1989) and 1492R: 5'- GGTTACCTTGTTACGACTT-3' (Weisburg et al 1991) primers in the PCR. Ten rumen bacteria were screened with specific primers (Table 1) using the metagenomic DNA. PCR mixtures contained (per 40 µL) 1 µL of metagenomic DNA, 10 pmol of each primer, 250 µM deoxyribonucleoside triphosphates (Vivantis, Malaysia), 4 µL of 10X PCR buffer and 0.5 units of Taq DNA polymerase (Vivantis, Malaysia). Amplification was carried out in a Bio-Rad thermocycler using an initial denaturating step of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at the appropriate annealing temperature, 1 min at 72°C, and 1 cycle of 5 min at 72°C for final extension. PCR products were loaded and then visualized on 1% agarose (Sigma) gel. All PCR procedures were performed in duplicate.

Restriction analysis

The nucleotide sequences of 16S rDNA of 10 rumen bacteria were obtained from NCBI and restriction enzyme map was analyzed using Clone Manager 9 (Scientific & Educational Software, USA) program. The 16S rDNA PCR products were digested using SphI (New England Biolabs, 10 U) for overnight at 37°C according to manufacturer's protocol. The restriction products were visualized on 1% agarose gel.

Results

Metagenomic DNA isolations were performed from stool samples taken from sheep. Stool samples dried rapidly and 53.72% of the weight was lost in 24 h. In the following days, no change was observed in the fecal mass (Table 2). Metagenomic DNA isolations were performed from the interior parts of fresh and dried feces. Although fresh stool were easily suspended in lysis buffer, it was quite difficult to suspend the dried samples. However, this case showed no negative effects on DNA concentrations and purity (Table 2). DNA concentrations and purity was varied between 25.60-59.50 ng/µL and 1.72-1.90, respectively.

Amplification of 16S rDNA region was observed in both fresh and dried samples, and this result showed that the inhibitors were successfully eliminated during DNA isolation. Then PCR was carried out with specific rumen bacterial primers. PCR process did not reveal the presence of *R. albus, R. amylophilus,* and *S. dextrinosolvens*. By using the other primers, PCR products with the expected sizes were obtained (Figure 1). No PCR product was amplified after 7 and 8 week of drying for *S. ruminantium* and *S. bovis,* respectively, while the PCR products were obtained from all other bacteria until the end of drying period
(Table 3).

Specific primers of *R. amylophilus*, *R. albus* and *S. dextrinosolvens* amplified no PCR products and this result indicated that these three bacteria did not found in the stool. To confirm this state, restriction enzymes have been investigated to distinguish these bacteria from others. It was found that SphI digests the 16S rDNA regions of *R. amylophilus, R. albus* and *S. dexrinosolvens* from about 460, 1320 and 525. bp, respectively. However, there is no SphI site in 16S rDNA of the other bacteria. Therefore SphI restriction reaction was carried out and any digestion was observed with 16S rDNA (Figure 2). This result is consistent with the PCR results and restriction analysis confirmed the PCR analysis.

Discussion

Culture-based methods are used as intensively for the determination of microorganisms (Cotta et al 2003, Tewari et al 2013, de Aguiar et al 2014). The microbial diversity of an environmental sample can be also investigated using culture-independent techniques by analysing the 16S rDNA region of metagenomics DNA (Han et al 2015).

Metagenomic analysis of fecal samples is based on DNA, which is isolated directly from stool. Various DNA isolation methods from stool specimens were tested (Yu and Morrison 2004, McOrist et al 2002, Zhang et al 2006, Fliegerova et al 2014). Organic matter is the source of inhibitors (Yeates et al 1998) and inhibitors that may be co-extracted with DNA from fecal samples, effects the subsequent enzymatic reactions, such as PCR or restriction analysis (Monteiro et al 1997, Wilson 1997). In this study, sufficient concentration of DNA with high purity was obtained, and subsequent PCR and restriction analysis showed that inhibitors were removed from DNA. In the present study, inner parts of fecal samples were used for DNA extraction and good PCR results were obtained for microbial diversity. Wehausen et al (2004) compared the outer and inner fecal pellet parts, and they observed that PCR success for four loci of Bighorn Sheep de-

Table 2. Mass of air-dried fecal samples and concentrations and purity of DNA which is isolated from fecal samples.

