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Correlation Between the Transcriptional Level of *iss* and *bor* Virulence Genes of Avian Pathogenic *Escherichia Coli* O78 Inoculated to Chicken Serum

Somaye Abbasi[1], Saeed Salari[*], Ahmad Rashki[1], Mohsen Najimi

Department of Pathobiology, Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran

Abstract

BACKGROUND: *Blue Open Reading* (*bor*) and *increased serum survival* (*iss*) genes, with intense structural and possible functional similarities, engage in the serum resistance of Avian Pathogenic *Escherichia coli* O78 (APEC-O78).

OBJECTIVES: This research aimed to determine whether there is a correlation between the transcriptional level of *bor* and *iss* of APEC-O78 inoculated in serum.

METHODS: Total RNA was extracted 24, 28, and 32 hrs after the inoculation of an APEC-O78 isolate, χ1378, to chicken serum. The fluorescence intensities related to the bands of gel electrophoresis of *bor* and *iss* were computed after cDNA synthesis and reverse transcription -PCR reaction assay. Using Pearson's partial correlation tests, the relationship between the transcriptional levels of *bor* and *iss*, and the influence of the selected time points on the possible relationship were respectively measured at $P<0.05$ for statistical significance.

RESULTS: A correlation was observed between the transcription levels of *bor* and *iss* ($P=.012$), which was not influenced by the selected time points ($P=.001$).

CONCLUSIONS: The *bor* must be carefully weighed in the transcriptional analysis including *iss* and vice versa. The number of transcripts/alleles, upstream sequence, regulation process at the pre-transcriptional phase, and the location of *bor* and *iss* in χ1378, and taking samples from different time intervals may explain our result. Our findings emphasizes the multifactorial and complex mechanism of APEC-O78 serum resistance, and can lay the foundation for further studies on the transcription of *bor* and *iss,* particularly in the specific strain and the three time points.

KEYWORDS: APEC; *bor*; *iss*; semi-quantitative Reverse Transcription PCR; serum resistance

Correspondence

Saeed Salari, Saeed Salari; Mailing Address: Department of Pathobiology, Faculty of Veterinary Medicine, University of Zabol, P.O. Box 98615–538, Zabol Tel: +98 (054) 31232250, Fax: +98 (054) 31232251, Email: saeed-salari@uoz.ac.ir

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Introduction

Avian Pathogenic *Escherichia coli* (APEC), the causative agent of poultry colibacillosis, expresses some response genes when exposed to serum stress, encoding protective mechanisms, thereby neutralizing the stress and survival in the serum (Li *et al.*, 2011). Among various serotypes of APEC, the O78 is the major serotype of poultry colibacillosis in Iran (Salari, Zahraei Salehi, Nayeri Fasaei, & Karimi, 2014). Many investigations have emphasized serotype difference in pathogenesis and the impact of serum stress, particularly on the genes responsible for serum resistance. However, the exact mechanism of the serum resistance of Avian Pathogenic *Escherichia coli* serotype O78 (APEC-O78) is still unknown (Li *et al.*, 2011; Ma *et al.*, 2018; Mellata *et al.*, 2003; Salari *et al.*, 2014; Xu, Li, & Fan, 2018).

