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Genotyping of avian pathogenic *Escherichia coli* by DNA fragment analysis for the differences in simple sequence repeats

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Abstract

Avian pathogenic *E. coli* (APEC) causes severe economic losses in the poultry farms, due to systemic infections leading to lethal colisepticemia. It causes a variety of diseases from air sac infection to systemic spread leading to septicemia. Secondary infection contains opportunistic infections due to immunosuppression disease. Collibacillosis causes the great problems in the poultry industry in Korea. Thus, it is necessary to identify and classify the characteristics of *E. coli* isolate of chicken origin to confirm the diversity of symptoms and whether they are transmitted among the farms. Fragment analysis is identify the difference in the number of Variable-Number Tandem-Repeats (VNTRs) for genotyping. VNTRs have repeating structure (Microsatellite, Short tandem repeats; STR, Simple sequence repeats; SSR) in the chromosome. This region can be used as a genetic marker because of its high mutation rate. And various lengths of the amplified DNA fragment cause the difference in the number of repetition of the DNA specific site. The number of repetition sequences indicates the separated size of fragments, so the each fragments can be distinguished by specific samples. The results of the sample show that there is no difference in six microsatellite loci (*yjiD*, *aidB*, *molR_1*, *ftsZ*, *b1668*, *yibA*). There are differences among the farms in relation of the number of repetitions of other six microsatellite loci (*ycgW*, *yaiN*, *yiaB*, *mhpR*, *b0829*, *caiF*). Four (*ycgW*, *yiaB*, *b0829*, *caiF*) of these six microsatellite loci show statistically significant differences ($P < 0.05$). It means that the analysis using four microsatellite loci including ycgW, yiaB, b0829, and caiF can confirm among the farms. Five *E. coli* samples in one farm have same SSR repetition at all markers. But, there are significant differences from other farms at Four (*ycgW*, *yiaB*, *b0829*, *caiF*) microsatellite loci. These results emphasize again that the four microsatellite loci makes a difference in the amplified DNA fragments, enabling it to be used for *E. coli* genotyping.

Key words : Avian *pathogenic E. coli*, Simple sequence repeats, DNA fragment analysis, Sequencing, Genotyping

INTRODUCTION

Escherichia coli are Gram-negative bacteria consisting with numerous strains and serotypes that present in the normal microflora of the Avian gastrointestinal tract (Delicato et al, 2003).

Avian pathogenic *E. coli* (APEC) causes severe economic losses on farms, through the both primary and secondary infections (Lutful Kabir et al, 2010). It causes a variety of diseases such as air sac infection, omphalitis, respiratory tract infection, swollen head syndrome, septicemia, polyserositis, coligranuloma, enteritis, cellulitis and salpingitis (Calnek et al, 1997). Secondary infection contains opportunistic infections due to immunosuppression after the infection with chicken anemia virus (CAV), infectious bronchitis virus (IBV), and in-

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fectious bursal disease virus (IBDV) outbreaks (Kemmett et al, 2013).

Colibacillosis causes economic problems in the poultry industry in Korea. Thus, it is necessary to identify and classify the characteristics of *E. coli* isolated from chickens with colibacillosis to confirm the diversity of symptoms and whether they are transmitted among the farms (Oh et al, 2011).

In this study, we use the fragment analysis to identify the difference in the number of Variable-Number Tandem-Repeats (VNTRs) for genotyping. VNTRs have repeating structure (Microsatellite, Short tandem repeats; STR, Simple sequence repeats; SSR) in the chromosome. This region can be used as a genetic marker because of its high mutation rate. And various lengths of the amplified DNA fragment cause the difference in the number of repetition of the DNA specific site. The number of repetition sequences indicates the separated size of fragments, so the each fragments can be distinguished by specific samples (Zhou et al, 2013).

This study was conducted to identify the diversity of short tandem repeats occurred in APEC and to assess whether the genetic diversity can be applied for usage in the *E. coli* genotyping.

MATERIALS AND METHODS

APEC (Avian pathogenic *E. coli*) sampling

All bacteria used for this study were isolated from chickens with colibacillosis. The swap samples obtained from the infected tissues showing fibrinous polyserositis and the swabs were subjected to APEC isolation procedure with MacConkey agar cultivation at 37°C. We analysed the samples that were classified as the farm unit. Laboratory *E. coli* strains, such as W3110 and DH5αF', were obtained from the Korea Collection for Type Cultures.

