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<Original Article>

Virulence factors, antimicrobial resistance patterns, and genetic characteristics of hydrogen sulfide-producing *Escherichia coli* isolated from swine

Hyun-Eui Park[1], Min-Kyoung Shin[1], Hong-Tae Park[1], Seung Won Shin[1], Myunghwan Jung[1], Young Bin Im[1], Han Sang Yoo[1,2,*]

[1]*Department of Infectious Disease, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea*
[2]*Institute of Green Bio Science and Technology, Seoul National University, Pyeongchang 232-916, Korea*

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Abstract : *Escherichia* (*E.*) *coli* is commensal bacteria found in the intestine; however, some pathogenic strains cause diseases in animals and humans. Although *E. coli* does not typically produce hydrogen sulfide (H_2S), H_2S-producing strains of *E. coli* have been identified worldwide. The relationship between virulence and H_2S production has not yet been determined. Therefore, characteristics of H_2S-producing isolates obtained from swine feces were evaluated including antibiotic resistance patterns, virulence gene expression, and genetic relatedness. Rates of antibiotic resistance of the H_2S-producing *E. coli* varied according to antibiotic. Only the *EAST*1 gene was detected as a virulence gene in five H_2S-producing *E. coli* strains. Genes conferring H_2S production were not transmissible although the *sse*A gene encoding 3-mercaptopyruvate sulfurtransferase was detected in all H_2S-producing *E. coli* strains. Sequences of the *sse*A gene motif CGSVTA around Cys238 were also identical in all H_2S- producing *E. coli* strains. Diverse genetic relatedness among the isolates was observed by pulsed-field gel electrophoresis analysis. These results suggested that H_2S-producing *E. coli* strains were not derived from a specific clone and H_2S production in *E. coli* is not associated with virulence genes.

Keywords : *sse*A, antibiotic resistance, *Escherichia coli*, hydrogen sulfide, virulence factor

Introduction

Hydrogen sulfide (H_2S) is the colorless gas, but has distinct odor like rotten egg. The gas can be synthesized by reduction of thiosulfate in some enteric bacteria such as *Salmonella* species, *Edwardsiella tarda*, *Citrobacter freundi,* and *Proteus* species. Therefore, production of H_2S has been used as a distinct characteristics in the identification of the enteric bacteria. *Escherichia* (*E.*) *coli* does not produce H_2S generally even though the bacterium is the commensal in the intestine of animal and human. However, H_2S producing strains of *E. coli* have been isolated in worldwide from animal and human [4, 7, 16, 17, 24]. Clinical and biochemical properties and antimicrobial susceptibility properties of H_2S producing variants of *E. coli* were described [6, 16]. H_2S producing *E. coli* isolates were reported in poultry, healthy pigs and clinical urine samples [4, 16, 24]. The ability to produce H_2S in several *E. coli* isolates was transmissible and the transmissible plasmid were also characterized [9, 11, 16].

H_2S can be produced by three enzymes, cystathionine β-synthase, cystathionine, γ-lyase, and 3-mercaptopyruvate sulfurtransferase (3-MST) [14]. Bacterial H_2S is produced by 3-MST in many Enterobacteriaece including *E. coli* [22]. Substitution of Ser240 in catalytic motif CGSGVTA with lysine or alanine altered affinity of 3-MST to thiosulfate [3]. H_2S has been recognized as highly toxic gas to animals and human. But it was discovered that H_2S have cytoprotective functions in mammals [13, 14, 25]. Although physiological function of H_2S as a metabolic end product in bacteria was unclear, a universal function of H_2S against antibiotics was reported [22]. So, H_2S producing *E. coli* was expected to have more antibiotic resistance than non-producing strain. As described above, characteristics of H_2S producing *E. coli* were already investigated [4, 7, 16, 17, 24]. But some characteristics including virulence genes and genetic relatedness were not reported in previous researches. Therefore, virulence genes, antibiotic resistance pattern, and genetic relatedness of our isolates were investigated.

