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Epidemiological Prevalence of Avian Pathogenic *Escherichia coli* Differentiated by Multiplex PCR from Commercial Chickens and Hatchery in Korea

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We examined 216 *Escherichia coli* (*E. coli*) isolated from chickens and environmental specimens from hatcheries between 2005 and 2006 in order to evaluate the epidemiological prevalence of avian pathogenic *E. coli* (APEC) in Korea tentatively by multiplex PCR. The multiplex PCR which was used as tentative criteria of APEC targets 8 virulence-associated genes; enteroaggregative toxin (*ast*A), increased serum survival protein (*iss*), iron-repressible protein (*irp*2), P fimbriae (*pap*C), aerobactin (*iuc*D), temperature-sensitive hemagglutinin (*tsh*), vacuolating autotransporter toxin (*vat*), and colicin V plasmid operon (*cva*/*cvi*) genes. The number of detected genes could be used as a reliable index of their virulence. It was demonstrated that *E. coli* strains already typed as APEC always harbor 5 to 8 genes, but non-APEC strains harbor less than 4 genes. Assuming the criteria of APEC is a possession of more than 5 virulence-associated genes, we discriminated 24 APEC strains among the 216 *E. coli* strains. Contamination rates of APEC in the field were 31.3% in layers, 14.0% in broilers, 2.7% in broiler breeders, and 0.0% in environmental specimens from hatcheries. The combinational tendency of APEC examined is a fundamental possession of *ast*A, *iss* and *iuc*D genes and addition of *cva*/*cvi*, *tsh*, *vat*, and *irp*2 genes which have a critical importance for virulent traits of APEC. Compared with intravenous chicken challenge or embryo lethality assay, multiplex PCR method could be useful to discriminate APEC rapidly for convenient diagnosis.

Key Words: Avian pathogenic *E. coli*, Multiplex PCR

INTRODUCTION

Colibacillosis is pathogenic to mammals and fowls primarily or secondarily as a mixed infection in which symptoms are complicated. Especially, avian pathogenic *E. coli* (APEC) strains are the etiologic agents of colibacillosis in birds and are an important problem for the poultry industry (2,16,21). APEC strains fall under the category of extraintestinal pathogenic *E. coli*, which are characterized by the possession of virulence factors that enable to live extraintestinal life (3,13,23). These virulent factors have been identified (3,6,13,27). However, no specific virulent factor that contributes entirely to the pathogenicity of APEC has been discovered (20,23). Thus a lack of diagnostic tests

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to determine degrees of the virulence which are primary pathogens (highly virulent), secondary pathogens (moderately virulent), or nonpathogenic (avirulent) makes it difficult to control the colibacillosis (28). And the lack of diagnostic tests also results in the scarce knowledge about epidemiology of APEC. Especially in Korea, there is no available information of epidemiology of APEC. Take these circumstances into consideration, we carried out epidemiological study of APEC in chicken farms and hatcheries using multiplex PCR, which could tentatively differentiate APEC from field isolates of *E. coli*. This multiplex PCR was reported in a previous study (6), and it was proved that virulent strains which are already typed as APEC had 5 to 8 virulence-associated genes but avirulent strains which are already typed as non-APEC strains had at most 4 virulence-associated genes (6).

