Korean Journal of Microbiology (2013) Vol. 49, No. 4, pp. 415-418
DOI http://dx.doi.org/10.7845/kjm.2013.3080

| 단 보 |

Toward The Fecal Microbiome Project

Tatsuya Unno

College of Molecular Life Sciences, Jeju National University, Jeju 690-756, Republic of Korea

분변 미생물군집 프로젝트

운노 타쯔야

제주대학교 생명자원과학대학 생명공학부

(Received November 25, 2013 / Accepted December 17, 2013)

Since the development of the next generation sequencing (NGS) technology, 16S rRNA gene sequencing has become a major tool for microbial community analysis. Recently, human microbiome project (HMP) has been completed to identify microbes associated with human health and diseases. HMP achieved characterization of several diseases caused by bacteria, especially the ones in human gut. While human intestinal bacteria have been well characterized, little have been studied about other animal intestinal bacteria. In this study, we surveyed diversity of livestock animal fecal microbiota and discuss importance of studying fecal microbiota. Here, we report the initiation of the fecal microbiome project in South Korea.

***Keywords*:** 16S rRNA, environment, fecal bacteria, microbiome, MiSeq

Since the development of next generation sequencing (NGS) technology, 16S rRNA gene sequencing has become a more preferred method to the culture-based microbe identification. Recently MiSeq (Illumina Inc.) has been upgraded to be capable of sequencing longer read length (250 × 2 bp) with outputs maximum 8.5 Gb, which is approximately 18 times more than that of the pyrosequencing device, GS FLX Titanium (450 Mb). Accordingly, MiSeq-based microbial community analysis protocol was developed recently (Kozich *et al.*, 2013), in which 386 samples were subjected to 16S rRNA gene sequencing for microbial community analysis. Until recently, most of the microbial ecology studies were reported based on pyrosequencing results, however, MiSeq now offers far more outputs and far lower cost of sequencing. In addition, new implementation on MiSeq sequencing also includes sequencing of the dual indexed PCR amplicons, reducing number of primer sets required for hundreds samples. For example, combinations of 16 indexes on forward primers and the same 24 indexes on reverse primers allow 384 (16 × 24) samples (Kozich *et al.*, 2013).

The human microbiome project (HMP) initiated by US National Institute of Health (NIH) discovered how microbes were related to our health such as diabetes (Cani *et al.*, 2008), cancer (Li *et al.*, 2012), and obesity (Greenblum *et al.*, 2012). Considering the use of pyrosequencing with low cost effectiveness and low multiplexing capability, unreasonable amount of time and cost have been spent to complete HMP. On the other hand, livestock microbiome has been less focused compared to human microbiome. Table 1 summarizes on-going studies related to fecal microbes.

As shown, not only human fecal microbes, but livestock fecal microbes has been applied in various research fields. Despite the importance of livestock fecal microbes, it has never been intensively studied. In this study, we surveyed diversity of livestock fecal microbiota using MiSeq. Here we demonstrate MiSeq-based highly multiplexed 16S rRNA gene sequencing and report the initiation of the fecal microbiome project in South Korea.

Fecal materials were obtained from pig, cow, dog, horse and chicken farms in Jeju and Gwangju, South Korea (Table 2). DNA was extracted using PowerFecal DNA isolation kit (MO BIO Laboratories Inc., USA). Obtained DNA was further PCR amplified and purified using gel extraction kit (Bioneer, Korea). Resulting DNA concentration was normalized and pooled prior to sequencing. MiSeq sequencing was carried out

*For correspondence. E-mail: tatsu@jejunu.ac.kr; Tel.: +82-64-754-3354; Fax: +82-64-756-3351

Table 1. Examples of fecal bacteria studies related to our life

Host animals	Study contents	References
Human	Cure to Crohn's disease	Floch (2010)
Human	Causes of diabetes	Serino *et al.* (2012)
Human	Causes of intestinal cancers	Fox *et al.* (2010)
Pig	Probiotics development	Ihara *et al.* (2013)
Pig	Growth promoter	Zhou *et al.* (2011)
Cow	Green House Gas emission	Bell *et al.* (2011)
Cow	Environmental pathogen dissemination	Edrington *et al.* (2009)
Livestock	Fecal pollution source tracking	Unno *et al.* (2010)
Livestock	Pharmaceutical pollution treatment	Topp *et al.* (2013)
Panda	Alternative energy cellulase	Fan *et al.* (2012)

by Macrogen (Macrogen, Korea) according to the manufacturers' instruction. Obtained sequence data was processed using free software, mothur (Schloss *et al.*, 2009). Table 2 summarizes the number of fecal samples and sequences obtained in this study.

Total 157 fecal materials are collected in this study. All samples were separately PCR-amplified, then pooled into one prior to MiSeq sequencing, which costs less than 5 million South Korean won. In addition, the number of reads obtained for each sample is not low. Results in Figure 1 show that nearly 10,000 reads were obtained for each sample at least. Although concentrations of PCR amplicons were normalized prior to pooling, resulting sequence number varied independently from types of source animals or locations. It may be qualities of the extracted DNA or the conditions of fecal samples used in this study. Further studies may clarify the problem, nonetheless we were able to obtain more than enough sequences for the downstream analyses. Besides, number of reads or the sequence depth is not critical factors especially for beta diversity analyses (Caporaso *et al.*, 2012).