Materials and Methods

Isolation and identification of *E. coli*

Fifty six fecal samples from pig with diarrhea were inoculated to MacConkey agar plate (Oxoid, USA). After incuba-

*Corresponding author
Tel: +82-2-880-1263, Fax: +82-2-874-2738
E-mail: yoohs@snu.ac.kr

Table 1. Oligonucleotide sequences of primers used for virulence genes and *sse*A gene amplification in this study

Target gene		Primer sequence (5'→3')	PCR product size (base pair)	Reference
Stx1	F	TTAGACTTCTCGACTGCAAAG	530	[26]
	R	TGTTGTACGAAATCCCCTCTG		
Stx2	F	CTATATCTGCGCCGGGTCTG	327	[26]
	R	AGACGAAGATGGTCAAA		
STa	F	TCCCCTCTTTTAGTCAGTCAACTG	163	[23]
	R	GCACAGGCAGGATTACAACAAAGT		
STb	F	GCAATAAGGTTGAGGTGAT	368	[15]
	R	GCCTGCAGTGAGAAATGGAC		
F4	F	ATCGGTGGTAGTATCACTGC	601	[20]
	R	AACCTGCGACGTCAACAAGA		
F5	F	TGGGACTCCAATGCTTCTG	450	[20]
	R	TATCCACCATTAGACGGAGC		
F6	F	ATGAGAATGAAAAATCCGCA	567	[19]
	R	CGAATAGTCATTACTGCACT		
F18	F	GTGAAAAGACTAGTGTTATTTC	510	[10]
	R	CTTGTAAGTAACCGCGTAAGC		
F41	F	GAGGGACTTTCATCTTTTAG	431	[20]
	R	AGTCCATTCCATTTATAGGC		
EAE	F	CATTATGGAACGGCAGAGGT	790	[5]
	R	ATCTTCTGCGTACTGCGTTCA		
LT	F	TTACGGCGTTACTATCCTCTCTA	275	[8]
	R	GGTCTCGGTCAGATATGTGATTC		
AIDA-I	F	ACAGTATCATATGGAGCCA	585	[6]
	R	TGTGCGCCAGAACTATTA		
EAST1	F	TCGGATGCCATCAACACAGT	125	[21]
	R	GTCGCGAGTGACGGCTTTGTAG		
PAA	F	ATGAGGAACATAATGGCAGG	360	[2]
	R	TCTGGTCAGGTCGTCAATAC		
sseA	F	GCCTGGACAGGAGGATCGTA	343	In this study
	R	CTCTCCTTCCGGCAGCTCTA		
sseA-seq	F	TGATCACACTTCCCCGCTTC	603	In this study
	R	TTCACGTTTGGCACATCCAG		

F, forward; R, reverse.

tion of the plates for 24 h at 37ºC, pink colored colonies were picked up and subcultured to Muller-Hinton agar (Oxoid) supplemented with 0.68% of sodium thiosulfate and 0.08% of ferric ammonium sulfate. H_2S production of the isolates was confirmed by using TSI agar (Oxoid). The isolates were identified as *E. coli* by using the VITEK-2 System (bioMérieux, France).

Analysis of virulence genes and *sse*A gene

Genes encoding virulence factors and enzymes which involved in H_2S production were investigated by PCR using primers (Table 1). PCR was performed as described previously, with some modifications [19]. DNA templates were prepared by collection of supernatants of bacterial suspension after centrifugation of bacterial suspensions heated at

95°C for 10 min. PCR reaction mixtures were composed of 1 μL of DNA template (10 ng/μL), 4 μL of dNTP (10 mM), 5 μL of 10x PCR buffer, 10 μM of each primer, and 1.25 Unit of *Taq* polymerase (Intron, Korea) and distilled water up to 50 μL. The PCR was carried out by using the following conditions: denatured at 94°C for 3 min, followed by 35 cycles of 94°C for 30 sec, 60°C (virulence genes) and 57°C (*sse*A) for 30 sec, 72°C for 30 sec, and final extension at 72°C for 5 min. The PCR products were visualized by electrophoresis in 1.5% agarose gel with ethidium bromide staining.