These genes are enteroaggregative toxin (*ast*A), increased serum survival protein (*iss*), iron-repressible protein (*irp*2), P fimbriae (*pap*C), aerobactin (*iuc*D), temperature-sensitive hemagglutinin (*tsh*), vacuolating autotransporter toxin (*vat*), and colicin V plasmid operon genes (*cva*/*cvi*) (6). *Ast*A gene encodes for EAST1 (enteroaggregative *E. coli* heat-stable enterotoxin 1), which was first observed in EAEC (enteroaggregative *E. coli*) strains, which had been recognized as an agent of diarrhea (15). Although the role of EAST1 in pathogenesis was not fully established, presence of this toxin was found in high frequency (17,26,29). *Iss* gene attributing to increased serum survival, has been described as an important feature of virulent *E. coli* in chickens (5). Previous studies reported that *iss* and the protein which *iss* encodes were useful targets of colibacillosis (6,19). Location of *iss* gene tends to exist in both ColV plasmid and bacterial chromosome (13,19,27). Among 8 virulence-associated genes, genes related to iron acquisition system are *irp*2 and *iuc*D. *Irp-fyu*A gene cluster encoded for yersiniabactin, were first detected in *Yersinia species* (10,25). In addition, it also has been found in *E. coli* isolated from humans and birds (10,24,25). The other gene encodes for iron acquisition system, *iuc*D gene, codes for aerobactin has been described that it is located in both ColV and bacterial chromosome (10,24,25). P fimbriae was first identified in *E. coli* isolates pathogenic to human urinary tract (1), and the pathogenesis of this gene in avian strains has not been fully understood. Several studies have shown the frequent existence of *pap*C in APEC (1,4,6,12). *Tsh* and *vat* genes both code for serine protease autotransporter and they have 75% protein homology (18). However, only *tsh* is on a ColV-type plasmid, and it is located near the colicin V genes in many of APEC strains (3,11). Gene responsible for vacuolating cytotoxin was first detected in septicemic chickens (22), and their location in PAI (pathogenicity island) was first described in avian *E. coli* that encoded an autotransporter protein (18). *Cva* A/B and *cvi*/*cva*C genes are related to ColV plasmid. Possessing *iss*, *iuc*D, *tsh* and *cva*/*cvi*, ColV plasmids have been considered to be a defining feature of the APEC strains (13,25).

In this study, we screened 216-field *E. coli* isolated from the liver of debilitated chickens and environmental specimens from hatcheries between 2005 and 2006 in order to evaluate the epidemiological prevalence of APEC in Korea by the multiplex PCR. We temporarily assumed that the criteria of APEC were a possession of 5 to 8 virulence-associated genes, and strains which had less than 4 virulence-associated genes considered to be non-APEC based on the results of previous study (6). According to these criteria, we tentatively differentiated APEC and examined their distribution in Korea. We also obtained detection rates of each virulence-associated genes and combinations of detected genes. Moreover, we could also reevaluate the validity and rapidity of multiplex PCR in terms of application to field samples.

MATERIALS AND METHODS

1. Bacterial strains

A total of 216 *E. coli* strains were collected from chickens and hatcheries. All of *E. coli* strains had been isolated from each liver surface of debilitated chickens submitted to our laboratory from chicken farms for autopsy and environmental specimens from hatcheries. Each swabbed specimen from liver surface of chickens was smeared on MacConkey agar plate (Difco, Detroit, MI, USA), and

then incubated at 37℃ for approximately 18 h in aerobic conditions. Specimens from hatcheries were collected from the MacConkey agar plates which were left for 10 minutes in the incubation room, hatching room and packing/dispatching room, and these agar plates were submitted to our laboratory to evaluate the cleanliness level of operation room in hatcheries. Colonies expressing bright pink and surrounded by precipitate in MacConkey agar were selected and tested for screening biochemical traits by using the API 20E (BioMérieux, Marcy l'Etoile, France). The selected *E. coli* strains include 129 strains from broilers, 16 strains from layers, 37 strains from broiler breeders, and 34 strains from environmental specimens of hatcheries.

2. DNA preparation

Each of glycerol stock was incubated in Nutrient broth (Difco) and grown overnight in shaking incubator at 37℃. After that, the enriched medium in a 1.5 ml-effendorf tube was microcentrifuged in 1,500 rpm for 10 min, and the supernatant was discarded. Autoclaved distilled water (DW) or TE buffer (10 mM Tris-HCl, 1 mM EDTA at pH 8.0) was added to the pellet. This tube was freezed for 10 min at -80℃, and then, incubated (boiled) for 5 min at 95℃. After repeating this procedure 3 to 4 times, the tube was centrifuged again in 1,500 rpm for 10 min. Amount of DNA in supernatant was evaluated by Nanodrop (ND-1000, Nanodrop Technologies, Wilmington, DE, USA). For adjusting the suitable amount of template DNA, 1 μl, 2.5 μl, 5 μl and 10 μl of each the templates were used for PCR.

