Original Article

Comparison of *iss* Virulence Gene Prevalence in Milk Samples From Clinical and Subclinical *Escherichia coli* Mastitis Cases

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ABSTRACT

Background: Coliform mastitis is common in dairy herds, especially in high-producing cows. The isolation rate of the increased serum survival (*iss*) gene from mild and severe cases was compared in this study as an indicator of *Escherichia coli* virulence factor.

Objectives: This study aimed to evaluate the importance of the *iss* gene and some affecting factors in bovine coliform mastitis caused by *E. coli.*

Methods: The milk samples of 80 clinical and 20 subclinical mastitis cases infected with *E. coli* were collected from Holstein dairy farms. In the laboratory, the colony-forming unit (CFU) of isolated *E. coli* was determined, and the presence of the *iss* gene in the bacteria was analyzed with polymerase chain reaction (PCR). The isolates were divided into the control (*E. coli* count<500 CFU) and the case (*E. coli* count >500 CFU) groups.

Results: The *iss* gene was detected in 13 of 45 isolates (29%) in the control group and 11 of 55 isolates (20%) in the case group (P>0.05). Mastitis grade, *E. coli* colony count, and somatic cell count (SCC) did not show any difference between the groups with and without the *iss* gene (P>0.05). The average milk production levels before the presence of mastitis in cases with the *iss* gene were lower than those without the *iss* gene only in the case group (P<0.05). In the case group, a correlation was found between *iss* gene expression and hot summer months, when heat may kill bacteria or inhibit their growth (r=0.465, P=0.01). There was no correlation between the expression of the *iss* gene and hot summer months in the control group (r=-0.334, P=0.054).

Conclusion: This study concluded that the role of the *iss* gene in the occurrence of severe mastitis requires further investigation. However, the presence of the gene under conditions of high milk-production stress in cows, along with the threat to bacterial growth posed by summer heat, was important in the development of mastitis cases.

Keywords: Coliform mastitis, Dairy cows, *E. coli*, Serum survival virulence gene (*iss*)

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Introduction

Mastitis is considered the most common and costly disease in the dairy cattle industry (Guimarães et al., 2017; Verma & Ambatipudi, 2016; Dalanezi et al., 2020).

Coliform clinical mastitis is the most important mastitis problem in well-managed dairy cattle, which generally have low somatic cell counts (SCCs) (Constable et al., 2017; Maity & Ambatipudi, 2019; Maity & Ambatipudi, 2021; Algharib et al., 2024), and is one of the most common causes of peracute and fatal mastitis in dairy cows. *Escherichia coli* not only has other bacterial effects on reproduction (Constable et al., 2017), but, according to the findings of Ibrahim et al. (2023), it also reduces progesterone through lipopolysaccharides, which in turn decreases fertility. The increased possibility of bacterial proliferation in wet cow bedding in cases of *Klebsiella pneumoniae*, as well as the presence of stressful conditions such as parturition in cases of *E. coli*, contribute to increased incidence and severity of coliform mastitis (Constable et al., 2017; Zigo et al., 2019). Different serotypes of *E. coli* are responsible for coliform mastitis in cattle (Constable et al., 2017). The ability of *E. coli* to acquire exogenous DNA, including virulence genes, contributes to the development of pathogenic strains adapted to the mammary gland and potentially causes mastitis. Mammary pathogenic *E. coli* (MPEC) has been proposed as a new pathotype responsible for causing mastitis in dairy animals (Goulart & Mellata, 2022).

Some differences between environmental factors (the ineffective in causing mastitis) and mastitis-causative *E. coli* have been reported in many studies (Zigo et al., 2020). Mastitis causative isolates are significantly different from the fecal isolates in adonitol fermentation, production of aerobactin, and ability to grow in 90% mastitis whey (Nemeth et al., 1994). *E. coli* isolates from clinical mastitis cases can resist phagocytosis by neutrophils, multiply faster, and ferment lactose more quickly than environmental *E. coli* isolates (Blum et al., 2015). Virulence factors of MPEC strains, causing acute (VL2874 and P4) or persistent (VL2732) mastitis, are different from an environmental (K71) strain (Blum et al., 2015). It is found that the three mastitis pathogenic strains can metabolize D-serine and uric acid; however, the metabolism of uric acid is completely negative in strain K71 (Blum et al., 2015). Uric acid has a role in the oxidative antimicrobial defense of milk in the mammary alveoli during mastitis (Blum et al., 2015). It is reported that an environmental isolate termed *E. coli* K71 is incapable of causing experimental mastitis in either mice or cows (Blum et al., 2015). The ferric citrate system is present in all mastitis isolates, while it is absent from some other strains, such as the K71 strain (Kempf et al., 2016). It is found that only the fec locus (fecIRABCDE) for ferric dicitrate uptake is present in the core genome of MPEC, and that it is absent in the dairy-farm" *E. coli* (DFEC) genomes (Blum et al., 2018).

Besides, a MPEC P4 mutant lacking fec loses its ability to induce mastitis, whereas the fec_ DFEC K71 mutant can trigger intramammary inflammation (Blum et al., 2018). Coliforms also have somatic and capsular factors that affect their resistance to bovine bactericidal activity. Long polar fimbriae and an enteroaggregative heat-stable enterotoxin are also prevalent in the majority of strains isolated from cattle with clinical *E. coli* mastitis (Blum et al., 2018). The fibronectin-binding property of *E. coli* from bovine mastitis may be an important virulence factor that allows the organism to adhere to the ductular epithelium (Blum et al., 2015).