Antibiotic susceptibility test

Antibiotic susceptibility test was performed by disk diffusion method according to the recommendations by the National Committee for Clinical Laboratory Standards [18]. The antibiotics included in this study were ampicillin (10 μg/disc), amoxicillin/clavulanic acid (30 μg/disc), cefoxitin (30 μg/disc), cephalothin (30 μg/disc), ceftiofur (30 μg/disc), cefepime (30 μg/disc), chloramphenicol (30 μg/disc), florfenicol (30 μg/disc), colistin (10 μg/disc), gentamycin (10 μg/disc), kanamycin (30 μg/disc), streptomycin (10 μg/disc), nalidixic acid (30 μg/disc), ciprofloxacin (5 μg/disc), enrofloxacin (5 μg/disc), sulfamethoxazole/trimethoprim (25 μg/disc), imipenem (10 μg/disc), and tetracycline (30 μg/disc) (all Oxoid).

Conjugation assay

Transferability of H_2S production was carried out by conjugation assay [12]. Sodium azide resistant *E. coli* J53 strain was used as a recipient. Donor and recipient strains were incubated for 12 h and diluted to 1.0 McFarland scale. Donor and recipient suspensions were mixed at 1 : 5 ratio. The mixtures were incubated at 37°C for 20 h. Mixed cultures were plated into Muller Hinton agar (Oxoid) supplemented with 0.68% of sodium thiosulfate, 0.08% of ferric ammonium sulfate, and 200 μg/mL of sodium azide (Sigma, USA) and incubated for 20 h and observed for production of H_2S through presence of black precipitation on colony.

Pulsed-field gel electrophoresis (PFGE)

PFGE was conducted as previously described with some modifications [12]. In brief, all *E. coli* strains were incubated on blood agar plate at 37°C for 16 h and suspended in 3 mL of cell suspension buffer (100 mM Tris : 100 mM EDTA, pH 8.0) for 4.0 McFarland turbidity standard. 200 μL of bacterial suspensions were mixed with 200 μL of 1% Seakem Gold PFGE agarose (Lonza, USA) and poured into the molds. After bacterial plug became solid, lysis reaction was conducted with cell lysis buffer (50 mM Tris : 50 mM EDTA, pH 8.0, 1% Sarcocyl) at 55°C for 2 h. After lysis reaction, plugs were washed twice with distilled water at 55°C for 15 min and washed 4 times with TE buffer (10 mM Tris : 1 mM EDTA, pH 8.0) at 55°C for 15 min. Restriction enzyme reaction was carried out with 50 U of *Xba* I (Takara Bio, Japan) at 37°C for 2 h. Electrophoresis was performed by using the CHEF Mapper system (Bio-Rad Laboratories, USA). Electrophoresis were performed with 0.5× TBE buffer at 14°C for 18 h. Lamda ladder PFGE marker (New England BioLabs, USA) was used as a standard. After electrophoresis, gel images were obtained by Gel Doc system and analyzed using the GelCompar II software (Applied Maths, Belgium).

Sequencing of *sse*A gene

Sequencing of *sse*A gene was performed with PCR amplified products using a 3730xl DNA analyzer (Applied Biosystems, USA). The sequences of *sse*A gene were compared to GenBank database using Blast/blastn algorithm (National Center for Biotechnology Information, USA).

Statistical analysis

Differences of antibiotic resistance rate between H_2S-producing and H_2S-non producing *E. coli* were statistically analyzed using the Chi-square test with EXCEL software (Microsoft, USA). *P* values of < 0.05 were considered as statistically significant.

Results

Isolation and identification of *E. coli*

Sixteen H_2S producing and 32 H_2S non-producing *E. coli* were isolated from 56 swine fecal samples (Table 2). H_2S production of the isolates was different depending on the media. The production was detected with all H_2S producing *E. coli* in Mueller Hinton agar supplemented with sodium thiosulfate and ferric ammonium sulfate. But the production was not observed in some isolates with triple sugar iron (TSI) agar.