The *Blue Open Reading* (*bor*) gene, encoding BOR lipoprotein, can alter the serum sensitivity of the lysogenic host and is the only phage gene recognized to neutralize the serum stress (Barondess & Beckwith, 1995; Lynne, Skyberg, Logue, & Nolan, 2007). In fact, *bor* has been known as the first unique example associated with phage, mediating the resistance to immune response. The in-vitro effect of *bor,* which assists in the survival of *E. coli* K-12 lysogen in animal serum, is the same as *increased serum survival* (*iss*) gene (Lynne, Skyberg, Logue, & Nolan, 2007). The *iss* gene is a conserved virulence gene that encodes Iss lipoprotein, engages in the activity for resistance against serum, and routinely exists in avian-origin *E. coli*, particularly in different APEC serotypes (Lynne, Skyberg, Logue, & Nolan, 2007; Mohamed, Shehata, & Rafeek, 2014; Paula Signolfi *et al.*, 2015; Radwan, Salam, Abd-Alwanis, & Al-Sayed, 2014). Salari *et al.* (2014) showed that the sensitivity to the serum of *iss* deleted mutant of APEC- O78 strain was not statistically different from its parent. Therefore, it is logical to conduct follow-up investigations to evaluate whether the genes of the parent are differentially transcripted/ translated/expressed in serum when differences in the mutants and the wild type are not detected (Salari *et al.*, 2014; Skyberg, Johnson, & Nolan, 2008; Xu *et al.*, 2018). Furthermore, Li *et al.* (2011) did not detect any upregulation in the *iss* of APEC O1 incubated with 100% chicken serum, despite the fact that *iss* was, at the time, known to contribute to the serum resistance of *E. coli*. They concluded that the conditions used in that study or some virulence genes were responsible for that observation. Notably, *bor* was not included in their study (Li *et al.*, 2011).

The similarity of nucleotide sequence and even the predicted protein of *bor* and *iss* were reported. Their product is involved in serum resistance. Both Bor and Iss were found to be surface-exposed proteins (Lynne, Skyberg, Logue, & Nolan, 2007). These two genes have structural and possibly functional similarities (Timothy J. Johnson, Wannemuehler, & Nolan, 2008). Furthermore, Nayeri Fasaei *et al.* (2019) showed that *bor* and *iss* were homologous genes in an APEC O78 strain. Therefore, investigation about the interaction between the functions of *bor* and *iss*, especially on a molecular level, could be advisable to increase the knowledge of APEC pathogenesis and *bor* characteristic (Nayeri Fasaei, Zahraei Salehi, Salari, Ranjbar, & Yousefi, 2019).

The present work aimed to evaluate the transcription level of *iss* and *bor*, two similar virulence genes of APEC involved in serum resistance, under serum stress conditions to specify any association between the transcriptional levels of *bor* and *iss*.

Materials and Methods

Bacterial Strain and Oligonucleotide Primers:

This in-vitro experimental study did not include any experiments on human participants or animals. We selected an APEC-O78 isolate, χ1378, which was recovered from chicken colibacillosis in Iran and studied previously (Nayeri Fasaei *et al.*, 2019; Salari *et al.*, 2014), representing a virulent wild strain of APEC-O78 in Iran. For the present investigation, two genes of χ1378, *iss* (Accession No FJ416147.1) and *bor* (Accession No KC253896.1), were selected. A considerable similarity between *iss* gene and *bor* gene was reported in χ1378 (Nayeri Fasaei *et al.*, 2019). *gapdh* was selected as housekeeping gene (Fan & Liu, 2011; Stürzenbaum & Kille, 2001), indicating the metabolic activity of the strain.

Stress Exposure, Sampling, and RNA Isolation

Strain χ1378 was cultured in 9 tubes, containing 8 ml Luria-Bertani (LB) broth (Difco) with 50% chicken serum (Li *et al.*, 2011; Lynne *et al.*, 2007a; Salari *et al.*, 2014), and incubated at 37˚C. 24 (time 1; n=3), 28 (time 2; n=3), and 32 (time 3; n=3) hrs following χ1378 inoculation, total RNA was extracted from χ1378. Three replicates were considered in each time. The procedure of chicken blood collection for serum preparation was in accordance with the Animal Care and Use Guidelines of the Ethic committee of Faculty of Veterinary Medicine, University of Zabol, Iran (certification number: IR-UOZECRA.2015.001).