Table 1. A set of radioactive primers for twelve Short tandem repeats amplification region

Locus	Simple Sequence Repeats region	Radioactive primers (5' to 3')	Amplicon size (bp)
ycgW	G	**VIC**-GATTTTGCATATGAGTATATTAC TTAATTACAGGATGTTCAGTC	200～204
yaiN	C	**6'FAM**-AATTTATCCGGTGAATGTGGT CAACTTAATCTCGGGCTGAC	193～195
YjiD	T	**VIC**-TACATGGCTGATTATGCGG TCGCTATGAATATCTACTGAC	184
aidB	GT	**6'FAM**-GTCAGAGCAGATCCAGAATG TCTACAGCAAATGAACAATG	168
molR_1	TC	**NED**-GGTCATCAGGTGAAATAATC CGTCCTGATAGATAAAGTGTC	183
ftsZ	CGG	**6'FAM**-CAATGGAACTTACCAATGAC TACCGCGAAGAATTCAACAC	114
b1668	GGT	**6'FAM**-AGCATCAGCGCACAATGCAC TGTATGCAGGCTGGCACAAC	143
yiaB	ATTA	**6'FAM**-ATAACGATCTCCATATCTAC CTCTATCAGCAACTTCTGCC	200～202
mhpR	GC	**NED**-AATCACCCGTTGTTCACT CGGAACAAGACCGCAAGGA	201
b0829	C	**PET**-ACCGCAACATCCTTACAC TGACAAGATTACGCACTC	188～192
yibA	A	**VIC**-AATCGGACTTTCCTACAGA AACTCACGCTATGAACGC	214
caiF	T	**6'FAM**-TGAATGCCGATGCGACTG GTATGCAACTTCACCGTC	231～232

Preparation DNA and polymerase chain reaction (PCR)

Five colonies were collected and dissolved in sterile saline to be used for DNA/RNA extraction. To obtain results of the same conditions, DNA/RNA was extracted with the viral DNA/RNA Extraction kit (INTRON, KOREA) according to the manual method. The extracted DNA/RNA were stored in a −20℃ freezer until use.

A set of radioactive primers for the amplification region of DNA include the twelve SSR of E.coli (Table 1) and primers were synthesized at BIONEER.

2～3 μL of DNA extract (～50 ng), 5 μL of primer mix (Table 2), 10 μL of 2X MasterMultiMAXPCR solution (INTRON, KOREA) and the distilled water were added into the tubes to make total volume 20 μL. The cyclic conditions for PCR consisted of denaturation at 95℃ for 5 min, followed by 5 cycles (1 min at 95℃, 1 min at 55℃, and 1 min at 72℃), 20 cycles (1 min at 95℃, 1 min 50℃, and 1 min at 72℃), a final step of 7 min at 72℃, and cooling to room temperature (Gur-Arie et al, 2000).

DNA fragment analysis

Labeling of DNA fragments with fluorescent dyes were achieved by using polymerase chain reaction (PCR). Multiple different colored fluorescent dyes can be detected in one sample. One of the dye colors was used for a labeled size standard present in each sample. The size standard (GeneScan™ 500 LIZ® Size Standard, Life Technology, USA) was used to extrapolate the base-pair sizes of the sample product peaks.

Ten μL diluted size standard (add size standard 20 μL into 1 mL HI-DI formide) added to final PCR products (3～5 ng/μL). Those samples were injected at AB3500XL (Applied Biosystems 3500 XL Genetic Analyzer, Life Technology, USA).

Table 2. PCR primer mix combination

Primer mix 1		Primer mix 2	
Locus	100 pmol Primer (μL)	Locus	100 pmol Primer (μL)
ftsZ	1.5	aidB	1.5
b1668	1	molR	1
yjiD	1.5	ycgW	1
b0829	1	mhpR	1.5
yaiN	1	yibA	1.5
yiaB	1.5	caiF	1.5

※Add 90 μL Rnase free water to the mixing primer.