Many phylogenetic subgroups and virulence genes have been detected in bovine mastitis *E. coli*. Phylogenetically, *E. coli* can be subdivided into several groups called phylogroups A, B1, B2, C, D, E, and F4 (Clermont et al., 2013). Ghanbarpour and Oswald (2010) revealed that 38.58% of 127 *E. coli* isolates in bovine mastitis belonged to group B1, and the remaining isolates fell into four phylogenetic subgroups: A0, 18.1%; A1, 26.8%; D1, 6.3%; and D2, 10.2%. Fernandes et al. (2011) showed that 23 out of 27(85%) isolates of *E. coli* in clinical bovine mastitis belonged to phylogenetic group A, and 4 out of 27(14.8%) belonged to B1. It has been shown that phylogroup A and phylogroup B1 of *E. coli* were the main strains from cases of bovine mastitis (Zhang et al., 2018; Aslam et al., 2021; Bag et al., 2021), and phylogroup B2 was mostly isolated from extra-intestinal *E. coli*. An association between *E. coli* phylogenetic group and its antibiotic resistance was found in some studies. Liu et al. (2014) reported that 67.1% of *E. coli* isolates in their study were resistant to streptomycin and that group B1 isolates were more resistant to streptomycin than group A isolates (Liu et al., 2014). Among the many *E. coli* virulence genes, *traT*, *ler*, *eae*, *flic*, and *iutA* (in persistent intramammary infections) (Dego et al., 2010), *fimH* (Fernandes et al., 2011), *f17A* and *irp2* (in phylogenetic group A), *colV* (in phylogenetic group B1) (Liu et al., 2014), and *f17A* (Ghanbarpour et al., 2010) were among the most common genes identified in isolates from bovine mastitis. Keane (2016) showed that the isolates encoded few virulence genes; the most com-

mon included *iss* (increased serum survival, 41%), *lpfA* (long polar fimbriae, 19%), and *astA* (enteroaggregative heat-stable toxin, 14%) *E. coli* strains with specific virulence factors selected within the mammary gland have been reported to be better suited to cause bovine mastitis (Sun et al., 2021; Zhou et al., 2021). Some studies have found that *E. coli* isolated from cows with mastitis is less genotypically diverse than environmental strains and that they mostly lack known *E. coli* virulence factors (Aslam et al., 2021). One of the bacterial genes involved in *E. coli* mastitis is *iss*. Most coliform isolates that cause severe mastitis are serum resistant, a trait associated with the Increased serum survival (*iss*) gene. Serum-sensitive organisms cannot multiply in normal glands because of the activity of milk bactericidins originating from blood serum (Constable et al., 2017). However, some observations indicate that serum-resistant coliforms have no selective advantage over serum-susceptible coliforms in naturally occurring intramammary infections (Constable et al., 2017). *E. coli* isolates from bovine mastitis milk have been opportunistic pathogens and represent several different strains that mostly lack known *E. coli* virulence factors (Silva et al., 2013; Blum et al., 2015). This suggests that specific cow factors and stressful conditions probably play a more important role than *E. coli* virulence factors in determining the severity of clinical signs after intramammary infection (Silva et al., 2013). An *E. coli* isolate is considered the causative agent of mastitis (pathogen colony count) when more than 500 colony-forming unit (CFU) are isolated from a mastitis milk sample, either alone or with two other bacterial colonies. Samples with fewer than 500 *E. coli* colonies or with three or more types of isolated bacteria are considered to have secondary contamination (nonpathogenic colony count) (Hogan et al., 1999).

This study aimed to compare the isolation rate of *iss* virulence gene in milk samples from cases of clinical and subclinical mastitis with varying degrees of severity caused by *E. coli*.

Materials and Methods

Farm selection and herd management

This study was carried out from July to December 2022 on a commercial milk-producing dairy farm with 1000 milking cows and stable management and nutritional conditions. Garmsar is located about 95 kilometers (59 mi) southeast of Tehran, the capital of Iran, at a latitude of 35° 07’ 60.00” N and a longitude of 52° 11’ 60.00” E. Its average altitude is 756.00 m/2480.31 ft. The region has a sub-tropical climate. During the study period, the ambient air temperature was 25.7±13.6 ˚C (maximum–minimum: 6.4–40.8), rainfall was 10.6±5.3 mm, relative humidity was 36±17%, and the number of sunny hours per month was 313.83±48.97. Relative humidity and ambient temperature data for the study period were obtained from the Meteorological Organization of Iran. Thus, the period spanned conditions ranging from hot and dry (June to September) to cold and rainy (October to May). Parity ranged from 1 to 5, body condition score was 3.25±0.5, and days in milk averaged 40±10 days. The bedding consisted of dry manure and clay. The cattle were kept in a free-stall system. The research farm is a commercial milk-producing farm where observing animal rights is mandatory, both from a business perspective and as an ethical obligation in the treatment of animals. The research used a case-control design and methodology, with no interference in the animals’ daily lives. The cows were kept in a free-stall barn and had free access to drinking water. All cows were fed with a balanced total mixed ration (TMR) twice a day that met the recommended requirements of cows based on the National Research Council (2000). They were milked two times a day, at 0500 h and 1500 h. During milking in the morning, the troughs were closed for cleaning for 30 minutes.