3. Multiplex PCR

Test organisms were examined for *ast*A, *iss*, *irp*2, *pap*C *iuc*D, *tsh*, *vat*, and *cva*/*cvi* genes using the following procedures. Optimal amount of template decided by Nanodrop was added to the reaction mixture containing 0.4 μl of each primer pair (50 pmol; Bioneer, Daejeon, Korea), 5 μl of dNTP (2 mM; Enzynomics, Daejeon, Korea), 5 μl of 10× PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl; Enzynomics) and 0.5 μl of Taq polymerase (5 U/μl; Enzynomics). The remainder portion was filled with autoclaved DW up to 50 μl. Amplifications were performed in a 50 μl reaction by using PCR cycler (PTC-220, MJ

Table 1. Sequence and specificity of PCR primers and their product sizes

No	Virulent genes	Primer sequence (5' - 3')	Location within gene	GenBank Acc. No	Size (bp)
1	*ast*A	TGCCATCAACACAGTATATCC	797~817	AF143819	116
		TCAGGTCGCGAGTGACGGC	912~894		
2	*iss*	ATCACATAGGATTCTGCCG	(–)10~(–)28	X52665	309
		CAGCGGAGTATAGATGCCA	282~264		
3	*irp*2	AAGGATTCGCTGTTACCGGAC	22~42	L18881	413
		AACTCCTGATACAGGTGGC	434~416		
4	*pap*C	TGATATCACGCAGTCAGTAGC	1284~1304	Y00529	501
		CCGGCCATATTCACATAA	1784~1767		
5	*iuc*D	ACAAAAAGTTCTATCGCTTCC	239~259	M18968	714
		CCTGATCCAGATGATGCTC	952~934		
6	*tsh*	ACTATTCTCTGCAGGAAGTC	132~151	AF218073	824
		CTTCCGATGTTCTGAACGT	955~937		
7	*vat*	TCCTGGGACATAATGGTCAG	1076~1095	AY151282	981
		GTGTCAGAACGGAATTGT	2056~2038		
8	*cva* A/B	TGGTAGAATGTGCCAGAGCAAG	10745~10764	AJ223631	1,181
	*cvi cva*C	GAGCTGTTTGTAGCGAAGCC	11925~11904		

Research, Wilmington, DE, USA). The cycling conditions were the following: t_1, 4 min at 94℃; t_3, 30 sec at 58℃; t_4, 3 min at 68℃ (t2-t4, repeated 25 times); t_5, 10 min at 72℃.

4. Primers

Primers were obtained from Bioneer and the primer sequences used in this study were decided as described previously by Ewers *et al*. (6). Primers, their position within the target sequences, and size of amplicons are presented in Table 1.

5. Agarose gel electrophoresis

Seven μl samples of reaction mixtures were analyzed by gel electrophoresis in 0.8% agarose, dissolved in 1× TAE (40 mM Tris-Acetate, 1 mM EDTA at pH 8.3) for 60 min at 90V. After staining with ethidium bromide, the amplicons were photographed by Gel Documentation Analyzer (Vilber Lourmat Trance, Marne-la-Vallée, France).

6. Sequence analysis

The specificity of multiplex PCR was evaluated by direct sequencing of all amplicons from 24 APEC isolates and 16 strains of non-APEC which had dominant combinations of virulence-assiciated genes. The target bands of expected sizes were excised with a blade and purified using Genomed Jetsorb Jet Extraction Kit (Genomed, Inc., St. Louis, MO,

