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Comparative study of *Clostridium perfringens*, *Salmonella* spp. and *E. coli* focused on characteristics of *E. coli* O157 isolated from pigs of HACCP- and non-HACCP-accredited swine farms in Korea

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Abstract : To determine the prevalence of *Escherichia* (*E.*) *coli* O157 : H7 from pigs after the Hazard Analysis and Critical Control Point (HACCP) system has been applied to Korean swine farm since 2006, 291 fecal samples were tested between May and December in 2008. Four *E. coli* O157:non-H7 (1.4%) were isolated from 4 different non-HACCP-accredited farms and they didn't have virulent genes which can cause illness for human. Also, *Clostridium* (*C.*) *perfringens*, *Salmonella* spp. and *E. coli* enterotoxins were tested using multiplex PCR. The positive rate for these pathogens of non-HACCP-accredited farms was higher than that of HACCP-accredited farms, and especially in case of *C. perfringens*, *E. coli* enterotoxins LT and STa, it was statistically significant ($p < 0.05$). Thus, the early implementation of the HACCP program is expected to greatly contribute to the safety of livestock products as well as food hygiene.

Keywords : *Escherichia coli* O157, HACCP, pig

Escherichia (*E.*) *coli* O157 : H7 has been recognized as an important cause of diarrhea, hemorrhagic colitis and hemolytic-uremic syndrome in human as well as an important food-borne pathogen [9]. This pathogen has several virulence factors which are related to the pathogenicity. Verocytotoxin (VT)1, VT2 and its variants, attaching-and-effacing (*eaeA*) and hemolysin (*hlyA*) are the most frequently identified [9, 10]. *E. coli* O157 : H7 has been isolated from a variety of animals and the outbreaks are associated with the consumption of food and water contaminated with feces of infected animals [7, 10, 12]. Among the animals, cattle are now considered to be the principal source of *E. coli* O157 causing human disease. Even though some of non-pathogenic *E. coli* O157 have been isolated from pigs [2, 5], swine have been proved a potential reservoir of *E. coli* O157 [3, 7, 8, 11]. In Korea, there has been reported on the *E. coli* O157 : H7 isolated from pigs during 2000-2002 [7, 8]. But after that, there has been no reported on it to date. The present swine breeding environment of Korea has been remarkably enhanced since the Hazard Analysis and Critical Control Point (HACCP) system for livestock products was introduced in 1997. The system has been applied to slaughter house mandatorily in 2004, and to swine farms optionally in 2006 due to the efforts of governments to ensure the food safety. Thus, the main purpose of this study was to investigate the patterns and characteristics of *E. coli* O157 isolated from pigs recently after the HACCP system application. Additionally, since *Salmonella* spp. is an important pathogen to be controlled in the HACCP-accredited swine farm, commercial multiplex PCR including *Salmonella* spp. was also performed.

A total of 291 porcine fecal samples were collected between May and December in 2008, from 69 swine farms in eight provinces (excluding Jeju province) of Korea. Age groups ranging from nursing to adult pigs were represented among the samples. Among 69 swine farms, 20 farms (138 samples) were HACCP-accredited farms, which were certified from the Korea Livestock Products HACCP Accreditation Service, and 49 farms

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(153 samples) were non-HACCP-accredited farms. One hundred and sixty six pigs (50 from HACCP-accredited and 116 from non-HACCP-accredited farms) showed signs of diarrhea at the time of sampling, and all others appeared clinically normal. Fresh fecal samples were collected from individual pigs and prepared as 10% suspension in phosphate-buffered saline (pH 7.2). One milliliter of 10% suspension was incubated in modified *E. coli* (mEC) broth containing 1% novobiocin (Difco, USA) at 37°C for 24 h. After centrifugation (3,000 rpm, 10 min), one loop of mEC pellet was streaked onto MacConkey Sorbitol Agar plate (Difco, USA) and incubated at 37°C for 24 h. Non sorbitol-fermenting, colorless colonies (up to 5 per sample) on the SMAC were selected and then streaked onto MacConkey Agar plate (Difco, USA) and Eosin Methylene Blue Agar plate (Difco, USA) to confirm a typical colony of *E. coli*. Further confirmation was done by Triple Sugar Iron (Oxoid, England) test and Vitek2 GN (bioMérieux, USA) test. To identify serogroup/type, serum agglutination test was performed using O157 antigen and H7 antigen antisera (Difco, USA). All isolates confirmed to be *E. coli* O157 or O157 : H7 were subjected to a polymerase chain reaction (PCR) assay (Table 1) for the detection of flagellin subunit-encoding $fliC_{H7}$ (*H7*) and other virulence genes: *VT1*, *VT 2*, *eaeA* and *hlyA*. *E. coli* O157 : H7 ATCC43894 was used as a control strain. In the meantime, to examine the presence of some bacterial pathogens, *Clostridium* (*C.*) *perfringens*, *Salmonella* spp., *E. coli* LT (Heat-labile toxin), *E. coli* STa (Heat-stable toxin a) and *E. coli* STb (Heat-stable toxin b), Seeplex Porcine Diarr-B Detection Kit (Seegene, Korea) was used according to the manufacturer's instructions. The data were analyzed using the Pearson's chi-square test in order to determine whether there were any differences in their detection rate between HACCP-accredited and non-HACCP-accredited farms.