Sequencing

PCR was performed by using a PCR PreMix Kit (Maxime PCR PreMix Kit, INTRON, KOREA) with DNA template (~50 ng) and each 12 pairs of non-dying labeled primers. PCR final products ran on AB3500XL for sequencing after pre-treatment of cleaning (ExoSAP-IT™ kit, Life Technology, USA) dye labeling (BigDye®-Terminator v3.1 kit, Life Technology, USA), and purification (Bigdye® XTerminator purification kit, Life Technology, USA).

Data Analysis

Panel of test DNA samples ensure that expected repetitions are detected for each marker. Each panel made by comparing the number of repetition of the sequencing results with the position of DNA fragment analysis peak (DNA fragment size).

The data of results were analysed by software (GeneMapper®, life technologies) that include panel.

Each sample includes size standard to create a standard curve. Each dye-labeled fragment indicated the peak in software. The peak represent relative size of fragment in the sample comparing with the standard curve. E.coli was classified as the results of the analyzed data in GeneMapper.

Statistical analyses

Data are presented as grouping of the number of repetition. They were subjected to statistical analysis by dividing the 17 farms including 2 standard *E. coli*. Data were analyzed using one-way analysis of variance test (IBM SPSS software, USA, version 21). Differences between farms were considered significant at $P<0.05$.

RESULTS

Validation and establishment of analytical methods

Primers were divided into two groups to avoid interference and overlap of product sizes (Table 2). And, multiple experiments were carried out to identify six distinct bands in the electrophoresis (Fig. 1).

The number of SSR was checked in sequencing data to find the correlation among the SSR Panels formed to detect a valid peak positions. Peak appears in the Gene mapper and a panel was formed on that place (Fig. 2) and those were adjusted by repeated experiments of various samples. When the number of repeats and the position of peaks were same tendency, they were marked as the number of repeats sequence (Table 3) at panels of genemapper.

Diversity of twelve SSR loci in APEC isolates

In all 30 samples containing laboratory (non-pathogenic) *E. coli* strains DH5αF'and W3110, 6 out of 12 selected SSR loci showed the same number of repeats. The number of six loci was 9 in yjiD (T), 4.5 in aidB (CT), 4.5 in molR_1 (TC), 5 in ftsZ (CGG), 4 in b1668 (GGT), 9 in yibA (A).

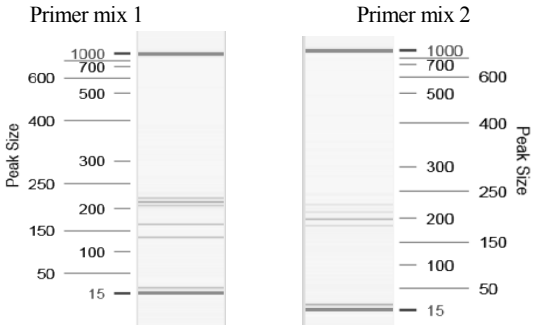

Fig. 1. Result of five distinct bands in the electrophoresis.