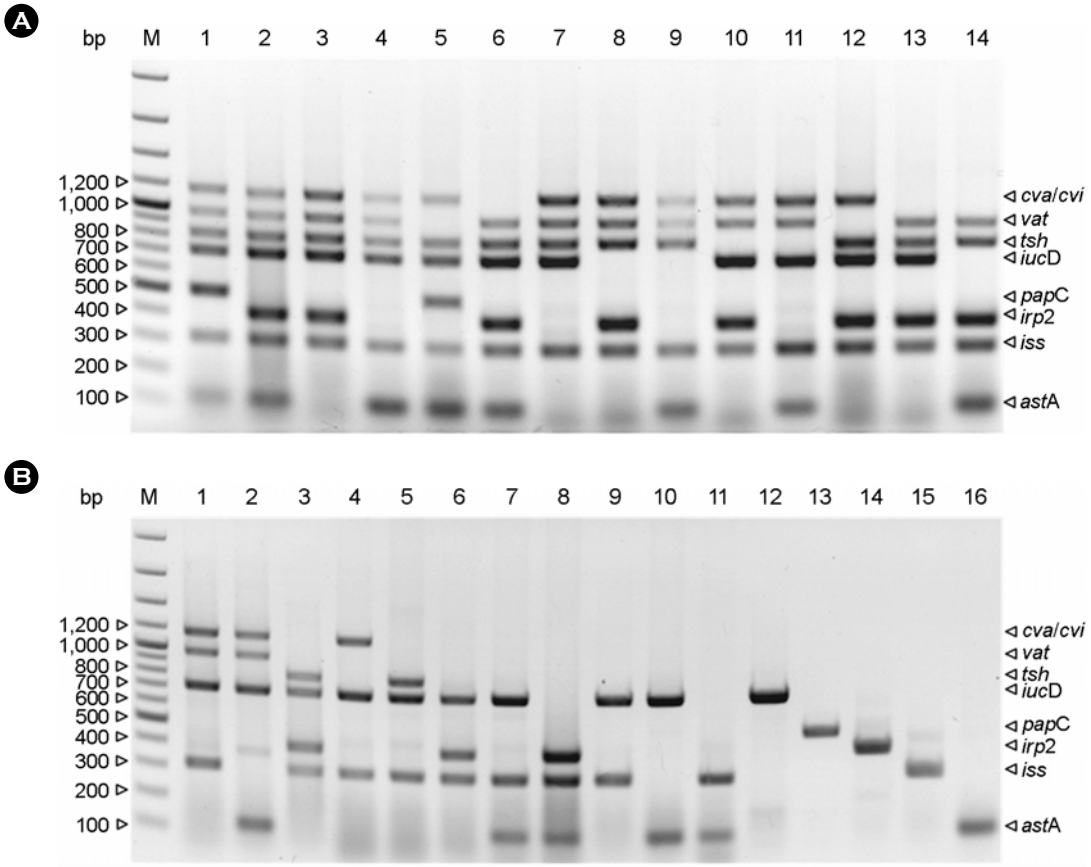

Figure 1. Amplicon patterns of virulence-associated genes by multiplex PCR. **(A)** Combinations of virulence-associated genes of APEC differentiated by multiplex PCR. There were 14 combinations of virulence-associated genes in 24 APEC strains. M: 100 bp ladder; Lanes 1~2: gene combinations of strains which had 7 virulence-associated genes; Lanes 3~6: gene combinations of strains which had 6 virulence-associated genes; Lanes 7~14: gene combinations of strains which had 5 virulence-associated genes. **(B)** Combinations of virulence-associated genes of non-APEC differentiated by multiplex PCR. There were 16 combinations of virulence-associated genes which match to more than 2 non-APEC strains. M: 100 bp ladder; Lanes 1~3: main gene combinations of strains which had 4 virulence-associated genes; Lanes 4~8: main gene combinations of strains which had 3 virulence-associated genes; Lanes 9~11: main gene combinations of strains which had 2 virulence-associated genes; Lanes 12~16: main gene combinations of strains which had 1 virulence-associated genes.

USA). Purified DNA products were sequenced in both directions using the Applied Biosystems 3130/3130xl Genetic Analyzer with the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. Sequences were examined for identity with published sequence data (Table 1) from the National Center for Biotechnology Information (NCBI). Sequence alignment and comparison were performed using the GENETYX version 6.1 (Software Development, Tokyo, Japan) with manual adjustment.