Total RNA from each tube was extracted using the RNAX™-plus (Cinnagen, Tehran, Iran) as stated in the instructions of the manufacturer. The RNA concentration was assessed using the spectrophotometer (ND-1000, NanoDrop, Thermo Fisher Scientific Inc., USA).

cDNA Synthesis and Semi-quantitative Reverse Transcription-PCR

The transcription levels of three genes, *gapdh*, *iss,* and *bor* were determined for three time points by semi-quantitative Reverse Transcription-PCR (RT-PCR).

Two micrograms of total RNA were used for cDNA synthesis with 2-Step RT-PCR Kit (Vivantis, Malaysia) according to the manufacturer's recommendations. PCR condition was the same for all three genes.

PCR reaction consisted of 200 *ng* cDNA, 12.5 *µl* 2-red mastermix (Pishgam, Tehran, Iran), and 1 *µ*M of each primer in a total volume of 25 *µl*. We used primers *iss* F: 5'- GTGGCGAAAACTAG-TAAAACAGC-3'; *iss* R: 5'- CGCCTCGGGGT-GGATAA-3'; *bor* F: 5'- CTCGATGCAAAA-TAC-ACGAAGGAGTTAGCT-3'; *bor* R: 5' TAATTTT-CTACACATACGATTCTGCGAACT-3' (Lynne *et al.*, 2007a; Salari *et al.*, 2014); *gapdh F:* 5'-TAGGTATCAACGGTTTTGGC-3'; and *gapdh R:* 5'- CGAACACGGAAGTGCAA-3'.

The PCR program included incubation at 94˚C (4 min), followed by 35 cycles of 40 sec at 94˚C, 30 sec at 57.2˚C, and 80 sec at 70˚C. Eventually, a final extension was performed at 72˚C (8 min) (gradient Eppendorf's Master cycler® pro, Eppendorf, Hamburg, Germany).

Gels were pictured using a UV transillumination (Gel doc, Cambridge, USA) and the fluorescence intensities of the bands were computed with ImageJ2x 2.1.4.5 O software (Wayne Rasband, National Institute of Health, USA).

To verify that the RNA samples were DNA-free, the RNA samples without reverse transcription served as PCR template (data not shown).

RNA experiments were confirmed using the *gapdh* as control (Fan and Liu, 2011; Sturzenbaum and Kille, 2001).

Data and Statistical Analysis

The image of RT-PCR work in the current study is available in the Mendeley Data repository at Mendeley Data, V3, doi: 10.17632/p4ct92vxnr.3.

Mean fluorescence intensities of *iss* and/or *bor* bands, obtained by RT-PCR, were calculated for each gene in each time. The fluorescence intensity of *iss* and/or *bor* bands, obtained by RT-PCR, was not comparable with the *gapdh* because the role of *gapdh* was different from that of *iss* and *bor,* particularly under serum stress. The band intensity of *gapdh* was applied to control the quality of the RT-PCR and show the metabolic activity of the bacterial population.

Statistical Package for Social Sciences (IBM Statistics, USA), set at P-value<0.05 for statistical significance, was used to statistically analyze the hypothesis.

One-way analysis of variance was applied to show the impact of time points on the transcriptional levels of the studied genes. Pearson's correlation was run to determine any relationship between the transcriptional levels of *bor* and *iss*. To examine whether the time points (24/28/32 h after inoculation) influenced the observed relationship, partial correlation was run to measure the strength and direction of the linear relationship between the transcriptional levels of *bor* and *iss* while controlling for the effect of time points, also known as 'covariates' variables (Field, 2018). We considered the time points as 'covariates' variables and control covariates to be excluded from the statistical analysis; this means that the effect of time

points on the transcriptional levels of *bor* and *iss* was omitted from the statistical analysis.

