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Different Loci and mRNA Copy Number of the Increased Serum Survival Gene of
Escherichia coli

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Abstract

The increased serum survival gene (*iss*) has been identified as a virulence trait associated with the virulence of *Escherichia coli*, causing colibacillosis in poultry. However, it remains unclear as to whether *iss* mRNA copy number and sequence affect the virulence. To examine these influences, we assessed the presence of *iss*, sequence analysis, *iss* mRNA copy number, and serum resistance. *iss* was detected in (all) 88 *E. coli* isolates from different sources, and sequencing identified 16 alleles (32 different loci) and 10 amino acid sequences (10 different loci). Nested polymerase chain reaction improved *iss* detection. The isolates from sick chicken had >68% livability in serum resistance tests and higher *iss* mRNA copy number. *iss* mRNA copy number highly correlated with mortality and *E. coli* livability. T-tests confirmed the relationship between the different loci to *iss* transcription, serum resistance, and virulence. These data suggest that *iss* mRNA copy number and different loci affect the virulence and serum resistance. These findings could be useful in further studies on the prevalence of *iss* among *E. coli* isolates and other virulence factors.

Key words: Colibacillosis, different loci, increased serum survival gene, real-time quantitative PCR, serum resistance

Introduction

Colibacillosis, a major pathogenic disease in poultry, is caused by some strains of *Escherichia coli*. Several factors, such as F1 fimbriae (type 1), P fimbriae, curli, aerobactin iron-sequestering systems, capsules, temperature-sensitive hemagglutinin, and resistance to the bactericidal effects of serum, toxins, and cytotoxins, influence the virulence of avian *E. coli* (Ellis et al. 1988; Ike et al. 1992; Nolan et al. 1992; Dho-Moulin and Fairbrother 1999; Mellata et al. 2003). The bacteria must overcome host defense mechanisms in the bloodstream, including the bactericidal effects of the serum complement. The increased serum survival gene (*iss*), encoded on the ColV plasmid, was first discovered in 1979 and was considered to be associated with serum resistance (Binns et al. 1979). *iss*, spanning 309 bp, encodes an outer membrane protein. Many studies have suggested that *iss* is frequently expressed by virulent strains (Johnson et al. 2007; Bonnet et al. 2009; Jeong et al. 2012; Knöbl et al. 2012; Oh et al. 2012; Mohamed et al. 2014; Radwan et al. 2014; Cyويا et al. 2015). Its protein product (Iss) was predicted to target the bacterial outer membrane and contribute to serum resistance by restricting C3 deposition on the bacterial outer membrane, thus blocking the membrane attack complex (Binns et al. 1982). Using polymerase chain reaction (PCR) or multiplex PCR, the positive rates for the gene varied in most studies (Johnson et al. 2008; Johnson et al. 2008b; Knöbl et al. 2012; Oh et al.

2012; Radwan et al. 2014; Cyويا et al. 2015). However, we observed that all avian *E. coli* strains may carry *iss* gene and that *iss* may be ubiquitous in *E. coli*. It is unclear whether the presence of *iss*, its nucleic acid sequence, or its transcription level affects the role of this gene in *E. coli*.

This study documents the presence of *iss* and confirms the contribution of *iss* to the virulence and serum resistance of avian *E. coli*. To achieve the aims of this study, *E. coli* strains were isolated from chicken farms located in different parts of China, and nested PCR, sequencing, real-time quantitative PCR (RT-qPCR), and complement resistance assays were performed to examine the effects of the nucleic acid sequence and transcription level of the gene on avian *E. coli* pathogenicity.