RESULTS

1. The criteria of APEC

We tentatively determined that the criteria of APEC were harboring of more than 5 virulence-associated genes as previously reported (6). According to the criteria, we differentiated 24 APEC among 216-field *E. coli*. The rest of 192 *E. coli* isolates represented less than 4 virulence-associated genes or none of the targeted genes were detected in these isolates (Fig. 1 and Table 2).

2. Detection rates of APEC according to specimen origins (layers, broiler breeders, broilers and environmental objects)

Table 2 shows detection rates of APEC according to the specimen origins. Five APEC (31.3%) were detected in 16 strains from layers, only one APEC (2.7%) was detected in 37 strains from broiler breeders, 18 APEC (14.0%) were discriminated among 129 strains from broilers, and there was no APEC (0.0%) discriminated in 34 strains of environmental specimens from hatcheries. Although *E. coli* isolates of layers were not enough, it had the highest APEC detection rates among 4 specimen origins. Being in a relatively good environmental condition, strains from broiler breeders had lower APEC detection rates than other origins.

Table 2. Detection rates of APEC from the field isolates of *E. coli*

Origin	Total isolates	APEC	Detection rates (%)
Layer	16	5	31.3
Broiler breeder	37	1	2.7
Broiler	129	18	14.0
Hatchery	34	0	0.0
Total	216	24	11.1

3. Detection rates of virulence-associated genes

There was no strain that contained all 8 of virulence-associated genes. However, there were 3 strains that include

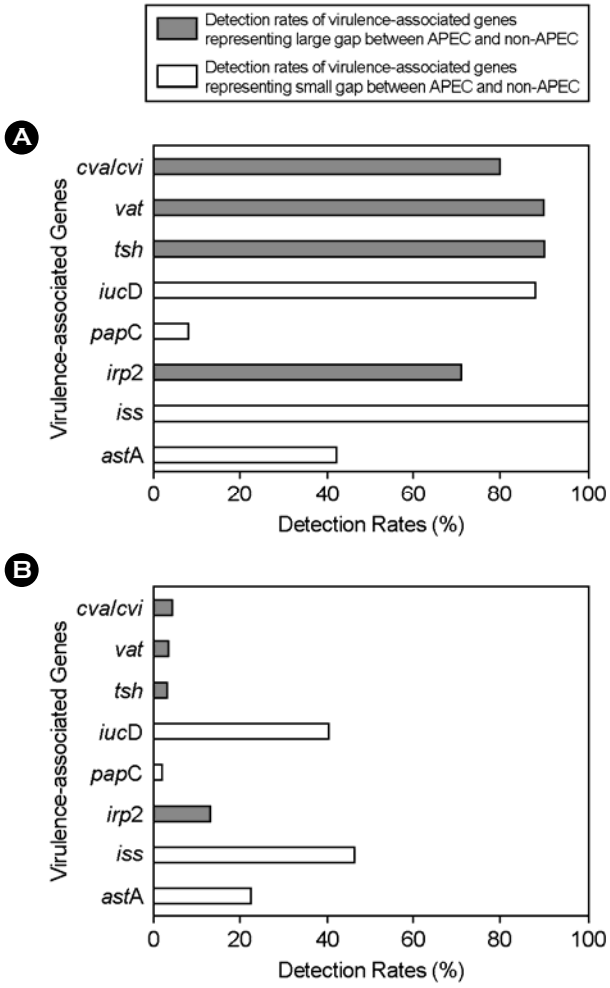

Figure 2. Total detection rates of virulence-associated genes between APEC and non-APEC. **(A)** Detection rates of virulence-associated genes in APEC. **(B)** Detection rates of virulence-associated genes in non-APEC. Taken altogether, virulence-associated genes were more frequently appeared in APEC than non-APEC. Detection rates of *irp*2, *tsh*, *vat* and *cva*/*cvi* in APEC were relatively higher than detection rates of the corresponding genes in non-APEC, respectively. On the other hand, detection rates of *ast*A, *iss* and *iuc*D were quite high in non-APEC as well as APEC strains. In addition, detection rate of *pap*C was very low in both APEC and non-APEC.