Sampling procedures

Milk samples from 80 cows with clinical mastitis and 20 cows with subclinical mastitis, all infected with *E. coli*, were collected from mastitis cases on the farm and sent to an equipped laboratory. Milk sample culturing, *E. coli* isolation and identification, and *E. coli* colony counts were performed in the laboratory. Finally, *iss* gene expression was detected in the samples using polymerase chain reaction (PCR).

Definition of clinical and subclinical mastitis cases

Clinical mastitis was diagnosed based on the presence of a clot or pus in milk and the presence of inflammatory symptoms in the mammary gland, with or without systemic signs in the cows (Constable et al., 2017).

Subclinical mastitis cases were detected on the farm using the California mastitis test (CMT) on milk samples from the affected quarters (CMT ≥1) (Constable et al., 2017; Kour et al., 2023). The samples were tested for somatic cell count (SCC) in the laboratory (Stanojevi´c et al., 2023). To determine the degree of subclinical mastitis, DeLaval solution (DeLaval; subclinical mastitis test solution; Poland) was used on the farm as a cell counter solution. DeLaval CMT solution indirectly estimates the SCC of milk samples and classifies them as grade 0 (nor-

mal, SCC <2×10^5 cells/mL), slight (trace) of subclinical mastitis (SCC: 150–500×10^3 cells/mL), 1+ (4–15×10^5 cells/mL), 2+ (8×10^5 – 5×10^6 cells/mL), to 3+ (SCC > 5×10^6 cells/mL). The somatic cells were counted directly, as previously described (Schalm et al., 1971).

Bacteriologic cultures

Bacteriological culturing of milk samples and interpretation of the results were performed (Hogan et al., 1999) and (Imam et al., 2021). *E. coli* isolates were identified using colony morphology, gram-staining characteristics (gram-negative), oxidase, and biochemical reactions in differential media (Markey et al., 2013). Glycerol-stored cultures maintained at −80 ˚C were subcultured on Luria-Bertani (LB) agar and used for further characterization. Ten microliters of each milk sample was spread on MacConkey's agar, incubated at 37 ˚C, and examined after 24 h. Probable lactose-positive colonies were inoculated into TSI, SIM, MR/VP, and urea agar using stab inoculation. Colonies showing the typical biochemical properties of *E. coli* were selected. To assess the colony counts (CFU) of the isolated *E. coli*, bacterial count were determined using the standard plate count method. MacConkey's and Luria-Bertani (LB) agar, and the other culture media were obtained from Merck, Germany.

DNA processing and PCR

For DNA isolation, a loopful of each confirmed *E. coli* isolate was picked from an overnight culture on Luria- Bertani (LB) agar (Merck, Germany) and extracted by boiling as described previously (Askari_Badouei et al., 2014). The *E. coli* isolates were subjected to specific PCR for the detection of the *iss* gene using the iss-F: (5'-CAGCAACCCGAACCACTTGATG-3') and iss-R: (5'-AGCATTGCCAGAGCGGCAGAA-3') primers (Askar Badouei et al., 2014). The PCR mixture contained 1.25 U of Taq DNA polymerase (Cinnagen, Iran), 60 ng of DNA, 0.7 µM of each primer, 200 µM of dNTP, 2 mM of $MgCl_2$, 2.5 µL of 10X PCR buffer, and sufficient ultrapure water to achieve a final reaction volume of 25 µL. The PCR program was as follows: initial denaturation at 95 ˚C for 4 min, followed by 30 cycles of denaturation at 94 ˚C for 30 s, annealing at 63 ˚C for 30 s, and extension at 72 ˚C for 1 min. A final extension was conducted at 72 ˚C for 5 min. A virulent O78: K80 APEC strain was used as the positive control, and sterile water was used as the negative control. The presence of the expected 323-base-pair (bp) product was recorded as a positive result (Askari Badouei et al., 2014). The gels were loaded with positive and negative controls and a 100 bp-plus DNA marker (Vivantis, Malaysia).

Definition of the case and control group

According to Hogan et al. (1999), pathogenic intramammary infection (IMI) with *E. coli* was defined by the isolation of more than 500 CFU of the bacterium, with fewer than three different types of bacterial colonies, and was considered a case group. Samples with more than three types of bacterial colonies were considered contaminated and excluded from the study. Milk samples with pure isolation of *E. coli* and colony counts of less than 500 CFU were classified as nonpathogenic isolation samples and considered a control group.

The design of the study and its hypothesis

This research was conducted as an observational, cross-sectional field study with a case-control design. The main hypothesis was that the proportion of severe clinical coliform mastitis cases in which the *iss* gene is detected is higher than that of mild or subclinical cases. The second hypothesis was that the proportion of *E. coli* cases carrying the *iss* gene in the case group (CFU >500) is higher than that in the control group (CFU >500) (CFU <500). The third hypothesis was that the isolation rate of *E. coli* carrying the *iss* gene is higher in mastitis cases with fewer stressors or predisposing factors, such as low milk yield. In highly stressed cases, in which the animals' susceptibility was increased, the presence of *iss* was lower. The fourth hypothesis was that, during summers with intense heat (more than 50 ˚C), when *E. coli* bacteria are damaged, the presence of the *iss* gene helps the bacteria proliferate in the host's mammary gland; therefore, mastitis cases involving the gene are more prevalent in summer.