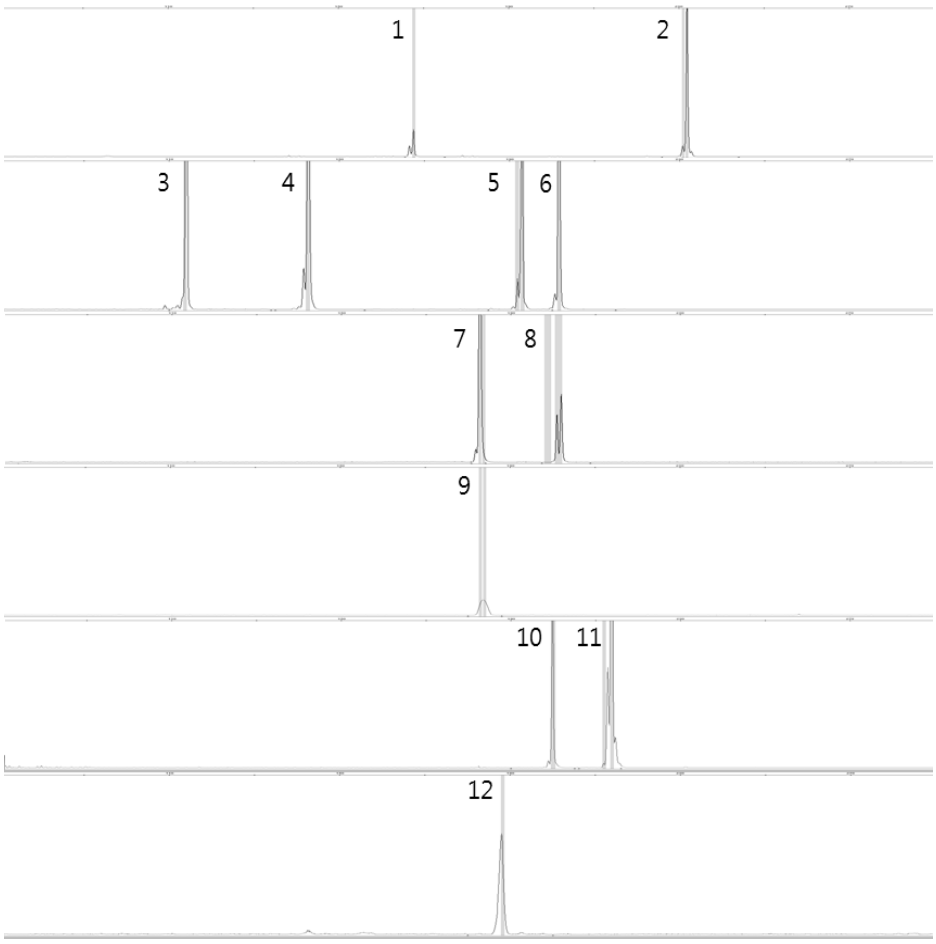

Fig. 2. The position of peaks appear in the Gene mapper and a panel was formed on each place. Each peaks represents aidB (1), caiF (2), ftsZ (3), b1668 (4), yaiN (5), yiaB (6), molR (7), mhpR(8), yjiD (9) ycgW (10), yibA (11) and b0829 (12).

Table 3. The position of peaks and the number of repeats at twelve loci

Sample	ycgW	yaiN	yjiD	aidB	molR_1	ftsZ	b1668	yiaB	mhpR	b0829	yibA	caiF
	G	C	T	CT	TC	CGG	GGT	ATTA	GC	C	A	T
DH5aF'	204	195	184	168	183	114	143	202	201	188	214	232
Marker	8	10	9	4.5	4.5	5	4	3	6	8	9	9

*Marker : the repeats number of SSR.

Table 4. E.coli diversity of SSR loci in two standard stains and sixteen Farm samples

Sample	ycgW	yaiN	YjiD	aidB	molR_1	ftsZ	b1668	yiaB	mhpR	b0829	yibA	caiF
	G	C	T	CT	TC	CGG	GGT	ATTA	GC	C	A	T
DH5αF'	8	10	9	4.5	4.5	5	4	3	6	8	9	9
W3110	8	10	9	4.5	4.5	5	4	3	6	8	9	9
1	4	8	9	4.5	4.5	5	4	3	N/A	8	9	N/A
2	4	8	9	4.5	4.5	5	4	3	6	8	9	8
3	4	8	9	4.5	4.5	5	4	3	6	8	9	9
4	4	8	9	4.5	4.5	5	4	3	6	8	9	8
	4	8	9	4.5	4.5	5	4	3	6	8	9	8
	4	8	9	4.5	4.5	5	4	3	6	8	9	8
	4	8	9	4.5	4.5	5	4	3	6	8	9	8
5	8	8	9	4.5	4.5	5	4	3	N/A	8	9	9
6	8	8	9	4.5	4.5	5	4	3	N/A	8	9	N/A
7	4	8	9	4.5	4.5	5	4	3	6	8	9	8
8	4	8	9	4.5	4.5	5	4	3	6	8	9	8
9	**4**	8	9	4.5	4.5	5	4	**2.5**	6	**12**	9	**8**
	4	8	9	4.5	4.5	5	4	**2.5**	6	**12**	9	**8**
	4	8	9	4.5	4.5	5	4	**2.5**	6	**12**	9	**8**
	4	8	9	4.5	4.5	5	4	**2.5**	6	**12**	9	**8**
	4	8	9	4.5	4.5	5	4	**2.5**	6	**12**	9	**8**
10	4	8	9	4.5	4.5	5	4	3	6	8	9	8
11	4	8	9	4.5	4.5	5	4	3	6	8	9	9
	4	8	9	4.5	4.5	5	4	3	6	8	9	9
	8	**10**	9	4.5	4.5	5	4	3	6	8	9	9
	4	8	9	4.5	4.5	5	4	3	6	8	9	9
12	4	8	9	4.5	4.5	5	4	3	6	8	9	8
13	4	8	9	4.5	4.5	5	4	3	6	8	9	8
	4	**10**	9	4.5	4.5	5	4	3	6	8	9	8
	4	8	9	4.5	4.5	5	4	3	6	8	9	8
14	N/A	8	9	4.5	4.5	5	4	3	N/A	8	9	8
15	4	8	9	4.5	4.5	5	4	3	6	8	9	8
16	4	8	9	4.5	4.5	5	4	3	6	8	9	8
	4	8	9	4.5	4.5	5	4	3	6	8	9	8
	4	**10**	9	4.5	4.5	5	4	3	6	8	9	8