7 genes (1.4%), 6 genes detected in 11 strains (5.1%), 5 genes detected in 10 strains (4.6%), 4 genes detected in 15 strains (6.9%), 3 genes detected in 24 strains (11.1%), 2 genes detected in 25 strains (11.6%), 1 gene detected in 68 strains (31.5%) and no virulence-associated genes were detected in 60 *E. coli* strains (27.7%).

4. Patterns of detection rates of virulence-associated genes

Patterns and combinations of virulence-associated genes for 24 APEC strains isolated in the present study are summarized in Fig. 1 and Table 4. Although the specimen origins of APEC strains were diverse, the existence of virulence-associated genes had similar patterns (Table 3). On average, detection rate of *ast*A gene showed that it frequently existed in non-APEC strains as much as APEC strains (Fig. 2). Especially in layer strains, detection rates of *ast*A represented apparently small gap between APEC and non-APEC (Table 3).

The *iss* was the highest detected gene among the 8 virulence-associated genes (Fig. 2). Furthermore, *iss* was located on entire 24 APEC strains in the present study. However, in strains from layers, detection rate of *iss* in non-APEC was as high as in that of APEC (Table 3). In the similar way, *E. coil* strains from broiler breeders, broilers, and environmental specimens from hatcheries also presented that *iss* gene was more frequently located on non-APEC than other virulence-associated genes (Table 3). In this regard, it was hard to discriminate APEC and non-APEC by *iss* gene.

*Irp*2 and *iuc*D, both related to iron acquisition system, demonstrated a different appearance in detection rates between APEC and non-APEC. *Iuc*D was detected nearly 90% in APEC, meanwhile, detection rates of *irp*2 was not as high as *iuc*D (Fig. 2). Our data also indicated that possessing of *iuc*D was not only a characteristic of APEC but also non-APEC strains, because detection rates of *iuc*D represented a small gap between APEC and non-APEC (Fig. 2). Furthermore, these two virulence-associated genes related to iron acquisition system tended to exist on the same strain. Among 24 APEC strains, we differentiated 23 APEC strains contained either or both of two iron acquisition systems (Table 4). Moreover, there are 15 APEC strains that possess both yersiniabactin system and aerobactin system, and 8 APEC strains which have either yersiniabactin system or aerobactin system (Table 4).

*Pap*C was barely detected in both APEC and non-APEC (Fig. 2). *E. coli* strains from layers and broiler breeders did not possess this gene at all (Table 3). Although *E. coli* strains from broilers and environmental specimens from hatcheries possessed *pap*C in several cases, this gene itself could not be a clue for differentiating between APEC among

Table 3. Detection rates of virulence-associated genes amomg 216-field isolates of *Escherichia coli*

Virulence-associated genes	Detection rates of virulence-associated genes (%)							
	Origins of *Escherichia coli*							
	Layer		Broiler breeder		Broiler		Hatchery	
	APEC	Non-APEC	APEC	Non-APEC	APEC	Non-APEC	APEC	Non-APEC
*ast*A	0/5 (0)	3/11 (27)	0/1 (0)	14/36 (39)	10/18 (56)	23/111 (21)	0/0 (0)	3/34 (8.8)
iss	5/5 (100)	9/11 (82)	1/1 (100)	13/36 (36)	18/18 (100)	62/111 (56)	0/0 (0)	5/34 (15)
*irp*2	4/5 (80)	4/11 (36)	1/1 (100)	9/36 (25)	12/18 (67)	9/111 (8.1)	0/0 (0)	3/34 (8.8)
*pap*C	0/5 (0)	0/11 (0)	0/1 (0)	0/36 (0)	2/18 (11)	1/111 (0.9)	0/0 (0)	2/34 (5.9)
*iuc*D	5/5 (100)	6/11 (55)	1/1 (100)	19/36 (53)	15/18 (83)	52/111 (47)	0/0 (0)	0/34 (0)
tsh	5/5 (100)	1/11 (9)	0/1 (0)	2/36 (5.6)	17/18 (94)	4/111 (3.6)	0/0 (0)	0/34 (0)
vat	5/5 (100)	2/11 (18)	1/1 (100)	0/36 (0)	16/18 (89)	5/111 (4.5)	0/0 (0)	0/34 (0)
cva/cvi	2/5 (40)	1/11 (9)	1/1 (100)	1/36 (2.7)	16/18 (89)	7/111 (6.3)	0/0 (0)	0/34 (0)