Table 2. H_2S production and virulence genes of *E. coli* isolates from swine feces

Strains	Production of H_2S		Number (%) of isolates which harbored virulence genes									
	MHA-H_2S	TSI	*Stx2*	*EAST1*	*F18*	*STb*	*AIDA-I*	*STa*	*eaeA*	*LT*	*F4*	*PAA*
H_2S producing strain	16 (100)	8 (50)	0 (0)	5 (31.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
H_2S non-producing strain	0 (0)	0 (0)	3 (9.38)	10 (31.3)	3 (9.38)	6 (18.8)	4 (12.5)	1 (3.1)	1 (3.1)	3 (9.38)	3 (9.38)	3 (9.38)

Prevalence of gene encoding virulence factors and *sse*A

EAST1 gene was detected in five H_2S producing *E. coli* isolates harbored (31.3%) (Table 2). Other virulence genes were not detected in H_2S producing *E. coli* isolates. But, 16 H_2S non-producing *E. coli* isolates harbored several virulence genes. The frequency of virulence genes was as below: *EAST1* (31.3%), *STb* (18.8%), *AIDA-I* (12.5%), *F18* (9.38%), *Stx2* (9.38%), *PAA* (9.38%), *LT* (9.38%), *F4* (9.38%), *STa* (3.1%), and *eae*A (3.1%). *Stx1, F5, F6,* and *F41* genes were not detected (data not shown). *sse*A gene was detected in both H_2S producing and non-producing strains. The sequences of *sse*A gene motif CGSVTA around the Cys238 were also identical regardless of H_2S production. Furthermore, the gene was not transmissible in the conjugation assay.

Antibiotic resistance rates

Generally, antibiotics resistance rates of H_2S producing *E. coli* isolates against each antimicrobial agent were various compared to H_2S non-producing *E. coli* isolates (Table 3). Higher resistance rate of H_2S producing *E. coli* isolates were observed in ampicillin, gentamycin, chloramphenicol, streptomycin, ciprofloxacin, enrofloxacin, sulfamethoxazole/trimethoprim, and tetracycline. The antibiotic resistance rates against amoxycillin/clavulanic acid, cephalothin, florfenicol, kanamycin, and nalidixic acid were higher in non-producing *E. coli* isolates than H_2S producing *E. coli* isolates. Only cephalothin showed statistically significant difference between H_2S producing and non-producing strains ($p < 0.05$). All strains were susceptible to second or third generation of cephalosporins such as cefoxitin, ceftiofur, and cefepime. In addition, none of the strains was resistance to colistin and imipenem.

Table 3. Antibiotic resistance of H_2S producing and non-producing *E. coli* isolates

Antibiotics	Number (%) of isolates resistant to antibiotics	
	H_2S producing strain	H_2S non-producing strain
Ampicillin	15 (93.8)	27 (84.4)
Amoxycillin/Clavulanic acid	8 (50)	20 (62.5)
Cefoxitin	0 (0)	0 (0)
Cephalothin	8 (50)	26 (81.3)*
Ceftiofur	0 (0)	0 (0)
Cefepime	0 (0)	0 (0)
Chloramphenicol	12 (75)	23 (71.9)
Florfenicol	11 (68.8)	23 (71.9)
Colistin	0 (0)	0 (0)
Gentamycin	7 (43.8)	8 (25)
Kanamycin	5 (31.3)	20 (62.5)
Streptomycin	16 (100)	26 (81.3)
Nalidixic acid	5 (31.3)	17 (53.1)
Ciprofloxacin	4 (25)	4 (12.5)
Enrofloxacin	4 (25)	5 (15.6)
Sulfamethoxazole/Trimethoprim	9 (56.3)	13 (40.6)
Imipenem	0 (0)	0 (0)
Tetracycline	13 (87.5)	24 (75)

*vs. H_2S producing strain

Genetic relatedness

Total 48 strains of H_2S producing and non-producing *E. coli* were analyzed for genetic relatedness by PFGE. Forty-one distinctive PFGE patterns were identified in 45 *E. coli* isolates (Fig. 1) and 3 strains were confirmed non-typable. PFGE pattern was not related with ability of H_2S production and virulence genes in this analysis. Strains which isolated in same region showed relatively similar PFGE pattern compared to other strains, but it was not observed in all strains.

