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Brief Note

Molecular detection by analysis of the 16S rRNA gene of fecal coliform bacteria from the two Korean *Apodemus* species (*Apodemus agrarius* and *A. peninsulae*)

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ABSTRACT. Wild mouse feces can disseminate zoonotic microorganisms throughout a farm, which is a great threat to human health and can lead to economic loss through contaminated agricultural produce. To assess the microbial communities, especially fecal coliform

bacteria, we used two methods. First, we isolated bacterial colonies onto the common media LB (lactose broth) agar, TSA (tryptic soy agar), and MRS (de Man, Rogosa, and Sharpe) agar, and then randomly select colonies from each plate and stocked them to the mother plate for genomic DNA isolation. Second, we analyzed bacterial colonies using the 16S rRNA gene molecular diagnostic method. Based on bacterial cultures and bacterial 16S rRNA gene markers, we detected four different bacterial species (*Bacillus amyloliquefaciens*, *Escherichia coli*, *Staphylococcus xylosus*, and *Serratia liquefaciens*) from fecal coliforms of the striped field mouse *Apodemus agrarius* and *A. peninsulae* in agricultural areas in South Korea. These results could help us to better understand the pathogen reservoirs of mice and initiate some preventive measures to mitigate the microbial risks associated with mouse fecal matter in agricultural production areas.

Key words: Striped field mouse; *Apodemus agrarius*; *Apodemus peninsulae*; Pathogenic bacteria; Fecal coliform

INTRODUCTION

Wild mice often live in close proximity to humans and livestock. These small mammals are competent reservoirs of a number of zoonotic diseases responsible for significant economic losses and public health hazards (Dabritz et al., 2008; Meerburg et al., 2009). Wild mice maintain pathogen transmission cycles in a number of different environments such as cultured areas and the wilderness (Meerburg et al., 2009). They can transfer pathogens via direct contamination (i.e. physical contact) or indirect contamination (i.e. feces). Thus, the early detection of pathogenic bacteria in wildlife is very important as a preventative step for the public health of farm workers.

Molecular markers are one of the molecular tools for the identification of various taxa from bacteria to mammals (Kim et al., 2012) and will also be useful for detecting pathogenic bacteria from the fecal matter of wild animals. The bacterial 16S rRNA gene has been used to detect bacterial pathogens from various environmental sources such as wildlife feces (Maciel et al., 2011), drinking water (Maheux et al., 2014; Zhang et al., 2015), agricultural vegetables (Gorski et al., 2011), and soil (Gorski et al., 2011). In South Korea, however, there have been few studies detecting pathogenic bacteria from fecal coliforms of wild mice using molecular markers such as the 16S rRNA gene. The objective of the present study is to detect pathogenic bacteria from bacterial colonies collected from fecal coliforms of wild mice using the bacterial 16S rRNA gene.

MATERIAL AND METHODS

Sample collection

We captured eight individuals of two species (*Apodemus agrarius* and *A. peninsulae*) of wild mice using a Sherman trap in agricultural areas near Odaesan National Park, South Korea in May 2016. Each individual mouse was moved into disposable vinyl zipper bags,

and then released after collecting their feces in the bags. The fecal samples were brought to the laboratory in ice boxes, where they were processed within 3 h. The sample collection was conducted with the permission and following the guidelines of local government.

Culture of fecal coliform

The fecal samples (0.1-1 g) were suspended in 9 mL of distilled water and mixed vigorously to produce a uniform suspension. Next, the fecal samples were serially diluted up to 10^8 and then 0.1 mL of aliquots were spread on nutrient agar plates of MRS (de Man, Rogosa, and Sharpe), LB (lactose broth), and TSA (tryptic soy agar), resulting in a total of 24 plates that consist of three kinds of media plates (Table 1). The plates were incubated at 37°C for 24 h. Bacteria were cultured following the procedure of Jolt et al. (1994) with a little modification.

For the preparation of the stock plate, a total of 52 colonies were picked out randomly from the three incubated plates per fecal sample of every individual. Thus, eight stock plates with the 52 colonies were prepared. For genomic DNA isolation, six colonies were randomly selected from every stock plate (a total of 48 colonies out of 416 colonies from eight individual fecal samples; Table 1). With the sterilized toothpick, the selected colonies were transferred to 5 mL LB and incubated for at 37°C for 18 h. Then they were maintained at -80°C.