Materials and methods

Bacterial strains

Strains of CVCC1551, CVCC1553, CVCC1558, and CVCC1568 were obtained from the China Institute of Veterinary Drugs Control and were stored in our lab and were respectively collected from Guangdong, Tianjin, and Guangxi regions of China. Other strains were acquired from Beijing, Xinjiang, Shandong, and Heilongjiang regions of China and stored in our lab. The strains CVCC1551, CVCC1553, CVCC1558, CVCC1568,

O1, O2, O78, Beijing1, Beijing3, Xinda, and Tianda were recovered from chickens with colibacillosis and were considered to be avian pathogenic *E. coli* (APEC) strains by the China Institute of Veterinary Drugs Control. Other strains recovered from healthy chickens, namely, HL10, HL16, SD2, SD6, SD26, and E11, were considered to be APEC strains by the Agricultural College of Liaocheng University; the remaining strains were not considered to be APEC strains (some of which have been described in a study by Fan et al. 2010). All strains had lethality ranging from 0% to 100% (previous data of our laboratory and some described by Fan et al. 2010). Chicken SPF (specific pathogen-free) serum was purchased from Taikang Biotechnology Co. Ltd, Xi'an, China.

Reagents

RNAprep pure Cell/Bacteria Kit was purchased from Tiangen Biotech (Beijing) Co., Ltd. SYBR® Green real-time PCR master mix was purchased from Toyobo Co., Ltd. Shanghai, China. LIVE/DEAD BacLight Bacterial Viability Kit was purchased from Thermo Fisher Scientific Co. Ltd, Shanghai, China.

Human and animal rights

This study did not include any experiments on human participants or animals performed by any of the authors.

iss sequencing

iss was amplified by nested PCR using primers previously described by Fan et al. (2010) (Table 1). The products of nested PCR were used for sequencing. The extraction of DNA templates was performed as follows. Single colonies were incubated in 40 µL of lysis buffer (10 mmol/L Tris-Cl, 1 mmol/L EDTA, 50 µg/mL proteinase K, pH 7.5). The mixture was treated at 55°C for 10 min and at 80°C for 10 min before being diluted with 80 µL of sterile ddH₂O. The one-step amplification program included a cycle of 97°C for 5 min; nine cycles of 94°C for 1 min, 47°C for 1 min, and 68°C for 30 s; 25 cycles of 95°C for 1 min, 47°C for 1 min, and 68°C for 30 s; and a cycle of 68°C for 10 min. The nested amplification program included a cycle of 95°C for 5 min; 28 cycles of 95°C for 1 min, 47°C for 1 min, and 68°C for 30 s; and a cycle of 68°C for 10 min.

Serum resistance assay

Chicken SPF plasma (final concentration, 10%) was mixed with washed cells from an exponentially growing bacterial culture (Gibbs et al. 2003). The bacteria in the plasma were incubated in a 37°C water bath for 1 h without shaking. Reactions were stopped in an ice bath, and bacterial cells were collected by centrifugation, washed, and resuspended in 0.85% NaCl. Cells were stained using a LIVE/DEAD BacLight Bacterial Viability Kit (Thermo Fisher Scientific Co. Ltd, Shanghai, China), and stained samples were examined using a Becton Dickinson FACSCalibur flow cytometer to determine the percentage of live bacteria (Jeffrey et al. 2002).

RNA isolation and RT-qPCR

RNA extraction was performed using an RNAprep pure Cell/Bacteria Kit (Tiangen Biotech Co., Ltd. Beijing, China) according to the manufacturer's instructions. Primers for RT-qPCR were referred from Skyberg et al. (2008) (Table 2). RT-qPCR was performed using the Bio-Rad MiniOpticon Real-Time PCR System. Twenty microliter reaction mixtures contained 10 µL of SYBR® Green real-time PCR master mix (Toyobo Co., Ltd. Shanghai, China), 0.6 µL of each primer (10 µM), 0.8 µL of cDNA, and 8 µL of ddH₂O. The amplification program included a cycle of 95°C for 3 min and 39 cycles of 95°C for 10 s and 56°C for 0.5 min. Melting curve analyses were performed after each reaction to ensure amplification specificity. RNA levels were normalized using the housekeeping gene GAPDH as a control.

Statistical methods

The chi-squared test was used to compare serum resistance, virulence, *iss* nucleic acid sequence, and *iss* mRNA copy number. Sequence analyses were performed using the *iss* aligning results. *iss* mRNA copy number analyses were performed using IgC. The t-test was used to determine the relationship between different loci to serum resistance, virulence, and *iss* mRNA copy number (Jeffrey et al. 2002).