Results

Figure 1 shows the transcription level of the examined genes based on the time points. The transcription level of *iss* decreased between 24 and 28 h but increased between 28 and 32 h after inoculation (Figure 1a; $F_{(2, 6)} = 0.846$, P=0.474). The transcription level of *bor* decreased between 24 and 28 h and between 28 and 32 h after inoculation (Figure 1b; $F_{(2, 6)} = 3.212$, P=0.113). The transcription level of *gapdh* decreased between 24 and 28 h and between 28 and 32 h after inoculation (Figure 1c; $F_{(2, 6)} = 89.844$, P=0.000). Albeit the level of transcription of *iss* fluctuated among three time points of the present study, its decrease between 24 and 28 h, and its increase between 28 and 32 h after inoculation were not statistically different (Figure 1a). Moreover, the decrease of transcription level of *bor* by time passing showed no significant difference, statistically (Figure 1b); indicating two virulence genes (*iss* and *bor*) of χ1378 were constitutively transcripted from 24 to 32 h after the inoculation of χ137 to serum. The transcription level of *gapdh* significantly decreased between 24 h and 28 h after inoculation. The decrease in the transcription level between 28 h and 32 h after inoculation was not statistically significant, which indicated that *gapdh* constitutively transcripted between 28 h and 32 h after inoculation (Figure 1c)

There was a strong, positive correlation between the transcriptional level of *bor* and *iss*, which was statistically significant ($r_{(6)}$= 0.786, n=9, P=0.012). Partial correlation was run in the present study to find if this relationship was affected by time points. The results of the partial correlation showed a strong, positive partial correlation between the transcriptional level of *bor* and *iss* while controlling for the time points of the present study, which was statistically significant (r(6) = 0.924, n=9, P=0.001). The observed correlation between the transcription levels of *bor* and *iss* was not influenced by the selected time points.

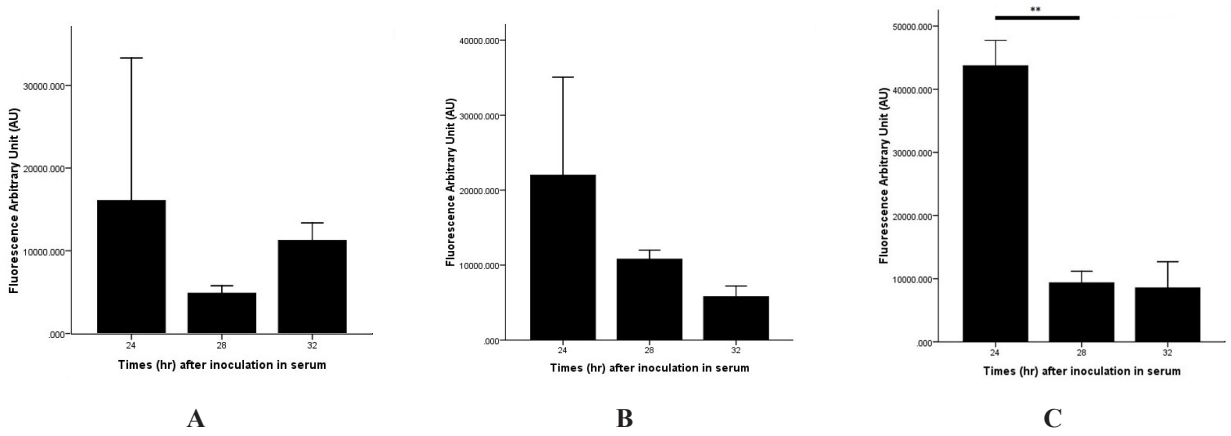

Figure 1. The fluorescence intensities (Mean) of *iss* (a), *bor* (b), and *gapdh* (c) of χ1378, estimated by ImageJ2x, after 24, 28 and 32 h growth in LB containing 50% chicken serum. Error Bars show 95% CI of Mean; ** indicated P< 0.05.

Discussion

Although many studies have associated virulence genes to the serum resistance of APEC-O78, the serum resistance mechanism of APEC-O78 is still unknown.

