Genetic polymorphism and expression analysis of *cMBL* gene in Iranian native and commercial chickens

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Abstract The aims of this study were to compare the promoter sequence of the mannose-binding lectin (*cMBL*) gene in Iranian native and commercial chicken strains; as well as to compare the *cMBL* gene expression in crossbred and inbred chickens. In total 79 native (Western Azerbaijan native fowls, WANF) and 49 commercial (Arian Commercial Strain, ACS) birds were reared as parents under same management practices. Then, four genotypes of F_1 offspring (purebreds: ACS and WANF, and crossbreds: ACS roosters × WANF hens and WANF roosters × ACS hens) were produced using artificial insemination. Sequence analysis of the promoter and exon 1 of the *cMBL* gene on the WANF and ACS parents was carried out; then, gene expression was analyzed in 4 genotypes of offspring. A valuable SNP (T>C) was found in −185 position of the *cMBL* promoter in the native birds. The mutation resulted in the modification of the promoter pattern to attachment of the *c-Jun* transcription factor. Due to the similarity of the *c-Jun* with the product of Avian Sarcoma Virus, it seems that the native birds are immunologically more resistant. Gene expression analysis revealed no significant differences between *cMBL* transcripts of 4 different genotypes; however, gene expression in crossbreds was slightly higher than in purebreds. The results showed that the promoter sequence of the *cMBL* gene in Iranian native and commercial birds is variable and is necessary to be investigated in further studies.

Keywords: native chickens, commercial strain, gene expression, mannose-binding lectin gene

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Introduction

Consumer demand for poultry meat and eggs is increasing (Arthur and Albers, 2003). Therefore, during the last decades the chicken meat production has shown a dramatic increase (Zhou et al., 2008). Since the early 1950s, poultry breeding has focused on increasing profitability, with little regard for the effect on the skeletal, respiratory or cardiovascular systems or the well-being (Whitehead et al., 2003), which resulted in increased disease susceptibility (Gabler and Spurlock, 2008). A variety of methods are available to combat the avian diseases in the commercial setting, including improved farm management practices, use of antibiotics, selection for disease resistance, and manipulation of the chicken immune system via vaccination (Lillehoj et al., 2004). Breeding chickens for producing strains resistant to infectious diseases would be beneficial, because the use of antibiotics is under pressure and will be forbidden in the near future (van Hemert et al., 2004). Hence, it would be useful to consider genes involved in disease susceptibility as a trait in new breeding plans (Tohidi et al., 2013).

Mannose-binding lectin (*MBL*), a glycoprotein and a member of the collectin family of proteins, is an important constituent of the innate immune system in vertebrates such as birds (Laursen and Nielsen, 2000). There is a basal level of chicken MBL (*cMBL*; Juul-Madsen et al., 2003); however, serum *cMBL* concentration in chickens increases due to infection. Serum *cMBL* concentration in chickens is, in general, significantly higher than in humans. However, no *cMBL*-deficient chickens have so far been found, which may indicate the importance of *cMBL* in chickens (Schou et al., 2010). There is variation between baseline serum *cMBL* concentrations in chickens (approximately 2.5 to 7 µg/mL), however, the concentration of *cMBL* increased 1.5-3 folds after infection challenge with a maximum levels of approximately 7 to 14 μg/mL (Laursen and Nielsen, 2000). Serum concentration of *cMBL* may be controlled in transcription or translation processes. Therefore, regulatory parts in the promoter may have a key role in *cMBL* gene expression. Nevertheless, different gene expressions of *cMBL* gene may be due to breed/strain specific reasons. In humans, four regulatory sites were reported on the promoter of *MBL* gene and most of the mutations occurred in the promoter region and exon 1 (Laursen and Nielsen, 2000). There are few reports on the regulatory elements and mutations in the promoter and exons of chickens, with most studies focusing on serum *cMBL* concentrations (Norup et al., 2009). There are both native and commercial chicken strains in Iran, but, these genetic resources have not received sufficient attention to compare the potential of their innate immunity performance for producing of crossbreds. The aims of this study were to compare the promoter sequence of the mannose-binding lectin (*cMBL*) gene in Iranian native and commercial chicken strains; as well as to compare the *cMBL* gene expression of crossbred and inbred chickens.

Material and Methods

Birds rearing, production and management practices

A total number of 49 ACS birds and 79 WANF birds (10-week old) were transferred from the Arian Commercial Broiler Production and Breeding Center and the Western Azerbaijan Native Fowl Production and Breeding Center, respectively, to the Poultry Research Station of Tarbiat Modares University. All birds were kept under the same management practices. The birds, identified with numbered wing tags, were kept in individual wire cages and had full access to antibiotic-free feed and water. The energy content of the diet for the birds before production, after production for females, and after production for males were 2650, 2745 and 2920 Kcal/kg, respectively. The protein contents for the birds at the same periods were 14.4, 14.3 and 13.3%, respectively. Artificial insemination method was used for producing F_1 offspring from each WANF and ACS birds by allocating 3-5 hens per rooster. Mating of WANF roosters with WANF or ACS hens generated 131 WANF and 35 (WANF × ACS) offspring. Mating of ACS roosters with ACS or WANF hens produced 37 ACS and 95 (ACS × WANF) offspring. All chickens were produced in 3 hatches. All chickens were randomly allocated in groups with 20 chickens, and reared until slaughtering at 7 weeks of age. The mash starter and the pelleted grower and finisher diets were corn-soybean diets that met or exceeded the National Research Council (NRC, 1994) recommendations. None of the parents or offspring were vaccinated in this study.