Table 4. Combinations of virulence-associated genes in 24 isolates of APEC

No	Origin	Strain	*ast*A	*iss*	*irp*2	*pap*C	*iuc*D	*tsh*	*vat*	*cva/cvi*
1	Layer	L/06/2		+	+		+	+	+	
2	Layer	L/06/3		+	+		+	+	+	
3	Layer	L/06/4		+			+	+	+	+
4	Layer	L/06/8		+	+		+	+	+	+
5	Layer	L/06/14		+	+		+	+	+	
6	BB[a]	PS/06/1		+	+		+		+	+
7	Broiler	B/06/6		+	+		+	+	+	+
8	Broiler	B/06/7		+	+		+	+	+	+
9	Broiler	B/06/13	+	+			+		+	+
10	Broiler	B/06/16		+	+		+	+	+	+
11	Broiler	B/06/17		+	+		+	+	+	+
12	Broiler	B/06/19	+	+			+	+	+	+
13	Broiler	B/06/21		+	+		+	+	+	+
14	Broiler	B/06/25	+	+	+		+	+	+	
15	Broiler	B/06/31	+	+	+		+	+	+	+
16	Broiler	B/06/34	+	+		+	+	+	+	+
17	Broiler	B/06/63	+	+			+	+	+	+
18	Broiler	B/06/77	+	+	+		+	+	+	+
19	Broiler	B/06/78		+	+		+	+	+	+
20	Broiler	B/06/79		+	+			+	+	+
21	Broiler	B/06/80	+	+				+	+	+
22	Broiler	HC/06/1		+	+		+	+		+
23	Broiler	HC/06/8	+	+		+	+	+		+
24	Broiler	HC/06/9	+	+	+			+	+	

[a] Broiler breeder

non-APEC.

In the total isolates of field *E. coli*, *tsh*, *vat* and *cva*/*cvi* were presented as much as *iss* and *iuc*D. However, *tsh*, *vat* and *cva*/*cvi* were relatively more limited than other targeted genes in APEC (Fig. 2). In strains from layers and broilers, it was obvious that the gap of detection rates of these genes between APEC and non-APEC was much larger than other virulence-associated genes (Table 3). In strains from broiler breeders and hatcheries, the detection rates of *tsh*, *vat* and *cva*/*cvi* were apparently low in non-APEC (Table 3), and these results indicated that these genes might be relatively exclusive in APEC strains.

Taken altogether, detection rates of 8 virulence-associated genes in APEC were remarkably higher than non-APEC (Fig. 2). In particular, detection rates of genes *iss*, *iuc*D, *vat*, *tsh* and *cva*/*cvi* were nearly 90% in APEC. However, detection rates of genes *iss* and *iuc*D were relatively high not only in APEC but also in non-APEC.

5. Sequence analysis

Eight amplicons represented expected sequences in accordance with published data (Table 1) from NCBI (data not shown).

DISCUSSION

We examined field *E. coli* by the multiplex PCR which presumably enabled us to investigate the severity of APEC contamination of chicken farms and hatcheries in Korea. In addition, the rates of virulence-associated genes and the combinational tendency of APEC within targeted 8 virulence-associated genes were also investigated. Although the specimens from four origins were not collected from the same numbers of chickens, the APEC detection rates (31.3%) were the highest in layers (Table 2). The lowest APEC detection rates (0.0%) were observed in environmental specimens from hatcheries (Table 2). These results indicated that APEC was widely spread in farms rather than in hatcheries of Korea. Furthermore, being in a relatively good environmental condition, strains from broiler breeders (2.7%) had lower APEC detection rates than strains from broilers (14.0%) (Table 2).