Different studies have performed serum resistance assay with different final concentrations of serum, including 10% (Xu *et al.*, 2018), 90% (Mellata *et al.*, 2003), 100% (Li *et al.*, 2011), and 50% (Lynne, Skyberg, Logue, Doetkott, *et al.*, 2007). Notably, various concentrations of chicken serum should be considered in analyzing the transcription levels; however, only one concentration of chicken serum (50%) was selected in the present investigation which was the continuation of our previous relevant studies (Naroyi & Salari, 2017; Salari *et al.*, 2014).

Different studies have employed serum resistance assay at different incubation times, including 1 h (Xu *et al.*, 2018), 3 h (Mellata *et al.*, 2003; Nayeri Fasaei *et al.*, 2019), 4 h (Salari *et al.*, 2014), 4 and 6 h (Lynne, Skyberg, Logue, & Nolan, 2007), and 8 and 9 h (Li *et al.*, 2011). We concerned about the time points selected in the present work. The time points selected in the present study may cover mid and late exponential phases and the early stationary phase of χ1378 growth state. They were chosen based on our expert opinion although scientific evidence was required (Naroyi and Salari, 2017; Nayeri Fasaei *et al.*, 2019; Salari *et al.*, 2014). This might be explained by *gapdh*, which is a metabolic gene. The activity of *gapdh* may demonstrate the role of bacterial population in metabolism (Figure 1c). The authors expected most strains of *E. coli* to be in stationary phase by 24 h. Our findings indicated that *gapdh* constitutively transcripted between 28 h and 32 h after inoculation (*P*=000; Figure 1c) portending the χ1378 was evidently not in death phase. Based on the transcription levels of g*apdh* the population of the tested bacteria was active and in neither mid/late stationary nor death phase. Together, mid and late exponential phase and early stationary phase for the three time points in the present work may be in accordance with the rules of formal argument; moreover, we recommend taking samples from the lag phase, different time intervals in log phase, and stationary phase for further analysis.

The results of the present investigation can be considered as an addition to the related literature because it was the first to report the constitutive transcription of *bor* and *iss* from 24 to 32 h after the inoculation of χ137 to serum and the presence of statistical correlation between their transcriptional levels, which was not influenced by the selected time points, clarifying the characteristics of *bor* and *iss* in a major serotype of APEC in Iran inoculated to serum. We comment for and against our results. We give the reason for the results and hope that the argument provided in this section would be convincing.

Genes *iss* and *bor* of χ1378 were constitutively transcripted from 24 to 32 h after the inoculation of χ137 to serum. There is not enough data about the *bor* (Xu *et al.*, 2018). The *bor* gene was reported in other studies in different *E. coli* strains (Johnson *et al.*, 2008; Lynne *et al.*, 2007b) and appeared similar to the *lom* gene which helps bind the host to eukaryotic cells and is also known as a lysogenic conversion gene. The *bor* gene is an accessory gene and changes the host in some of its characteristics (Casjens and Hendrix, 2015). The results of Nayeri Fasaei *et al.* (2019) showed that there was a high similarity between the *bor* gene in *E. coli* bacteria as well as their phage and plasmid; furthermore, this gene is conserved in *E. coli* genomic DNA along with their phage and plasmid. Similarity analysis of the *bor* gene in *E. coli* strain χ1378 (O78:K80) isolated from avian colibacillosis in Iran to different bacterial species, their plasmid, and bacteriophage showed that *Paracoccidioides brasiliensis* was the most similar to O78:K80, followed by *Bacillus thuringiensis* CT43 plasmid pBMB0558, and *Salmonella enterica* subsp. *enterica* serovar Kentucky strain CVM29188 plasmid (Nayeri Fasaei *et al.*, 2019). Using transconjugant mutant, Lynne *et al.* (2007a) reported that *iss* played a significant role in serum resistance compared to *bor* in APEC-O2. According to our literature review, there is no transcriptional/expressional analysis about the *bor*. That the *bor* was transcripted, particularly in the specific serotype, is the first evidence indicated by the present study.