Discussion

Although H_2S was produced by several Enterobacteriace, *E. coli* has been recognized as H_2S non-producing bacteria. However, H_2S producing *E. coli* isolates were reported and characterized in several previous studies [4, 7, 16, 17, 24]. But still some characteristics were not investigated in the previous studies including genetic relatedness and virulence genes. Therefore, H_2S producing *E. coli* were isolated and investigated the presence of virulence genes, genetic relatedness, and antibiotic resistance pattern.

EAST1 gene was detected in five H_2S producing *E. coli* isolates. But *EAST1* gene was also detected in non-producing *E. coli* isolates. Other virulence genes were not detected in H_2S producing *E. coli* isolates. This result suggested that H_2S production was not associated with virulence genes.

H_2S production and transferability of H_2S producing ability were opposed with a previous study [9]. In our result, the ability of H_2S production was not transferable. This phenomenon might be the different location of the genes in our isolates such as chromosome or non-transmissible plasmid which are different with a previous research [9]. Difference in H_2S production was observed in TSI agar. Eight strains were not produced H_2S in TSI agar. This phenomenon is coincided with a previous study showing H_2S producing and lactose fermenting enteric bacteria cannot produce black colony in TSI agar due to acid production [16]. These findings suggested that bacterial H_2S production can be inhibited by fermentation of lactose.

PFGE analysis of 45 strains of *E. coli* isolates indicated diversity of the isolates not showing any relatedness in H_2S production. This diversity in genotypes of the isolates suggested that H_2S producing strains might not belong to specific genetic clone. This relatedness was also observed in virulence genes.

As described above, H_2S protects bacteria from antibiotics through inhibition of oxidative stress [24]. Thus, H_2S produc-