As we expected, there was no genes which exclusively detected in APEC and no exact combination which always apply to APEC. However, by means of this study, we detected the pattern of gene combination and estimated the importance of genes among the 8 virulence-associated genes.

In this study, *iss* was detected 100% and *iuc*D was detected nearly 90% in APEC strains. However, these strains also detected in non-APEC frequently. This indicates that each of *iss* and *iuc*D itself might not be a contributor to virulence and these genes merely support other essential virulent traits. In addition, detection rate of *pap*C was low in both APEC and non-APEC. Although P fimbriae promote evasion of the host's defenses, they might be not an essential virulence trait for APEC (25). On the other hand, *tsh*, *vat*, and *cva*/*cvi* appeared to be essential in APEC strains. Because their detection rates of *tsh*, *vat* and *cva*/*cvi* were almost 90% in APEC strains, while these genes existed less than 10% in non-APEC strains.

Gram negative bacteria have several methods for transporting their proteins to the external environment (11,14). *Tsh* and *vat* which are functioning as an autotransporter, and these system are capable of directing their own secretion across the outer membrane. *Tsh* coding for temperature sensitive hemmagglutinin has adhesive and proteolytic properties independently (3). *Tsh* has been treated as an important trait in avian pathogenesis of *E. coli* in many studies (3,6,25). In our data, significant difference in detection rates between APEC and non-APEC confirmed the importance of *tsh* in APEC.

Another autotransporter targeted in this study was *vat* encoding for vacuolating cytotoxin. Vacuolating cytotoxin was first described by Salvadori *et al*. (22) that cytotoxic substance in supernatant of APEC caused a cytotoxic effect resembling to morphological changes of *Helicobacter pylori* in target cells. Although a role of *vat* in pathogenesis for avian *E. coli* has not been elucidated, our results showed that it might function as an important virulent trait in APEC.

Our data also represented the importance of ColV plasmid. ColV plasmids were found primarily among virulent enteric bacteria and have been shown to encode several virulence related properties in addition to colicin V (9,13,27). Possessing *iss*, *iuc*D, *tsh* and *cva*/*cvi*, ColV plasmids have been considered to be a defining feature of the APEC strains (13,25).

Admittedly, using the multiplex PCR method which is only based on the number of detected genes (6) could not completely differentiated APEC from other *E. coli* strains in the field. However, in this study, this method was useful to evaluate the contamination of APEC of the chicken farms and hatcheries in Korea. On the basis of Ewers *et al*. study (6), we differentiated 24 APEC among 216-field *E. coli* existed in the liver surface of debilitated chickens. Our data showed that 11.1% of APEC was spread in chickens and environment of hatcheries and was primarily or secondarily related to colibacillosis. In the further study, the pathogenicity of APEC isolates would be evaluated by embryo lethality assay (ELA) which was considered to have a correlation with intravenous chicken challenge (7,8,28). ELA might be useful to reevaluate the pathogenicity of 24 APEC strains isolated in this study.

In consequence, the 'epidemiological prevalence of

APEC differentiated by multiplex PCR from commercial chickens and hatchery in Korea' was a first report to recognize the severity of APEC in Korea. In addition, it was also confirmed that there was no exact combination of virulence-associated genes that could differentiate between APEC and non-APEC. However, we could recognize the frequent combination pattern of APEC and evaluate the significance of virulence-associated genes which had been targeted for discriminating APEC among non-APEC. *Tsh*, *vat* and *cva*/*cvi* which were relatively exclusive to APEC could be considered as the essential virulence traits of APEC. In contrast, only *iss* and *iuc*D themselves could not be contributor of APEC. In this respect, addition of *irp*2, *tsh*, *vat* and *cva*/*cvi* to the fundamental possession of *ast*A, *iss* and *iuc*D was a frequent combinational tendency in 24 APEC isolates. Furthermore, finding this combinational tendency would be effective way to discriminate APEC among non-APEC strains in the field.

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