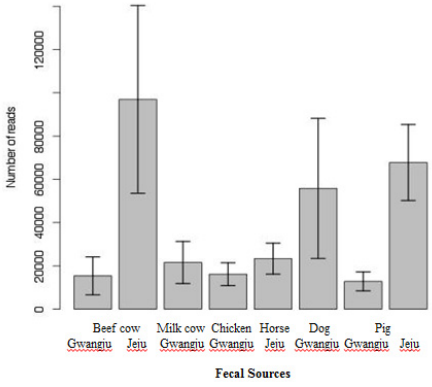

Fig. 1. Average number of sequences obtained for each livestock source animal in this study.

Table 2. Number of feces collected and MiSeq reads remained after trimmed with mothur

Host animal	Location	No. of samples	Total No. of reads
Beef cow	Gwangju	65	1,000,387
Beef cow	Jeju	26	2,520,918
Milk cow	Gwangju	14	301,843
Chicken	Gwangju	15	242,340
Dog	Gwangju	9	209,921
Horse	Jeju	5	279,046
Pig	Gwangju	17	217,817
Pig	Jeju	6	406,620
Total	NA*	157	5,178,892

* Not applicable

With the remained trimmed sequences, we have conducted cluster analyses. Since number of feces obtained for horse was too small (5), horse fecal samples were eliminated and the rest were clustered. As suggested previously, we have reduced sequence depth down to 1,000 to reduce computational burden (Caporaso *et al.*, 2012). Distance 0.03 operational taxonomic units (OTUs) were used to obtain the Yue & Clayton theta similarity coefficient.

Figure 2 shows the non-metric multidimensional scaling (NMDS) analysis. While beef cow fecal microbiota and milk cow microbiota are clustered together, results in Fig. 2 show that fecal microbiota is specific to types of source animals. Analysis of molecular variance (AMOVA) also showed significant separation among source animals (Fs=14.2907, P<0.001). In addition, source specific difference can be also confirmed in taxonomic bacterial compositions at the phylum level (Fig. 3).

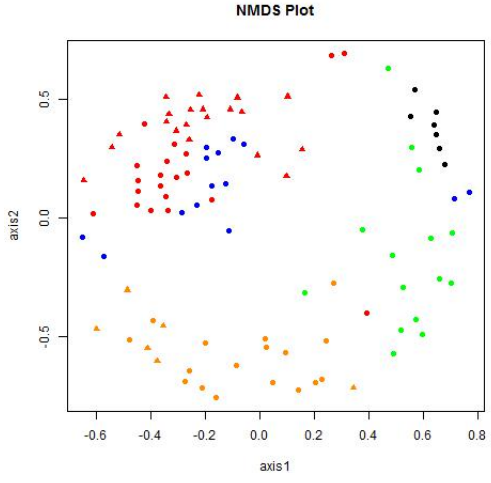

Fig. 2. NMDS analysis based on OTUs at distance 0.03: red, beef cow; blue, milk cow; green, chicken; black, dog; orange, pig; closed circle, Gwangju; and closed triangle, Jeju.

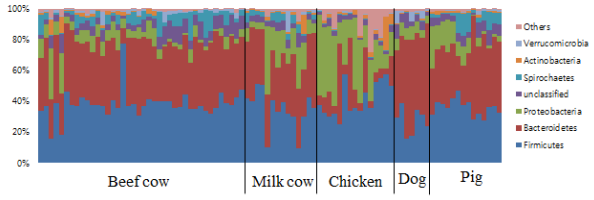

Fig. 3. Relative abundance of fecal bacteria in livestock animals.

When compared to other source of animals, statistically more abundant OTUs were selected using Metastats (Foster, 2003) and summarized in Table 3.

Higher number of abundant OTUs indicates high source-specificity of the fecal microbiota. This differential abundance test may help us to further study about the bacteria with source specific functions. In addition, the existence of those that represents type of source animals may lead better environmental pollution monitoring (i.e., microbial source tracking). Fecal microbes have been applied to several research areas including medicine development, alternative energy production, and environmental protection. The potential use of fecal microbes may have not been investigated enough. Now that microbial communities of a few hundreds samples can be analyzed with one MiSeq run, investigating livestock fecal microbiota further would lead us to new understanding of animals. Our results showed significant differences in fecal microbiota between types of source animals, suggesting that there are more diverse fecal bacteria species than found. Construction of fecal microbiome database, so called “Fecal Microbiome Project”, may bring us some more ideas for applications of fecal microbes in various research areas.

적 요

차세대 염기서열 분석(next generation sequencing, NGS) 기술의 발전으로 16S rRNA의 염기서열 분석이 미생물 군집 분석의 주된 방법으로 사용되고 있다. 인간의 건강과 질병에 관여하는 미생물들을 밝혀내기 위한 인간 미생물군집 프로젝트(human microbiome project, HMP)가 최근에 완료되었다. HMP는 세균에 의해 발생하는 여러 질병들의 특성들을 밝혀내었고, 특히 장에 서식하는 세균들에 대해 많은 연구가 수행되었다. 비록 인간의 장내 세균들에 대한 연구는 잘 수행되어왔지만, 다른 가축의 장내 세균에 대한 연구는 거의 이루어지지 않았다. 본 연구에서는 가축의 분변 미생물 다양성에 관해 조사하였고, 분변미생물 생태연구의 중요성을 제시 할 것이다. 한국에서의 분변 미생물 군집 프로젝트(fecal microbiome project) 시작을 본 연구논문을 통해 보고하고자 한다.

Table 3. Differential abundance test results

Source animals	Number of statistically abundant OTUs when compared to			
	Cow	Chicken	Dog	Pig
Cow	NA*	318	205	287
Chicken	275	NA	38	94
Dog	208	143	NA	167
Pig	440	247	155	NA

*NA, Not available

Acknowledgements

This research was supported by the 2013 scientific promotion program funded by Jeju National University.

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