Statistical analysis

The absolute and relative rates of the *iss* gene presence among the isolated *E. coli* were compared between the control and case groups using the chi-square or Fisher's exact test. The chi-square test was used to assess associations between nominal variables. A one-sample Kolmogorov-Smirnov test was used to evaluate the normality of the distribution of SCC and milk production. The Mann-Whitney test was applied to compare median SCC between *E. coli* cases with and without the *iss* gene because the distribution of SCC was not normal. Student's t-test was used to compare mean milk production between the *iss*-negative and *iss*-positive groups because the distribution of milk production was normal. SPSS software, version 24 was used for data analysis. The confidence level was set at 95%, and a $P<0.05$ was considered statistically significant.

Results

Based on the colony counts of the isolated *E. coli*, 45 out of 100 cases (11 subclinical, 12 subacute, 20 acute, and 2 per-acute) showed less than 500 CFU and were classified as the control group, and 55 out of 100 cases (9 subclinical, 26 subacute, 16 acute, and 4 per-acute) showed more than 500 CFU and were classified as the case group (Table 1). Fisher's exact test showed no difference in the distribution of mastitis severity between the control and case groups (P>0.05).

The *iss* gene (Figure 1) was isolated from 3 out of 9 (33.3%) subclinical, 6 out of 26 (23%) subacute, 0 out of 16 (0%), acute, and 2 out of 4 (50%) per-acute cases in the case group (more than 500 CFU) (Table 1). Interestingly, the *iss* gene was also isolated from the control group (fewer than 500 CFU): in 3 out of 11 (27.3%) subclinical, 2 out of 12(16.7%) subacute, 6 out of 20(30%) acute, and 2 out of 2(100%) per-acute cases. Finally, the *iss* gene was isolated from 13 out of 45 cases (29%) in the control group (P>0.05) and 11 out of 55 cases (20%) in the case group (P>0.05). However, per-acute mastitis in the control group was the only mastitis category in which *iss*-negative *E. coli* was not isolated (Table 1).

The one-sample Kolmogorov-Smirnov test showed that SCC was not normally distributed. The Mann-Whitney test also showed that that the median SCC in *E. coli* mastitis cases (Table 2) did not differ significantly between cases with (MED=2.93×10^6) or without (MED=3×10^6) the *iss* gene (P=0.8).

Comparison of peak milk production between cows with (26.7±2.6 kg) and without (34.17±7.4 kg) the *iss* gene showed a significant difference only in the case group (P=0.000). In the case group, milk production just before the onset of mastitis also differed significantly between cows with (25±1.7 kg) and without (33.9±1.5 kg) the *iss* gene.

Comparison of clinical mastitis cases in the control (CFU <500) and case (CFU >500) groups by season using the chi-square test showed that, in the control group, 12 of 34 cases (35.3%) occurred in summer and 22 of 34 cases (64.7%) occurred in autumn. In the case group, 2 out of 46 clinical mastitis cases (4.3%) occurred in summer and 44 out of 46 cases (95.7%) occurred in autumn (P=0.00) (Figure 2).

Comparison of clinical mastitis cases in cows with and without the *iss* virulence gene by season using Fisher's exact test showed that, among clinical mastitis cases without the *iss* gene, 6 of 62 cases (9.7%) occurred in summer and 56 of 62 cases (90.3%) occurred in autumn. Among clinical mastitis cases with the *iss* gene, 8 of 18 cases (44.4%) occurred in summer and 10 of 18 cases (55.6%) occurred in autumn (P=0.002) (Figure 3).

In a crosstabulation among the CFU groups, *iss* gene expression groups, and season of clinical mastitis onset in this study, 66 of 80 clinical cases (82.5%) occurred in autumn, and 14 of 80(17.5%) occurred in summer. In the case group, all 38 cases (100%) without *iss* expression occurred in autumn (Table 3), whereas only 6 of 8 cases (75%) with *iss* expression occurred in autumn (P=0.027). In other words, no cases without *iss* expres-