A total of 4 *E. coli* O157 (1.4%) were isolated from 291 fecal samples and they were from 4 different non-HACCP-accredited farms. The isolates were negative for H7 antiserum test and didn't have $fliC_{H7}$ gene. Three of them were negative for 4 virulence genes, *VT1*, *VT2*, *eaeA* and *hlyA*, and one strain was positive for *VT2* (Table 2). Microbial profiles were compared between HACCP-accredited farms and non-HACCP-accredited farms in Table 3. The positive rate for all pathogens tested of non-HACCP-accredited farms was higher than that of HACCP-accredited farms. Especially, the detection rate of *C. perfringens*, *E. coli* LT and STa in non-HACCP-accredited farms were significantly higher ($p = 0.0007$, $p = 0.004$ and $p = 0.03$, respectively) than that in HACCP-accredited farms. Although the detection rate of *Salmonella* spp. and *E. coli* STb was higher in non-HACCP-accredited farms, statistical significance could not be shown ($p = 0.07$ and $p = 0.076$, respectively).

The HACCP system is a systematic preventive approach to food safety that addresses physical, chemical, and biological hazards as a means of prevention rather than finished product inspection. It was introduced as an alternative plan in order to ensure the food hygiene and food safety since 1959, as hazardous factors in foods were increased, and food poisong caused by *E. coli* O157 : H7 broked out

Table 1. Oligonucleotide primers used in the study

Target gene	Primer name	Sequence, 5′-3′	Size	References
H7	H7-F*	GCGCTGTCGAGTTCTATCGAGC	625	[4]
	H7-R	CAACGGTGACTTTATCGCCATTCC		
VT1	VT1-F*	CGTACGGGGATGCAGATAAATCGC	210	KCDC‡
	VT1-R	CAGTCATTACATAAGAACGCCCAC		
VT2	VT2-F*	GTTCTGCGTTTTGTCACTGTCAC	326	KCDC
	VT2-R	GTCGCCAGTTATCTGACATTCTGG		
eaeA	eaeA-F†	GTGGCGAATACTGGCGAGACT	165	[1]
	eaeA-R	CCCCATTCTTTTTCACCGTCG		
hlyA	hlyA-F†	ACGATGTGGTTTATTCTGGA	890	[1]
	hlyA-R	CTTCACGTGACCATACATAT		

*Primers used in the H7/VTs multiplex PCR. †Primers used in the eaeA/hlyA duplex PCR. ‡KCDC: Korea Centers for Disease Control and Prevention.

Table 2. Characteristics of Escherichia (*E.*) *coli* O157 isolates from fecal samples in Korea

Name of sample	Serotype	Presence of the following genes					Collection date
		H7	VT1	VT2	eaeA	hlyA	
CN3835	O157 : nonH7	−	−	−	−	−	Jul. 24, 2008
JN3994_95	O157 : nonH7	−	−	−	−	−	Aug. 26, 2008
GN4779_80	O157 : nonH7	−	−	+	−	−	Dec. 1, 2008
CB4794_95	O157 : nonH7	−	−	−	−	−	Dec. 5, 2008

Table 3. Microbial profiles of fecal samples using multiplex PCR

Classification	Number of positive samples (%)				
	C. perfringens	*Salmonella* spp.	*E. coli* (LT)	*E. coli* (STa)	*E. coli* (STb)
HACCP farm*	1 (0.7)‡	6 (4.3)	19 (14)‡	21 (15)‡	49 (36)
non-HACCP farm†	15 (9.8)‡	15 (9.8)	42 (27)‡	39 (25)‡	70 (46)

*HACCP farm, HACCP-accredited farm. †Non-HACCP farm, non-HACCP-accredited farm. ‡Significant in Pearson's chi-square test ($p < 0.05$).

sporadically in the United Stated [6]. In Korea, the HACCP system for livestock products was introduced in Dec. 1997 for the first time. Since then, from the slaughter house, through food selling, distribution, feed, storage, transportation and to swine farms, cattle farms, chicken and duck farms, it has been extended upto now. HACCP checklist for swine fams consists of two parts. First part is composed of 70 items which are associated with hygiene and sanitation standards; isolation control, facilities and their management, sanitation, feed, veterinary medical supplies, water quality, disease control, shipping and so on. The other has 23 items associated with HACCP management. To be certified as HACCP farms, they should be corresponded with the standard based on that checklist settled by the Korea Livestock Products HACCP Accreditation Service.

In this study, four isolates were identified as *E. coli* O157, but non of them were H7. Three of them didn't have any virulence genes tested in this study, and one strain produced VT2 only. The production of VTs is not in itself sufficient to cause disease and other virulence factors are thought to contribute to the virulence of *E. coli* O157 [10]. Thus, these 4 strains are unlikely to be source of infection for human as previously reported [2, 5]. Previous work on *E. coli* O157 isolates in Korea during 2000-2002 reported virulence factors (*eaeA* and *hlyA*) that can cause human disease [7, 8]. Interestingly, all these isolates were from non-HACCP-accredited farms. As a result of multiplex PCR using Seeplex Porcine Diarr-B Detection Kit, all 5 pathogens were detected with lower prevalence in HACCP-accredited farms. Especially the detection rate of *C. perfringens* and *Salmonella* spp., which are the main cause of swine dysentery, was less than half compared to that of non-HACCP-accredited farms. Although detection rate of several enteric bacteria and toxins was significantly lower in HACCP-accredited farms, differential study about pathogenic or non-pathogenic bacteria for human infection was not included in this study. Therefore, further study on relationship between risk factors and other human pathogens should be needed to validate the HACCP system in swine farms.

In conclusion, despite of the limitation of time and number of farms, this study emphasizes the regular monitoring and surveillance of the hazard that can be brought at breeding steps among the HACCP-accredited and non-HACCP-accredited farms. Finally, the early establishment and implementation of the HACCP program is expected to greatly contribute to the safety of livestock products as well as food hygiene. Furthermore, the detail comparative study among these farms should be added in identifying the production environment, facilities management, sanitation and so on.

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