Three out of 12 selected SSR loci didn't show peak in some samples. There were strains which didn't show any peaks at ycgW (G), mhpR (GC) and caiF (T). The number of non-peak samples was 1 in ycgW, 4 in mhpR, 2 in caiF. The samples that did not show peak in caiF did not show peak in mhpR either.

Five out of 12 selected SSR loci presented two repeat types. The number of repetitions was 4 or 8 in ycgW, 8 or 10 in yaiN, 3 or 2.5 in yiaB, 8 or 12 in b0829 and 8 or 9 in caiF.

Comparing the difference of the five SSR of *E. coli*, The data were highly significant in ycgW, yiaB, b0829, caiF as confirmed by one-way analyses of variance ($P<0.05$). but, yaiN was $P>0.05$ and there was no stat-

istical significance among groups.

DISCUSSION

In chicken farms, colibacillosis causes severe economic losses due to systemic infection, leading to colisepticemia. Genotyping of APEC isolates can be used to identify the clonal lineage of APEC among the chicken origin isolates of *E. coli*. The purpose of this study is to establish genotypes of *E. coli* isolated from chickens with colibacillosis by using amplified DNA fragment analysis in the bacterial microsatellite loci.

For the establishment of the experimental methods, gradient PCR were performed with the twelve primers to find the appropriate annealing temperature 57°C. The primers were grouped so that the PCR product size did not overlap, and the volume of primers was controlled so that similar amount of PCR amplification product that appeared electrophoresis peaks size could be obtained. The similar amount of PCR products can block 1～2 overpeaks in fragment analysis by the same dilution. Experiment was validated by confirm the consistency of peak positions.

All samples were studied after the establishment and validation of the experimental methods.

The results of the sample show that there is no difference in six microsatellite loci (*yjiD*, *aidB*, *molR_1*, *ftsZ*, *b1668*, *yibA*). There are differences among the farms in relation of the number of repetitions of other six microsatellite loci (*ycgW*, *yaiN*, *yiaB*, *mhpR*, *b0829*, *caiF*). Four (*ycgW*, *yiaB*, *b0829*, *caiF*) of these six microsatellite loci show statistically significant differences ($P<0.05$). It means that the analysis using four microsatellite loci including ycgW, yiaB, b0829, and caiF can confirm the difference among farms.

On the 9th farm (Table 4), five *E. coli* samples have same SSR repetitions at all markers. but, there is a significant difference from other farms at four (*ycgW*, *yiaB*, *b0829*, *caiF*) microsatellite loci ($P<0.05$). These results emphasize again that the four microsatellite loci enable the *E. coli* genotyping.

Further analysis of general and pathogenic standard strains is needed, and more farms and samples are needed to find significance for differences by farm. We need to find more microsatellite loci that can be used as markers except for the 12 short tandem repeats used in this study. Once the markers with significant differences are established, the *E. coli* genotyping protocol will be established and can be used for analyzing farm propagation.

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