Results

Sequencing results

We detected *iss* in 88 *E. coli* strains by nested PCR and *iss* sequencing. The results showed that all strains carry the *iss* gene. Sequencing revealed 16 alleles (32 different loci) and 10 amino acid sequences (10 different loci) (Figs. 1-2).

Using one-step PCR to detect *iss* in *E. coli* strains, 67 strains were found to exhibit a 416-bp band, providing a positivity rate of 76.14% (67/88). In this study, these strains were described as *iss*⁺ strains. However, using nested PCR, 88 strains were found to exhibit a 363-bp band. Twenty-one strains were *iss*[−] as detected using PCR (one-step PCR) and *iss*⁺ as detected using nested PCR. In this study, these strains were described as *iss*^{*} strains. The amplified bands were identified as *iss* gene by sequencing. Thus, (all) 88 strains were found to possess *iss*, and *iss* sequences of 88 strains were obtained. In conclusion, all strains were confirmed to carry *iss*. However, the mean lethality of *iss*^{*} strains was 40.0%, which was lower than that of *iss*⁺ strains (65.2%) (Table 3). The t-test results revealed that this difference was not significant ($p = 0.127$).

Eighty-eight strains had 16 *iss* sequences, which were named types 1–16, and 10 amino acid sequences, which were named forms 1–10. The differences among the types and

forms are illustrated in Figs. 1-2. Meanwhile, the homology of the nucleic acid sequence exceeded 93.5% and that of the amino acid sequence exceeded 96.1%. The correlation between the sequence homology and lethality was found to be significant ($p = 0.003$) (Table 3).

Compared with the reference sequence (open reading frame of the *iss* sequence from GenBank accession number AF042279.1), 48/88 (54.5%) of the sequences were identical. In particular, of 11 sequences obtained from sick chickens, 10 (90.9%) were identical. The *iss* sequence is mostly conserved. The locations and types of changes were similar, mostly involving G to A, A to T, C to T, and G to C changes (Fig. 1). Moreover, only two kinds of nucleotides were present at each different loci, where the nucleotide of the reference sequence was the dominant one. For example, 87 strains had T at the second site (identical to the reference sequence); one strain had C. The results further showed that the *iss* sequence was conserved. Thirty strains were randomly selected from the 88 strains for serum resistance and mRNA copy number experiments. In these 30 random strains, the nucleotide of the reference sequence was still dominant. For example, 29 strains had T at the second site. There were 32 different loci in the nucleic acid sequence, leading to 10 different loci in the amino acid sequence. The changes in nucleic acid sequence resulted in the replacement of only one or two amino acids in each *iss* gene (Fig. 2). The reference sequence was dominant.

Because of the limitation of the sample number for statistical analyses, the t-test was partly performed with different loci. In the 13 sites assessed using t-test, the change in 264th nucleotide was corresponding to the change in the 88th amino acid. Remaining 12 different sites did not exhibit changes in amino acids. The mean lethality of strains with dominant nucleotide (identical to the reference sequence) was higher than that of other strains at each different loci. The t-test results showed that each different loci was significantly related to the virulence of strains (separately $p = 0.005$, $p = 0.002$, $p = 0.008$) (Table 4).

Serum resistance

We tested the serum resistance of the 30 strains using flow cytometry. The results were expressed as livability (Table 3). The strains from sick chickens had 68%–99% livability, and those from healthy chickens had 17%–90% livability. The correlation between livability and lethality was significant ($p = 0.011$). The serum resistance was not significantly associated with homology ($p = 0.105$). The mean livability of *iss** strains was 56% and was significantly lower than the mean livability of *iss*+ strains, which was 76% ($p = 0.039$) (Table 3). The mean livability of the strains with dominant nucleotide was higher than that of others. The t-test results showed that the 135th different loci was significantly related to the serum resistance of strains ($p = 0.003$) (Table 4).