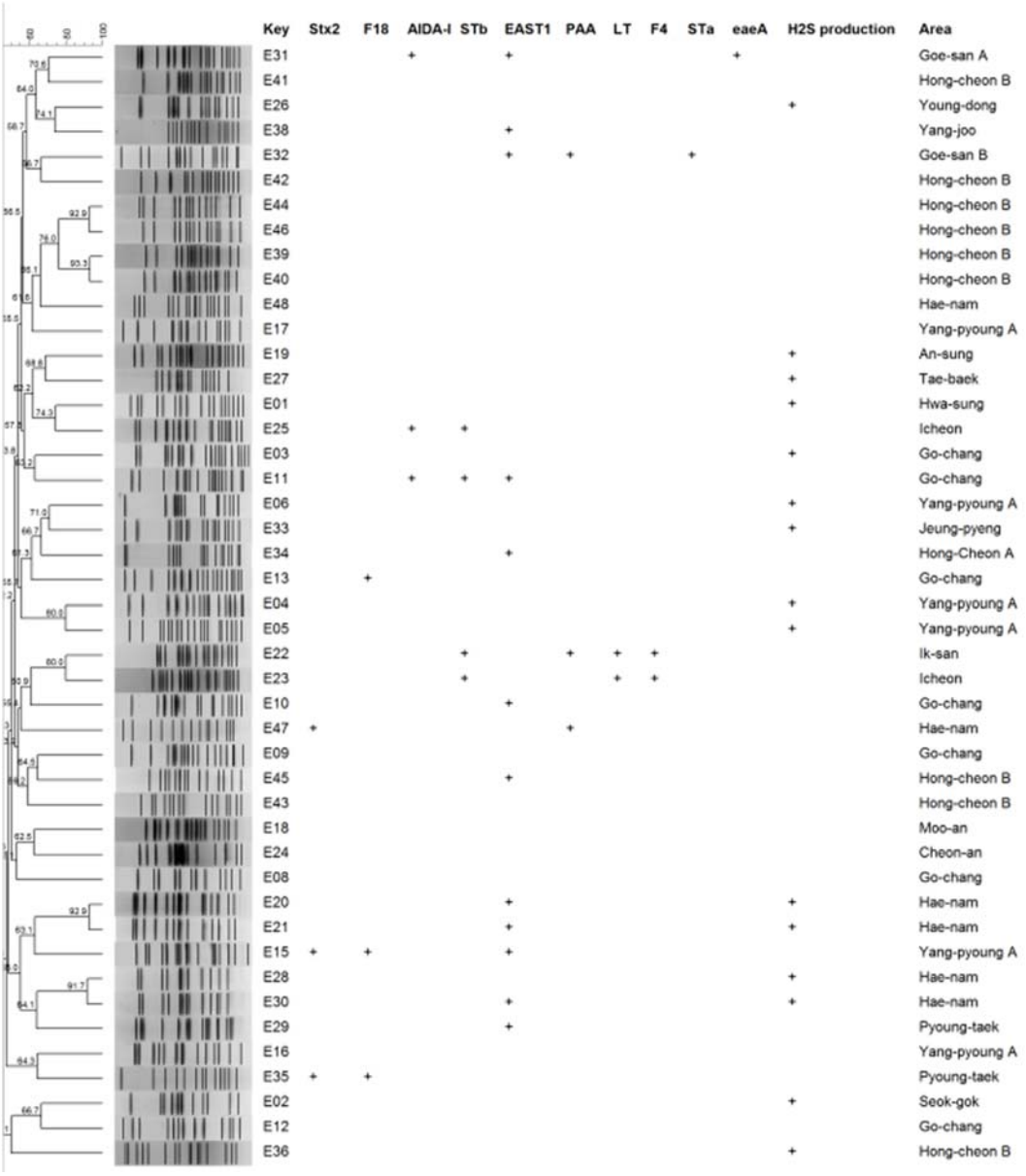

Fig. 1. Dendrogram of PFGE patterns of *E. coli* isolates from swine feces after digestion with *Xba* I.

ing strains were expected to more resistant to antibiotics than non-producing strain. Other study showed that H_2S producing strains have higher antibiotic resistance rate than non-producing strains [7]. But our result was not fully matched with previous study. Higher resistant rates were shown in 8 antibiotics (ampicillin, gentamycin, chloramphenicol, streptomycin, ciprofloxacin, enrofloxacin, sulfamethoxazole/trimethoprim, and tetracycline) in H_2S producing strains compared with non-producing strains. However, 5 antibiotics (amoxycillin/clavulanic acid, cephalothin, florfenicol, kanamycin, and nalidixic acid) were more resistant in non-producing *E. coli* isolates than H_2S producing *E. coli* isolates. In statistical analysis, only cephalothin showed significant difference between H_2S producing and non-producing strains. This phenomenon might be related to presence of antibiotic resistance genes. Cellular protection effect of H_2S against antibiotics can be covered due to influence of antibiotic resistance genes. Therefore, presence of antibiotic resistance genes in H_2S producing *E. coli* isolates should be investigated to clarify the relationship between antibiotic resistance and H_2S production. Furthermore, comparison of antibiotic susceptibility between *sse*A deficient *E. coli* and wild type *E. coli* should be needed to reveal the correlation between antibiotic resistance and H_2S production.

*sse*A gene was detected in all isolates regardless of H_2S production. Also, sequence of the *sse*A gene at motif CGSGVTA around the catalytic cysteine (Cys238) was identical. These findings are contrasted with a previous research show-

ing substitution of Ser240 with lysine in *sse*A gene causing increase of H_2S production with thiosulfate [3]. H_2S production in *E. coli* mainly occured through *sse*A dependent pathway, but there is an alternative pathway which regulated by other genes such as *cys*N, *cys*D, *cys*C, and *cys*JIH operon [1]. The genes *cyc*N, *cys*D, and *cys*C produce enzymes such as sulfate adenylyltransferase, subunit 1, sulfate adenylyltransferase, subunit 2, and adenosine 5'-phosphosulfate kinase, respectively. *cysJIH* operon produces enzymes such as sulfite reductase beta subunit (*cys*I), sulfite reductase alpha subunit (*cys*J), and phosphoadenosine phosphosulfate reductase (*cys*H) [1]. Mutation of gene can change activity of enzyme [3]. We assumed that production of H_2S in *E. coli* might be related to mutation of *cys* genes. Therefore, sequences of the genes such as *cys*N, *cys*D, *cys*C, and *cys*JIH operon should be investigated to understand the H_2S production *E. coli* in further research.

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