Waiting time for weighing	Temperature (°C)	Fecal Mass (mg)	Waiting time for DNA isolation	Concentration (ng/µL)	Purity
Fresh feces	37	678.71	Fresh feces	25.60	1.73
1 Day	35	314.07	1 Week	39.30	1.83
2 Day	35	322.00	2 Week	34.60	1.80
3 Day	34	319.20	3 Week	59.50	1.90
4 Day	34	315.27	4 Week	49.50	1.81
5 Day	34	317.27	5 Week	34.30	1.72
6 Day	34	314.26	6 Week	38.40	1.85
7 Day	32	313.70	7 Week	49.40	1.83
8 Day	33	314.34	8 Week	29.90	1.80

clined when any inner fecal material was used and excellent results were obtained when the very outer layer was used. PCR is a method often used to determine the ecology of gastrointestinal microbiome (Belanche et al 2014). Primers that are designed for a specific genus or species are used in detecting microorganisms (Wang et al 1996). In this study, detection of ten different rumen bacteria in sheep feces was investigated using specific primers. *R. flavefaciens* and *F. succinogenes* were detected by using the specific primers, while *R. albus* was not detected in fecal DNA samples. *R. flavefaciens, F. succinogenes* and *R. albus* are known as the main fibrolytic species (Sirohi et al 2012), however cellulolytic bacteria vary depending on the degradable starch content in the rumen, and *R. flavefaciens, F. succinogenes* and *R. albus* are reduced by lower pH as a result of high concentrate diet (Li et al 2014).

Rumen and omasum were major habitats for these three cellulolytic species, and *F. succinogenes* was the most abundant of them (Koike and Kobayashi 2001). *F. succinogenes* and *R. flavefaciens* were also detected in the colon and rectum, whereas *R. albus* was not detected in the colon and rectum (Koike and Kobayashi 2001). This could be the reason of the negative PCR result for *R. albus* in stool metagenomic DNA in this study.

Positive PCR results were observed with *S. bovis* and *S. ruminantium,* and negative PCR results were obtained from *R. amylophilus* and *S. dextrinosolvens.* It is necessary to note that *S. bovis* primers could be cross-reacted with *S. equinus*, since these strains are very similar (Tajima et al 2001). It has been reported that the number of amylolytic bacteria such as *R. amylophilus, S. bovis* and *S. ruminantium* decreased in sheep fed on high forage diet (Jiao et al 2014). *S. ruminantium* and *S. dextrinosolvens* showed positive interactions with cellulolytic rumen bacteria in fiber degradation (Koike et al 2003). *P. bryantii* and *P. ruminicola* were detected in feces by using PCR during the test period, in this study. Prevotella species

Table 3. PCR results by using specific primers and DNA which is isolated from fresh and air-dried fecal samples*.

Microorganisms	Fresh feces	2nd week	3rd week	4th week	5th week	6th week	7th week	8th week
A.lipolytica	+	+	+	+	+	+	+	+
F. succinogenes	+	+	+	+	+	+	+	+
P. bryantii	+	+	+	+	+	+	+	+
P. ruminicola	+	+	+	+	+	+	+	+
R. amylophilus	-	-	-	-	-	-	-	-
R. albus	-	-	-	-	-	-	-	-
R. flavefaciens	+	+	+	+	+	+	+	+
S. bovis	+	+	+	+	+	+	+	-
S. dextrinosolvens	-	-	-	-	-	-	-	-
S. ruminantium	+	+	+	+	+	+	-	-

*(+) PCR products with expected size, (-) No PCR product.

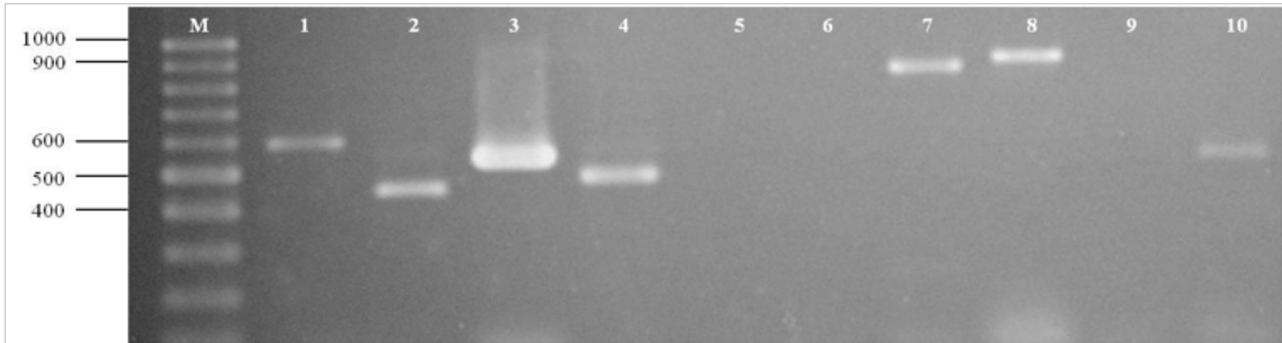

Figure 1. PCR products obtained from fresh fecal metagenomic DNA by using specific primers. (1) *A. lipolytica*, (2) *F. succinogenes,* (3) *P. bryantii,* (4) *P. ruminicola,* (5) R. *amylophilus,* (6) *R. albus,* (7) *R. flavefaciens,* (8) *S. bovis,* (9) *S. dextrinosolvens,* (10) *S. ruminantium.*