iss mRNA copy number

We tested the *iss* transcription of 48 strains (30 randomly selected strains and 18 strains covered all amino acid sequence Form1-10). lgC determines the relative mRNA expression. The lowest lgC was -7.34 in 48 strains. The lowest lgC was -4.55 in the strains from sick chickens, and 90.9% of the strains from sick chickens had greater than -2.72 lgC. The mean lgC of *iss*⁺ strains was -4.39 , which was lower than -2.31 (the mean lgC of *iss*⁺ strains) (Table 2). The t-test results revealed this difference to be significant ($p = 0.003$). The correlations between lgC (Table 2) and mortality (Table 3) ($p = 0.000$), between lgC and livability ($p = 0.000$), and between lgC and homology ($p = 0.008$) were found to be significant. The mean lgC of strains with dominant nucleotide was higher than that of others. The t-test results showed that each different loci was significantly related to lgC of strains (separately $p = 0.015$, $p = 0.008$, $p = 0.004$) (Table 4).

Discussion

iss is considered to be a feature of pathogenic *E. coli*, and the bacterium is classified as *iss*⁺ or *iss*[−] (Johnson et al. 2007; Bonnet et al. 2009; Jeong et al. 2012). Moreover, *iss* is

1 considered to enhance serum resistance, thereby enhancing virulence (Binns et al. 1979;
2 Binns et al. 1982; Gibbs et al. 2003; Bonnet et al. 2009). Most studies have focused on the
3 relationship between the presence of *iss* and the virulence of *E. coli*; however, the effect of
4 the sequence and transcription level of *iss* on the virulence of *E. coli* is unclear. This gene
5 was found in 72.2% of the examined extraintestinal pathogenic *E. coli* (ExPEC) strains and
6 0% of the avian pathogenic *E. coli* (APEC) strains (Mohamed et al. 2014). Jeong et al. (2012)
7 found that 78.2% of 101 *E. coli* strains carried *iss*. Most studies were based on PCR or
8 multiplex PCR. In our study, *iss* was found in 76.14% of strains using PCR (which was
9 similar to the results obtained by the above researchers) and in 100% of strains using
10 nested PCR. Amplification is higher in nested PCR than in PCR, and the inner nested
11 primers do not overlap with the outside primers, thus increasing the amplification of the
12 specific target fragment and reducing the false-positive result. Nested PCR can improve the
13 detection rate and accuracy. In addition, *iss* was found in 100% of strains (0%–100%
14 mortality), which is different from the current finding. We speculated that the sensitivity of
15 the detection method and conditions partly influence the results on the one hand. In our
16 previous study, KOD DNA polymerase was associated with higher detection rates than
17 rTaq DNA polymerase. On the other hand, because the copy number of *iss* encoded on the
18 ColV plasmid was different among strains, substantial differences in the amplification
19 template amount were observed. However, according to a t-test for the significance of *iss*+

strain (detected using PCR) in terms of transcription ($p = 0.003$) and serum resistance ($p = 0.039$), PCR can be acceptable in predicting the strain serum resistance. (Note that the *iss*-result indicates only low copy numbers and not the absence of the gene.) The isolates of this study were collected from Heilongjiang, Beijing, Xinjiang, Tianjin, Guangdong, and Guangxi, covering Northeast China, southern China, central China, East China, and other regions. The sources of the isolates included diseased and healthy chickens. Our results suggested that *iss* may be widely present in *E. coli* strains but with varying copy number.

The *iss* gene shared high identity and strong similarities with the *bor* gene (Johnson et al. 2008b; Salehi et al. 2013). To the best of our knowledge, there is no analysis on the relationship of different *iss* loci with virulence, serum resistance, and transcription. The aligning results in our study revealed that *iss* was conserved, which is in line with current research. We examined the influence of some different loci in the sequence, which were significantly related to transcription, virulence, and serum resistance. We speculated that the amino acid mutation results in a change in the position of the protein on the cell membrane due to some currently unknown mechanism that warrants further investigations. Moreover, most nucleotide sites did not cause changes in amino acids but still significantly reduced gene transcription, serum resistance, and virulence, such as those at 135th site.