Genomic DNA extraction, PCR, and sequencing

The 48 LB-cultured colonies (Table 1) were used for genomic DNA isolation with Qiagen DNeasy Blood and Tissue kit (Quagen, Valencia) according to the manufacturer's protocol. The universal 16S rRNA primer set for polymerase chain reaction (PCR) amplification was as follows: 27f (5'-GAGTTTGATYMTGGCTCAG-3' (Ludwig et al., 1993) and 1390r (5'-ACGGGCGGTGTGTRCAA-3') (Olsen et al., 1986). PCR amplification was performed in a final 25 μL reaction volume containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 4 mM $MgCl_2$, 200 mM each dNTP, 50 pmol each primer, 2 U ExTaq polymerase, and 1 μL of genomic DNA. The PCR was conducted under the following reaction conditions: an initial denaturation for 5 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and extension for 1 min at 72°C, and then a final extension of 10 min at 72ºC. The PCR products were run by electrophoresis with a 1.0% agarose gel and purified using a DNA gel extraction kit (Qiagen, Valencia, CA, USA). The purified PCR products were sent to Biomedic Co., Ltd. (Bucheon, South Korea) for sequencing.

The obtained bacteria 16S rRNA gene sequences were compared with other homologous sequences deposited in GenBank using BLASTN2.2.31+. Phylogenetic relationship was inferred using maximum-likelihood (ML) and neighbor-joining (NJ) analyses implemented in MEGA 7.0.14. The confidence of branches in ML trees was assessed using bootstrapping searches of 1000 replicates. A NJ tree was inferred using Kimura's two-parameter model, with bootstrapping searches of 1000 replicates.

RESULTS AND DISCUSSION

The number of colonies was similar among the three media, TSA, LB, and MRS, of each individual fecal sample. The average aerobic plate count (APC) colony numbers range

Table 1. Information on sample preparation, dilution series, bacterial stock per fecal sample, bacterial 16S rRNA gene sequenced colonies, and the detected pathogenic bacteria.

Species and individuals	Fecal sample ID	Dilution series	No. of bacterial colonies (CFUs) in three plates from each fecal sample				No. of colonies in each stock plate[a]	No. of LB cultures of the six colonies selected randomly from the 52 colonies on the stock plate for genomic DNA isolation	No. of 16S rRNA gene sequences obtained from PCR amplification of the genomic DNA	GenBank accession No.	Size (bp)	Similarity test in GenBank		Identification of bacteria from GenBank by the 16S rRNA gene sequences
			TSA	LB	MRS	Average No. (CFU/g)						Coverage (%)	Identity (%)	
Apodemus peninsuale	J1_M1_1	$1:10^{-5}$	125	112	110	115.7 (±8.1) x 10^{6}	52	6	1	KY047617	706	100	100	*Bacillus* sp
A. peninsuale	J2_M1_2	$1:10^{-9}$	1	2	1	1.3 (±0.6) x 10^{10}	52	6	1	KY047618	640	100	99	*Bacillus amyloliquefaciens* DSM7 (T)
A. peninsuale	J3_M1_6	$1:10^{-8}$	55	50	52	52.3 (±2.5) x 10^{9}	52	6	1	KY458798	644	100	100	*Bacillus amyloliquefaciens* SVN01
A.agrarius	M2_1	$1:10^{-4}$	101	100	95	98.7 (±3.2) x 10^{5}	52	6	1	KY458799	629	100	100	*Serratia* sp. K20-49
A. agrarius	M2_2	$1:10^{-10}$	1	2	1	1.3 (±0.6) x 10^{11}	52	6	1	KY458801	912	100	99	*Serratia* sp. UIWRF1065
A. agrarius	M2_3	$1:10^{-9}$	4	3	4	3.7 (±0.6) x 10^{10}	52	6	3	KY047619	770	100	99	*Staphylococcus xylosus* ATCC 29971(T)
										KY458800	1066	100	100	*Serratia liquefaciens* LZ-24
										KY458802	1068	100	100	*Escherichia coli* M9
A. agrarius	M2_4	$1:10^{-5}$	5	4	6	5 (±1.0) x 10^{6}	52	6	1	KY047620	683	100	99	*Serratia liquefaciens* ATCC 27592(T)
A. agrarius	M2_5	$1:10^{-8}$	19	17	15	17.0 (±2.0) x 10^{9}	52	6	3	KY458803	636	100	100	*Escherichia coli* TSA-3
										KY458804	693	100	99	*Serratia liquefaciens* ATCC 27592(T)
										KY458805	716	99	100	*Bacillus* sp. OSM29

[a]The stock plate contains 52 colonies that were selected randomly from TSA, LB, and MRS medium. - Media used in this study are TSA, LB, and MRS. - The numbers in parentheses in the average (CFU/g) indicate standard deviation. - There was no sequence difference among the sequences of the six colonies from same fecal samples. Thus, only single representative sequence of each bacterial strain was submitted to GenBank.

from 5 (SD = ±1.0, n = 3) x 10^5 colony-forming units (CFU)/g in the fecal coliform of *A. agrarius* (M2_4) to 1.3 (± 0.6, n = 3) x 10^{11} CFU/g in the fecal coliform of *A. agrarius* (M_2) (Table 1).