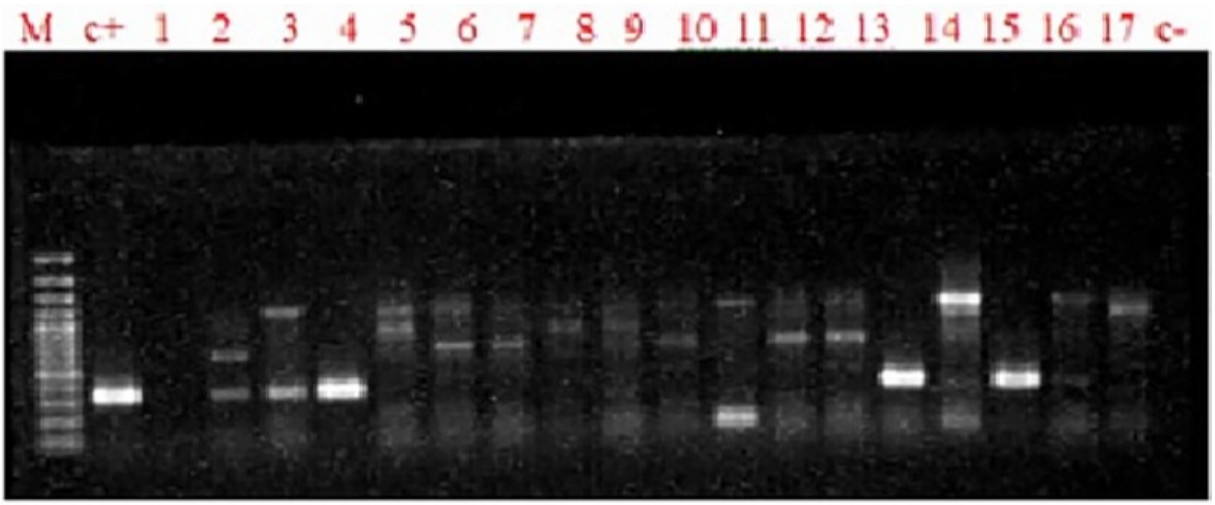


Figure 1. PCR detection of the *iss* gene in *E. coli*

Note: M: Marker 100+bp, +C: Positive control, -C: Negative control.

Table 1. Comparison of the absolute and relative numbers of *E. coli* isolates carrying the *iss* gene between the control (*E. coli* CFU<500) and case (*E. coli* CFU>500) groups and among the different mastitis grades

CFU Group	*iss* Gene Status	Statistic	Mastitis Grade				Total	P
			Subclinical	Clinical Grade 1	Clinical Grade 2	Clinical Grade 3		
<500 (control)	Negative	Count	8	10	14	0	32	0.074
		% within *iss* status	25	31.3	43.8	0	100	
		% within mastitis grade	72.7	83.3	70	0	71.1	
		% of total	17.8	22.2	31.1	0	71.1	
	Positive	Count	3	2	6	2	13	
		% within *iss* status	23.1	15.4	46.2	15.4	100.0	
		% within mastitis grade	27.3	16.7	30.0	100	28.9	
		% of total	6.7	4.4	13.3	4.4	28.9	
	Total	Count	11	12	20	2	45	
		% within *iss* status	24.4	26.7	44.4	4.4	100	
		% within mastitis grade	100	100	100	100	100	
		% of total	24.4	26.7	44.4	4.4	100	
>500	Negative	Count	6	20	16	2	44	0.119
		% within *iss* status	13.6	45.5	36.4	4.5	100	
		% within mastitis grade	66.7	76.9	100	50	80	
		% of *Total*	10.9	36.4	29.1	3.6	80	
	Positive	Count	3	6	0	2	11	
		% within *iss* status	27.3	54.5	0	18.2	100	
		% within mastitis grade	33.3	23.1	0	50	20	
		% of Total	5.5	10.9	0	3.6	20	
	Total	Count	9	26	16	4	55	
		% within *iss* status	16.4	47.3	29.1	7.3	100	
		% within mastitis grade	100	100	100	100	100	
		% of total	16.4	47.3	29.1	7.3	100	
Total	Negative	Count	14	30	30	2	76	0.102
		% within *iss* status	18.4	39.5	39.5	2.6	100	
		% within mastitis grade	70	78.9	83.3	33.3	76	
		% of total	14	30	30	2	76	
	Positive	Count	6	8	6	4	24	
		% within *iss* status	25	33.3	25	16.7	100	
		% within mastitis grade	30	21.1	16.7	66.7	24	
		% of total	6	8.0	6.0	4	24	
	Total	Count	20	38	36	6	100	
		% within *iss* status	20	38	36	6	100	
		% within mastitis grade	100	100	100	100	100	
		% of total	20	38	36	6	100	

Table 2. Difference in SCC between *E. coli* mastitis cases with or without the *iss* gene

Mastitis Grades	*iss* Expression	N	Mean	SCC	Median
				Std. Error of Mean	
Subclinical	-a	14	2.19×10^6	0.3×10^6	2.6×10^6
	+b	6	2.08×10^6	0.5×10^6	2.9×10^6
	Total	20	2.16×10^6	0.3×10^6	2.75×10^6
Subacute	-	30	6.31×10^6	1.1×10^6	4.1×10^6
	+	8	5.32×10^6	1.3×10^6	3.95×10^6
	Total	38	6.10×10^6	0.97×10^7	4.1×10^6
Acute	-	30	10.28×10^6	0.397×10^7	1.66×10^6
	+	6	13.07×10^6	7.7×10^6	1.24×10^6
	Total	36	10.75×10^6	3.5×10^6	1.62×10^6
Peracute	-	2	43.46×10^6	0.000	43.5×10^6
	+	4	23.95×10^6	0.6×10^6	2.4×10^6
	Total	6	16×10^6	8.6×10^6	3.5×10^6
Sum	-	76	8.1×10^6	1.8×10^6	3×10^6
	+	24	6×10^6	0.2×10^7	2.93×10^6
	Total	100	7.5×10^6	1.4×10^6	2.98×10^6

Note: a: *E. coli* without the *iss* gene; b: *E. coli* with the *iss* gene.

sion occurred in summer in the case group (Table 3), while 2 of 8 cases (25%) with *iss* expression occurred in summer (P=0.027). The correlation between *iss* expression and summer was significant in the case group (r=0.465, P=0.01).