We tested the transcriptional levels of the *iss* gene in 48 strains. The researchers observed reductions in virulence, with reducing transcription of *rfaH* gene, *curl* gene, and

LEE pathogenicity island in *E. coli* O157:H7 and APEC E058 strain (Gao et al. 2013; Hamner et al. 2013; Lee et al. 2014). The transcription level of some genes is suggested to affect the virulence of the strain. Similarly, we observed low virulence, with low transcription levels of *iss* gene in *E. coli* strains. Many studies deleted or replaced *iss* to test its effects (Skyberg et al. 2008; Salari et al. 2014). In our study, the logarithm of *iss* mRNA copy number highly correlated with mortality ($p = 0.000$) and *E. coli* livability ($p = 0.000$). Transcription may explain the mechanism by which *iss* influences serum resistance and virulence. The transcription level of *iss* mRNA could be a predictor of *E. coli* virulence.

Although the presence of *iss* is currently a good marker for virulence in *E. coli*, all strains tested in this study carried this gene. According to our results, the *iss* gene has a widespread presence and conserved sequence of the gene. *iss* mRNA copy numbers influence serum resistance and virulence. Further, the different loci of nucleotides affect the *iss* gene expression, which changes the virulence and serum resistance of the strains. Our findings offered new insights with some data for studying virulence genes. Further research on gene transcription and different loci is warranted, as it is more practical than simple gene detection, to study the relation between genes and virulence. This study emphasized that nested PCR is more accurate in prevalence investigation of a gene. Gene transcription and different loci could be useful for future virulence factor researches. A new perspective of implementing these findings will be useful in the poultry industry.

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Financial/Commercial Conflicts of Interest

There are no conflicts of interest to declare.

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Figure Legends

Fig. 1 Nucleotide alignment of the results of the *iss* sequence

Fig. 2 Alignment results of the amino acid sequence of *iss*

Draft

1 Table 1 Primer sequences for amplification and RT-qPCR

Name		Primers
<i>iss</i> primers in traditional PCR*	Forward	5'-AGCTATCGTTTAATTATTATCAC-3'
	Reverse	5'-GTAGGGAGCCCAGAAGTAT-3'
<i>iss</i> primers in nested PCR*	Forward	5'-CGCGGATCCAGGATTCTGCCGTTTTTA-3'
	Reverse	5'-CGCGTCGACCATATCGATGGGCAACTA-3'
<i>iss</i> primers in RT-qPCR†	Forward	5'-GCCGCTCTGGCAATGCTTATTACA-3'
	Reverse	5'-TCCTTTGGTGTTACTGCTGTCGGT-3'
GAPDH primers in RT-qPCR†	Forward	5'-CATCGTTTCCAACGCTTCCT-3'
	Reverse	5'-ACCTTCGATGATGCCGAAGTT-3'

2 *the primers used were as described by Fan et al. 2010.

3 †the primers used were as described by Skyberg et al. 2008

1 Table 2 RT-qPCR results

Strains	lgC	Form	Strains	lgC	Form	Strains	lgC	Form
O1	-2.24	1	HL17	-0.79	1	SD34*	-2.84	6
O2	-1.11	1	HL18	-0.94	1	SD35*	-7.34	6
O78*	-2.72	3	HL19	-1.66	1	E1*	-1.05	10
HL1	-5.98	6	HL26	-3.85	1	E4*	-6.02	4
HL2	-1.77	6	HL33*	-6.86	7	E10	-0.69	1
HL3	-0.71	1	SD2*	-1.56	8	E11	-2.43	1
HL4	-0.67	1	SD3	-0.03	7	E21	-2.58	2
HL5	-1.57	7	SD5	-4.08	1	SL3*	-5.14	6
HL6	-1.27	7	SD6	-3.02	6	CVCC1551	-1.47	1
HL7	-6.40	1	SD10*	-4.69	--	CVCC1553	-0.96	1
HL10	-1.28	1	SD11*	-4.63	--	CVCC1562	-1.10	1
HL11	-5.42	5	SD13	-2.15	7	CVCC1568	-4.55	1
HL12	-5.73	6	SD20*	-5.48	--	Beijing 1	-1.40	1
HL13	-6.14	6	SD26	-0.98	9	Beijing 3	-1.59	1
HL14	-5.29	6	SD29	-1.14	1	Xinda	-0.78	1
HL16	-1.38	1	SD31	-2.54	6	Tianda	0.04	1