The results of the present study, implying the multifactorial and complex mechanisms of the serum resistance of APEC-O78, corroborate the findings of the previous works (Li *et al.*, 2011; Lynne *et al.*, 2007a & b; Mellata *et al.*, 2003), particularly our previous investgation (Salari *et al.*, 2014) in the specific serotype. It seems that the number of transcripts associated with of *iss* and *bor* and/or the upstream sequence of these genes in specific strain is probably involved in the observed results. Previous studies suggested that the virulence and serum resistance of APEC might be affected by the copy number of mRNA of *iss* and *iss*'s different loci (Xu *et al.*, 2018). It could be assumed that the sequence of *bor* and/or *iss* probably contains a set of enhancers/inducers/suppressors influencing their promoter, increasing/decreasing/suppressing their transcription in specific situation and/or time point (Alberts *et al.*, 2002; Brown, 2018). Genes with similar functional annotations are also more likely to be bound by a

common transcription factor. It would also be useful, in a practical sense, for investigators to know what level of expression similarity was required for genes to have a certain probability a common regulatory mechanism (Allocco, Kohane, & Butte, 2004). More research is needed to confirm the previous points, particularly finding out the upstream sequence of *iss* and *bor* in specific strains (Alberts *et al.*, 2002; Brown, 2018; T. J. Johnson *et al.*, 2002; T. J. Johnson, Siek, Johnson, & Nolan, 2006).

A correlation was observed between the transcription levels of *bor* and *iss*, which was not influenced by the selected time points. This is a new outcome, regarding two similar genes involved in the serum resistance of APEC during in vitro growth under serum stress. Our finding suggests that the three time points in the present study had no influence on controlling for the relationship between the transcriptional levels of *bor* and *iss*. Therefore, further research should be conducted to reveal the effect of other factors, such as the number of *iss*/*bor* alleles (Xu *et al.*, 2018), the sequence of *iss*/*bor,* and their regulation process at their pre-transcriptional phase (Alberts *et al.*, 2014; Brown, 2017), possibly controlling their association.

The correlation between the transcriptional level of *bor* and *iss* might be related to the location of the studied genes in χ1378. *iss* was located on the plasmid of χ1378 (Derakhshandeh *et al.*, 2009) while *bor* was found on the chromosomal DNA of APEC (Johnson *et al.*, 2008). Relevant investigations have indicated that some virulence plasmids play more important roles than others in the pathogenesis process of bacteria and their products may influence the functions of other genes (Alberts *et al.*, 2014; Brown, 2017; Johnson *et al.*, 2006). This suggests more research to determine the characteristics of *bor* genes in APEC.

Moreover, based on some studies, certain genes are cryptic in some strains of bacteria. The expresion of these genes has been increased under certain circumstances, significantly (Gupta *et al.*, 2017). Further research is required to reveal if *bor* is a cryptic gene or not.

Conclusion

In conclusion, our study provides insight into the consideration required for the transcriptional analysis of *bor* and *iss* in the specific strain. As first evidence, we reported the constitutive transcription of *bor* and *iss* from 24 to 32 h after the inoculation of χ137 to serum and the association of the transcription level of *bor* and *iss*, particularly in the specific strain and the three time points, which was not influenced by the selected time points. The *bor* must be carefully weighed in the transcriptional analysis including *iss* and vice versa. The number of transcripts and alleles, the upstream sequence, the regulation process at the pre-transcriptional phase, the location of *bor* and *iss* in χ1378, and taking samples from different time intervals can explain our result, emphasizing the multifactorial and complex mechanism of APEC-O78 serum resistance. Our findings can lay the foundation for further studies on the transcription of *bor* and *iss* of APEC-O78.

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Ethics Approval and Consent to Participate

The procedure of blood collection from chickens performed in this study for serum preparation was in accordance with the Animal Care and Use Guidelines of the ethical committee of Faculty of Veterinary Medicine, University of Zabol, Iran (certifyication number: IRUOZECRA.2015.001).