In the control group, among clinical mastitis cases that occurred in the autumn, 18 of 24 cases (75%) involved *E. coli* without *iss* expression (Table 3), whereas 4 of 10 cases (40%) involved *E. coli* with *iss* expression (P=0.052). In the summer, 6 of 24 clinical mastitis cases (25%) involved *E. coli* without *iss* expression (Table 3), whereas 6 of 10 cases (60%) involved *E. coli* with *iss* expression (P=0.052). There was no correlation between *iss* expression and summer in the control group (r=-0.334, P=0.054).

Discussion

In this study, there was no difference in the distribution of mastitis severity or in the absolute and relative isolation rates of the *iss* gene between cows with *E. coli* mastitis and colony counts below or above 500 CFU. This finding rejects the first and second hypotheses of this research. It indicates that the *iss* gene alone did not affect the induction and/or development of *E. coli* mastitis as a virulence determinant. In addition, there was no significant difference in the absolute and relative isolation rates of the *iss* gene among the different degrees of mastitis. This finding shows that some of the hypotheses of this research are not confirmed, which reduces the importance of the *iss* gene as a factor in disease severity. However, an important finding of the present study was that, in cows with *E. coli* colony counts below 500 CFU, peracute mastitis cases were caused only by *iss*-gene-harboring *E. coli*. In other words, no cases of peracute mastitis caused by *iss*-gene-negative *E. coli* were observed in this group. This finding highlights the importance of the *iss* gene in the occurrence of more severe cases. Therefore, an *E. coli* pathotype containing the *iss* gene and present at counts below 500 CFU may be a mastitis pathogen. This may indicate the need to revise and reconsider the traditional definition of pathogenic *E. coli* colony counts based on Hogon et al. (1999).

Table 3. Comparison of the absolute and relative numbers of *E. coli* Isolates carrying the *iss* gene between groups with colony counts below (control) and above (case) 500 CFU and between summer and autumn among clinical cases

CFU Group	*iss* Gene Status	Statistics	Season		Total	P
			Summer	Autumn		
<500 (control)	Negative	Count	6	18	24	0.052
		% within *iss* status	25	75.0	100	
		% within season	50	81.8	70.6	
		% of total	17.6	52.9	70.6	
	Positive	Count	6	4	10	
		% within *iss* status	60	40	100	
		% within season	50	18.2	29.4	
		% of Total	17.6	11.8	29.4	
	Total	Count	12	22	34	
		% within *iss* status	35.3	64.7	100	
		% within season	100	100	100	
		% of total	35.3	64.7	100	
>500 (case)	Negative	Count	0	38	38	0.027
		% within *iss* status	0.0	100	100	
		% within season	0.0	86.4	82.6	
		% of total	0.0	82.6	82.6	
	Positive	Count	2	6	8	
		% within *iss* status	25	75	100	
		% within season	100	13.6	17.4	
		% of total	4.3	13	17.4	
	Total	Count	2	44	46	
		% within *iss* status	4.3	95.7	100	
		% within season	100	100	100	
		% of total	4.3	95.7	100	

CFU Group	*iss* Gene Status	Statistics	Season		Total	P
			Summer	Autumn		
Total	Negative	Count	6	56	62	0.002
		% within *iss* status	9.7	90.3	100	
		% within season	42.9	84.8	77.5	
		% of total	7.5	70	77.5	
	Positive	Count	8	10	18	
		% within *iss* status	44.4	55.6	100	
		% within season	57.1	15.2	22.5	
		% of total	10	12.5	22.5	
	Total	Count	14	66	80	
		% within *iss* status	17.5	82.5	100	
		% within season	100	100	100	
		% of total	17.5	82.5	100	

Most *E. coli* strains isolated from bovine mastitic milk have been found to carry genes encoding complement resistance protein (*traT*), Shiga toxin 1 (*stx1*), Shiga toxin 2 (*stx2*), and intimin (*eaeA*) (Aqeela Ashraf et al., 2018; Ismail et al., 2020).

Guerra et al. (2020) indicated that the expression of two genes, *traT* and *ompT*, is associated with the serum neutralization ability of extra-intestinal pathogenic *E. coli* strains responsible for mastitis. It can be inferred that these two genes may serve as representatives of this strain of *E. coli* in milk samples. Additionally, *traT* expression was detected in 15% of mild cases and 22.6% of moderate cases, suggesting that its expression increases in more severe cases of mastitis caused by extraintestinal strains Guerra et al.'s study did not investigate the importance of genes associated with serum neutralization in intestinal strains the authors concluded that these genes act as virulence factors in extraintestinal strains. In the present study, intestinal and extra-intestinal strains were not differentiated. However, consistent with Guerra et al.'s findings, when environmental or host-dependent predisposing factors for mastitis were absent, increased expression of the serum-survival gene was associated with the occurrence of clinical mastitis and the severity of its manifestations.