*Strains were *iss*[−] as detected using PCR (one-step PCR) and *iss*⁺ as detected using nested PCR. The strains with asterisk were described as *iss*[−]* strains in this study. (Other strains without an asterisk were *iss*⁺ as detected using one-step PCR. These strains were described as *iss*⁺ strains.)

IgC determines the base 10 of the relative mRNA expression value. Strains had 10 amino acid sequences, which were named forms 1–10. Strains were acquired from the Guangdong, Tianjin, Guangxi, Beijing, Xinjiang, Shandong, and Heilongjiang regions of China and were stored in our laboratory.

1 Table 3 Serum resistance, sequence, and lethality

Name	Mortality rates [†]	Livability	Type	Form	Name	Mortality rates [†]	Livability	Type	Form
O1	100%	68%	1	1	SD13	20%	90%	11	7
O2	100%	88%	1	1	SD26	80%	85%	15	9
O78*	100%	84%	3	3	SD29	60%	84%	1	1
HL1	0%	29%	13	6	SD31	20%	80%	13	6
HL7	40%	17%	1	1	SD34*	40%	34%	13	6
HL10	80%	90%	1	1	SD35*	0%	24%	13	6
HL13	20%	87%	13	6	E4*	20%	32%	4	4
HL14	60%	55%	13	6	E11	100%	81%	1	1
HL16	100%	84%	1	1	SL3*	20%	45%	13	6
HL19	40%	79%	1	1	CVCC1551	100%	77%	1	1
HL26	40%	89%	1	1	CVCC1553	100%	74%	1	1
HL33*	0%	86%	10	7	CVCC1562	100%	84%	1	1
SD2*	100%	87%	14	8	Beijing 1	80%	99%	1	1
SD5	0%	79%	1	1	Beijing 3	80%	88%	1	1
SD6	80%	63%	13	6	Xinda	100%	84%	1	1

2 *Strains were *iss*− as detected using PCR (one-step PCR) and *iss*+ as detected using
3 nested PCR. These strains with asterisk were described as *iss** strains. (Other strains
4 without an asterisk were *iss*+ as detected using one-step PCR. These strains were described
5 as *iss*+ strains.)

1 [†]Our previous laboratory data. Mortality rates of O1, O2, E11, Beijing3 were described
2 by Fan et al. 2010, others were not published yet.

3 Eighty-eight strains had 16 *iss* sequences, which were named types 1–16, and 10 amino
4 acid sequences, which were named forms 1–10. Strains were acquired from the Guangdong,
5 Tianjin, Guangxi, Beijing, Xinjiang, Shandong, and Heilongjiang regions of China and
6 were stored in our laboratory. Thirty strains were randomly selected from all the strains for
7 serum resistance and mRNA copy number experiments.

8

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1 Table 4 Different loci and t-test results

Loci	Mean of mortality*	T-test	Mean of livability*	T-test	Mean of lgC*	T-test
30, 129, 222	74.4%, 36.7%	0.005	76.7%, 63.8%	0.132	-2.31, -4.15	0.015
75, 195,	74.7%, 32.7%	0.002	77.2%, 61.8%	0.078	-2.24, -4.44	0.008
135	70.0%, 30.0%	0.008	78.6%, 52.1%	0.003	-2.42, -4.79	0.004

2 *The first value was the mean of strains with dominant nucleotide at different loci; the
 3 second value was the mean of other strains.

4

5

Fig. 1 Nucleotide alignment of the results of the *iss* sequence

182x261mm (300 x 300 DPI)

Fig. 2 Alignment results of the amino acid sequence of iss

182x66mm (300 x 300 DPI)