In 12 of 48 colonies, we collected 16S rRNA gene sequences of 629-1068 bp in length (Table 1). To identify them, the sequences were compared for similarity with those of bacteria deposited in GenBank, using the NCBI BLAST available at http://www.ncbi.nlm.nih.gov/. The bacterial colonies were identified by 99~100% identity of 100% sequence coverage in the sequence similarity comparison as the following species: *Bacillus* sp (= KY047617), *Bacillus* sp OSM29 (= KY458805), *B. amyloliquefaciens* DSM7 (T) (= KY047618), *B. amyloliquefaciens* SVN01 (= KY458798), *Staphylococcus xylosus* ATCC 29971(T) (= KY047619), *Serratia liquefaciens* ATCC 27592 (T) (= KY047620), *S. liquefaciens* LZ-24(= KY458800), *Serratia* sp K20-49 (= KY458799), *Serratia* sp UIWRF1065 (= KY458801) and *Escherichia coli* TSA3 (= KY458803), and *E. coli* M9 (= KY458802) (Table 1).

In the four bacteria species identified (*B. amyloliquefaciens*, *S. xylosus*, *S. liquefaciens*, and *E.coli*), *B. amyloliquefaciens* was detected from fecal pellets of *A. peninsulae*, while the other three bacterial species were identified from those of *A. agrarius* (Table 1).

Phylogenetic analysis revealed, with the exclusion of the genus *Bacillus* group, all species were well placed within their own genera (Figure 1). The group of the genus *Bacillus* were separated into two different clades. The two unidentified Korean *Bacillus* sp. were placed in the clade that includes the pathogenic *B. anthracis, B. cereus*, and *B. thuringiensis*. In the others, with the exclusion of *E. coli*, serious pathogenic bacteria were not detected from mouse fecal pellets (Figure 1).

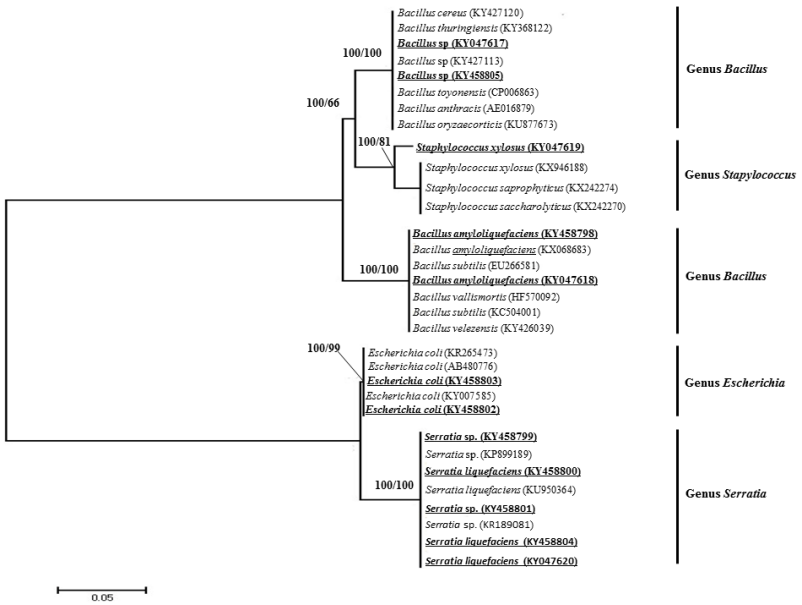

Figure 1. Phylogenetic relationship of fecal coliform bacteria detected from the two Korean *Apodemus* species (*A. agrarius* and *A. peninsulae*) based on the 16S rRNA gene sequences. Underlined species indicate fecal coliform bacteria from the Korean *Apodemus*. The numbers above branches indicate ML and NJ bootstrap values, respectively.

As part of the extended research of the wide agricultural area, in the present study we reported the bacterial data obtained from only a few individuals of two Korean wild mouse species. Thus, a larger collection of wild mice including other mouse species will provide information for the dynamics of the microbial community as well as screening of more microbial diversity. These results could help us to better understand the pathogen reservoirs of mice and initiate some preventive measures to mitigate the microbial risks associated with mouse fecal matter in agricultural production areas.

Conflicts of interest

The authors declare no conflict of interest.

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