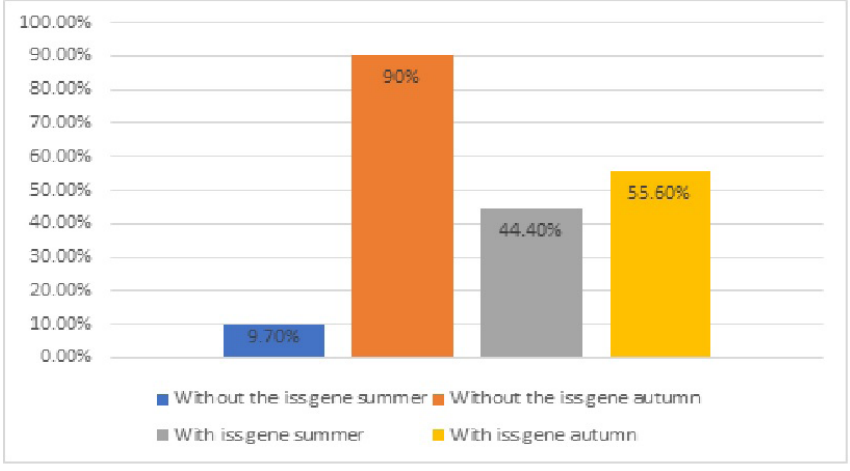


Figure 2. Comparison of clinical mastitis rates between the control (CFU<500) and case (CFU> 500) groups in summer and autumn using the chi-square test (P=0.00).

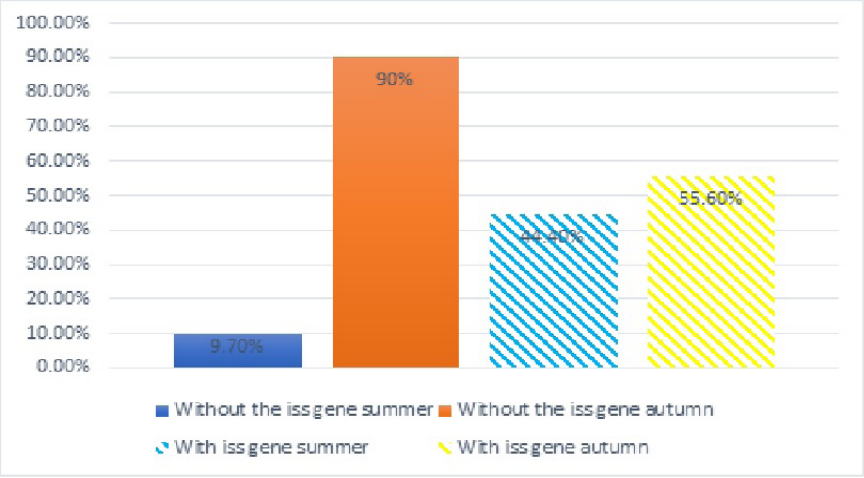


Figure 3. Comparison of clinical mastitis rates in cows with and without the *iss* virulence gene in summer and autumn using Fisher's exact test (P=0.002)

The *iss* gene has been isolated from clinical bovine mastitis by many researchers, consistent with the findings of the present study. One study showed that *iss*, together with *irp2*, *iucD*, and *papC*, was among the most common virulence genes detected in *E. coli* associated with clinical bovine mastitis (Suojala et al., 2011). However, none of the phylogenetic groups, virulence factors, or antimicrobial resistance traits studied was associated with clinical signs, persistence of intramammary infections, or clinical recovery from mastitis. The researchers concluded that mastitis-causing *E. coli* bacteria were typical commensal strains. According to Keane (2016) *iss* (41%), *lpfA* (long polar fimbriae, 19%), and *astA* (enteroaggregative heat-stable toxin, 14%) were among the most common virulence genes detected. Besides, opportunistic commensal organisms without known virulence genes (Keane et al., 2016) were found to cause many cases of clinical *E. coli* mastitis. Wenz et al. (2006) found that no predominant serotype was associated with a particular farm or with systemic disease severity, but the most frequent serotype, O158: NM, was identified in the group of cows with moderate disease severity. The virulence genes evaluated did not correlate with systemic disease severity (Silva et al., 2013), consistent with the findings of the present study. Finally, the researchers concluded that specific cow factors, rather than *E. coli* virulence factors, probably play a more important role in promoting systemic disease severity (Silva et al., 2013).

In a recent study, the molecular characteristics of mastitis-associated *E. coli* pathotypes were not distinguishable from those of common traits (Leimbach et al., 2017). In some studies, the resolution of clinical signs due to mastitis and the genetic similarity of isolates were not associated with phenotypic curli expression in isolated *E. coli*. Curli are adhesive surface structures that bind host proteins and activate inflammatory mediators (Leimbach et al., 2017). On the other hand, many studies have indicated that the pathogenicity of *E. coli* in bovine mastitis does not result from a specific *E. coli* virulence factor (Fernandes et al., 2011). Accordingly, the absence of specific virulence genes in commensal *E. coli* isolated from cases of clinical bovine mastitis has been reported in many articles, consistent with the findings of the present study. In the present study, if the presence of the *iss* gene as a virulence factor had a significant effect, the somatic cell count would have increased in cases with *iss* gene expression. Nevertheless, clinical severity in cases of bovine coliform mastitis has been reported to be associated with the coliform bacterial count in milk rather than with the somatic cell count (Nagasawa et al., 2019).