Consent to participate

Not Applicable.

Conflict of Interest

None declared.

Authors' contributions

SS, AR, and MN contributed to the conception and design of the work. SA contributed to the acquisition of data. SS contributed to the analysis and interpretation of data. SS and SA drafted the work and SS, AR, and MN substantively revised it critically for important intellectual contents. SA, SS, AR, and MN approved the submitted version. SA, SS, AR, and MN agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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همبستگی بین مقادیر رونوشت‌برداری ژن‌های حدت افزایش‌دهندهٔ بقای سرمی و خوانش باز آبی *اشریشیاکلی* بیماری‌زای طیور O78 تلقیح‌شده در سرم جوجه گوشتی

سمیه عباسی، سعید سالاری، احمد راشکی، محسن نجیمی

گروه پاتوبیولوژی، دانشکده دامپزشکی، دانشگاه زابل، زابل، ایران

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چکیده

زمینه مطالعه: ژن خوانش باز آبی (*bor*) و ژن افزایش‌دهنده بقای سرمی (*iss*) در مقاومت به سرم *اشریشیاکلی* بیماری‌زای طیور O78 نقش و ساختمان و احتمالا عملکردی مشابه دارند.

هدف: این مطالعه برای تعیین این که آیا بین مقادیر رونوشت برداری *bor* و *iss* همبستگی وجود دارد یا خیر، انجام شد.

روش کار: در سه زمان ۲۴، ۲۸ و ۳۲ ساعت بعد از تلقیح *اشریشیاکلی* بیماری‌زای طیور O78 (χ1378) در سرم جوجه، RNA تام استخراج شد. شدت فلورسانس باندهای ژل الکتروفورز *bor* و *iss* پس از ساخت cDNA و واکنش نسخه برداری معکوس زنجیره ای پلیمرازمحاسبه شد. در سطح معنی داری $P<۰/۰۵$، توسط آزمون‌های همبستگی پیرسون و پیرسون نسبی، به ترتیب، همبستگی بین مقادیر رونوشت‌برداری *bor* و *iss* و تأثیر زمان‌های منتخب فوق در همبستگی احتمالی ارزیابی شد.

نتایج: همبستگی قوی و مثبت بین مقادیر رونوشت برداری *bor* و *iss* مشاهده شد ($P=۰/۰۱۲$) که تحت تاثیر زمان های منتخب نبود ($P=۰/۰۰۱$).

نتیجه‌گیری نهایی: در مطالعات رونوشت‌برداری *bor*، به *iss* نیز توجه شود و بالعکس. یافته ما اولین گواه بر همبسته بودن میزان رونویسی *bor* و *iss* است که می‌تواند براساس تعداد رونوشت و آل‌ها، توالی بالادستی، فرایند تنظیم در مرحله پیش رونویسی، محل *bor* و *iss* در χ1378 و نیز گرفتن نمونه در زمان‌های دیگر توضیح داده شود. یافته‌های ما، علاوه بر تاکید بر چندعاملی بودن مکانیسم مقاومت سرمی *اشریشیاکلی* بیماری‌زای طیور O78، می‌تواند به‌عنوان پایه‌ای برای مطالعات بعدی در مورد رونویسی *bor* و *iss*، بویژه در χ1378 و زمان‌های منتخب استفاده شود.

واژه‌های کلیدی: *اشریشیاکلی* بیماری‌زای طیور، ژن افزایش‌دهنده بقای سرمی، ژن خوانش باز آبی، مقاومت سرمی، واکنش نیمه کمی نسخه‌برداری معکوس زنجیره‌ای پلیمراز

نویسندهٔ مسئول: سعید سالاری، گروه پاتوبیولوژی، دانشکدهٔ دامپزشکی، دانشگاه زابل، زابل، ایران ایمیل: saeedsalari@uoz.ac.ir