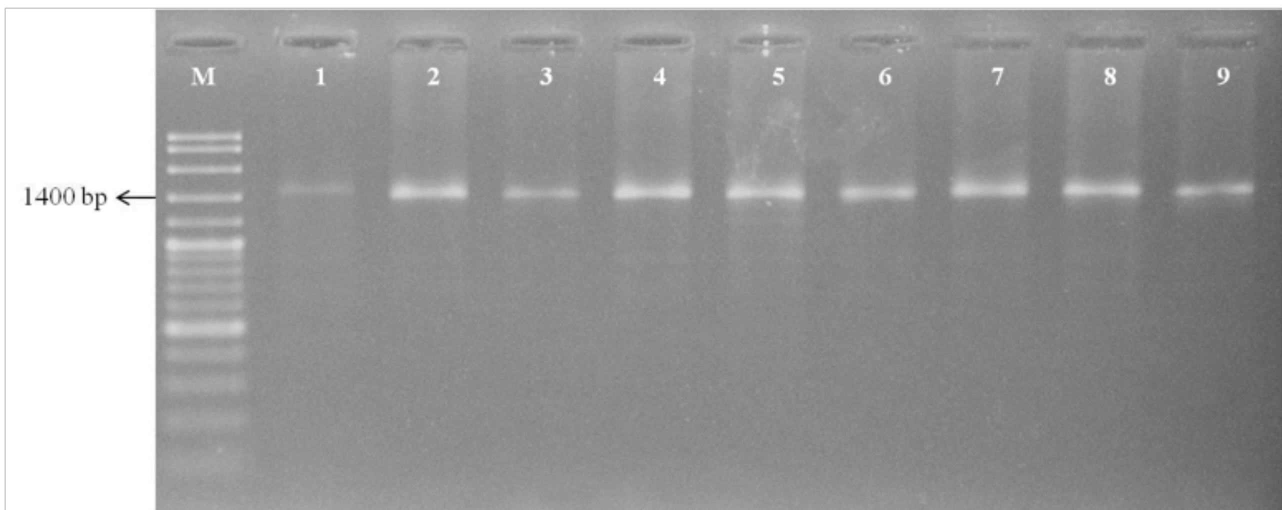

Figure 2. SphI digestion of 16S rDNA region. (1) 16S rDNA obtained from metagenomic DNA of fresh feces, (2-9) 16S rDNA region obtained from metagenomic DNA of dried feces from 1 week to 8 week. (M) 100 bp DNA ladder used as size marker (Vivantis, Malaysia).

are a major group of rumen and participate fiber breakdown possibly as oligosaccharide and xylan fermenters (Koike et al 2003). Prevotella/Bacteroides may account for 60-70% of ribosomal sequnce diversity in rumen samples (Ramsak et al 2000). The feeding regime of the ruminant animal results the presence of *A. lipolytica* in feces samples in the present study. *A. lipolytica* is sensitive to low pH and low pH reduces the number of *A. lipolytica* in the rumen (Gudla et al 2012). On the other hand, Tajima et al. (2001) found that *A. lipolytica* was not affected by diet since the variations in *A. lipolytica* DNA were not statistically significant.

Conclusion

There are important relationship between rumen micro-bial populations and feeding diet of ruminants. The use of metagenomics is increasing in determining the microbial populations of environmental samples. The microbiome of feces can give hints about the host and the most important advantage is the ease of sampling.

Feces from the animals, especially wild animals, can be sam-pled readily, and in this way fecal DNA can give important information about the animals. This study investigated the PCR detection of 10 rumen bacteria in feces and 7 bacteria were detected. Another objective of this study was to study the effects of drying of sheep feces in natural conditions on metagenomics DNA. In particular, fecal samples are of great help to study the microbial flora of the digestive systems of animals in wildlife. This study demonstrates that metagenomic DNA with a high purity can be obtained from air-dried stool and molecular detection of microorganisms can be carried out.

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