The decrease in milk production associated with *E. coli* carrying the *iss* gene, compared with bacteria without the gene, was observed only in animals with colony counts greater than 500 CFU. This finding may indicate that the high stress levels in high-producing cows (Constable et al., 2017) are sufficient to promote mastitis even when the *E. coli* are *iss*-negative. However, in low-producing cows, which do not experience the stress associated with milk production, *iss*-positive *E. coli* are more pathogenic than *iss*-negative *E. coli*. This finding supports the third hypothesis of this research.

More than 20 virulence genes play a role *E. coli* mastitis, and their upregulation and downregulation, together with the host response, are very important. Therefore, there was insufficient evidence to support the third hypothesis. Importantly, clinical symptoms of *E. coli* mastitis may be related to genes that have yet to be discovered

or to host-related characteristics (e.g. stage of lactation, number and function of circulating neutrophils, SCC, age of the cattle, nutritional and metabolic status, and genetic resistance) (Burvenich et al., 2003).

In the present study, *E. coli* mastitis involving the *iss* gene occurred more frequently during the hot months of the year among cases with *E. coli* colony counts greater than 500 CFU. This finding confirms the fourth hypothesis of this research. It has been suggested that coliform counts remain stable when bedding samples are incubated at 22 ˚C, increase at 30–44 ˚C, and the bacteria are killed at 50 ˚C (Constable et al., 2017). The absence, only during summer, of clinical mastitis cases involving *iss*-negative *E. coli* with colony counts greater than 500 CFU may suggest the importance of the *iss* gene in the induction and development of *E. coli* mastitis during the hot season in our study. The ambient temperature during the summer months of the present study ranged from 30 to 53 ˚C, which can kill *E. coli* bacteria. On the other hand, many studies have reported an increase in the incidence of coliform mastitis during the summer months (Constable et al., 2017). Although heat stress predisposes cows to mastitis in summer, intense heat brings *E. coli* closer to the point of death, and the presence of the *iss* virulence gene protects it from this stress. In other words, the presence of the *iss* gene may facilitate coliform mastitis during hot summer months, potentially reducing the number of stressors needed to induce mastitis in cows. In the present study, the correlation between *iss* gene isolation and season was not evaluated in subclinical mastitis cases because the necessary data on the season of occurrence were unavailable. Unfortunately, the small sample size is one of the limitations of the current study. Therefore, further evaluations are needed in future studies.

The *iss* gene, which is the focus of the present study, is not the only gene expressed by coliform bacteria in the host. Data from Yang et al. suggest that the *TLR4*, *NOD2*, *CXCL8*, and *OAS2* genes are key components of the host immune response to bovine mastitis. The lncRNAs MSTRG25101.2, MSTRG.56327.1, and MSTRG.18968.1, which are adjacent to the SCC QTL and SCS QTL, may potentially regulate the expression of these candidate genes (Yang et al., 2022).

Based on the findings of the present study, we suggest that *E. coli* mastitis should be defined not only by a bacterial count more than 500 CFU but also by considering three important criteria, including the organism's virulence potential, such as the presence of the *iss* gene, host conditions, including milk production and stress levels, and environmental factors, such as ambient temperature. For instance, strains carrying virulence genes may cause mastitis in high-temperature environments or during hot seasons rather than during mild or cold months. Alternatively, milk samples with fewer than 500 CFU could be classified as mastitis cases by taking into account the virulence potential of the isolated *E. coli* strains. Finally, despite unsuccessful attempts to define the *E. coli* strains causing mastitis, we believe that it may still be possible to determine their minimum predictive virulence potential by considering the interplay of other important factors, as described in the present study. Comprehensive data are necessary to change the current diagnostic criteria for *E. coli* mastitis.

Conclusion

The findings of the current study confirm that the *iss* gene may be important in the induction and development of *E. coli* mastitis cases only under stress-free host conditions (lower milk production) or in conditions that are lethal to the bacteria (hot seasons). Besides, *E. coli* pathotypes containing the *iss* gene and having a colony count of fewer than 500 CFU may be mastitis pathogens. Thus, the definition of *E. coli* mastitis may be revised by considering pathogen, host, and environmental factors. More studies are needed to test this hypothesis in the future.

Ethical Considerations

Compliance with ethical guidelines

The study used an observational design, and no interventions were applied to the herd or the animals. The animals were housed on a commercial dairy farm and were producing milk; they did not undergo any manipulation or injection. The only samples studied were milk samples.

The study plan and ethical considerations were approved by the Ethics and Animal Care and Use Committee of the Department of Clinical Sciences, Faculty of Veterinary Medicine, Garmsar Branch, Islamic Azad University, Garmsar, Iran, as part of a DVM thesis research project (No. 980- 11-08-1390).

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Authors' contributions

Conceptualization, study design, review, editing, and final approval: All authors; Sample collection and funding acquisition: Hamed Moosavi Anzahari; Laboratory analyses and data curation: Mehdi Askari Badouei; Methodology, software, validation, formal analysis, investigation, resources, visualization, supervision, data interpretation, project administration, and writing the original draft: Majid Mohammadsadegh;

Conflict of interest

The authors declared no conflict of interest.

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