DNA and RNA Extraction, cDNA Synthesis

Genomic DNA of parents was extracted from whole blood using phenol-chloroform extraction method. Concentration of DNA was measured by spectrophotometer and adjusted to a final concentration of 10 ng/μL. Offspring were slaughtered at 7 weeks of age, and liver samples from 6 individuals per genotype taken and immediately stored at −80 °C. Total RNA of the liver tissue was isolated using Trizol reagent (Sina Clone, Iran) according to the manufacturer's instructions (Figure 1). Ribonucleic acid pellets were dissolved in 20 μL of diethyl pyrocarbonate treated water, digested with DNaseI (Sina Clone, Iran) to remove any genomic DNA contaminants, and concentration was estimated by measuring the ultraviolet light absorbance at 260 nm. Reverse transcription was performed on 3 μg of total RNA using Oligo (dT) primers and SuperScrip II RNase H^- reverse transcriptase (Invitrogen Canada, Inc.) according to the instructions of the manufacturer. Aliquots of cDNA were subjected to semi-quantitative RT-PCR. The glyceraldehyde phosphate dehydrogenase (*GAPDH*) gene expression was measured from the same RNA samples as an internal control in all reactions.

Primers

Gene specific primers were designed using the Oligo software to amplify an 837-bp fragment located in the promoter and exon 1 of the *cMBL* gene (forward: 5′-TCT GAG GCA TAA TAC TGA AG-3′; and reverse: 5′-TGA TAA ATA CTC TGT ACC TGG-3′). In addition, primers specific for *cMBL* and *GAPDH* genes exp-

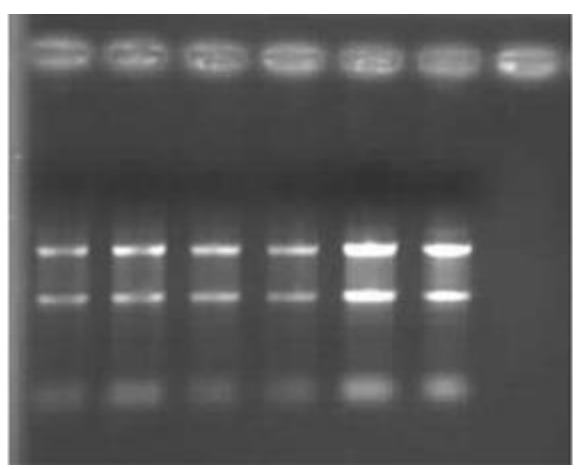

Figure 1. Samples of the RNAs of the liver tissues on 1% agarose gel electrophoresis.

ression were designed to produce PCR products of 198-bp and 288-bp in size, respectively (forward *cMBL*: 5′-AGA CCC AGG AGA AGG ACT TAG-3′ and reverse *cMBL*: 5′-GTA TCT GCT TAA GTC ATC TTC C-3′; forward *GAPDH*: 5′-TGA AGG GTG GTG CTA AGC GTG-3′and reverse GAPDH: 5′-GGA TGA TGT TCT GGG CAG CAC-3′).

PCR, sequencing and comparing the sequences

Each PCR reaction was performed in duplicate of individual's DNA. The PCR amplification was carried out in a final volume of 10 μL containing 0.04 μL of *Taq* DNA polymerase (5u/mL; Sina Clone, Iran), 0.25 μL $MgCl_2$ (100 mM), 1 μL PCR buffer (10X), 0.2 μl dNTPs (10mM), 1 μL of each primers (2.5 mM), 0.8 μL of DNA template. To detect DNA polymorphisms of the promoter and exon 1 of *ACS* and WANF parents, all PCR fragments were purified using a QIAquick PCR purification kit (Qiagen, GmbH, Hilden, Germany), and sequenced by the Macrogen (Macrogen, Seoul, South Korea) by dideoxynucleotide chain termination method (Sanger et al., 1977). The sequence was analyzed in the NCBI database using BLAST, the standard nucleotide-nucleotide homology search (http://www.ncbi.nlm.nih.gov/BLAST). For comparison of the sequenced regions, online PROMO software was utilized (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit. cgi ?dirDB=TF_8.3).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed on cDNA synthesized from 50 ng of total RNA extracted from liver samples of the offspring. The PCR products were run in a 2.2% agarose gel and amplification pattern intensities measured by Image J Software (Image J 1.44p, 2010). The relevant quantity for each genotype was analyzed using:

$$y_{ij} = \mu + S_i + e_{ij} \qquad (1)$$

where, y_{ij} = the j^{th} observation of the i^{th} genotype, μ = overall mean, S_i = the i^{th} genotype (ACS, WANF, ACS × WANF and WANF × ACS), and e_{ij} = residual. Statistical significance for each genotype was determined by one-way ANOVA. Probability values less than or equal to 0.05 were considered significant.

Results and Discussion

An 837-bp segment of the promoter and exon 1 of the *cMBL* of 14 ACS and 14 WANF birds were sequenced. There were no mutations in the exon 1; however, two mutations in −185-bp (T>C) and −269-bp (T>C) of the promoter of WANF birds were detected (Figure 2).

Several experiments have been carried out to dtermine serum concentration of *cMBL* (Juul-Madsen et al., 2011; Kjaerup et al., 2014; Kjaerup et al., 2013) and *cMBL* gene sequence (Laursen et al., 1998), but the sequence of this gene have been rarely compared between strains of chicken. Norup et al. (2008) reported that serum concentrations of *cMBL* in a crossbred strain (commercial × native) were higher than a commercial strain after *E. coli* infection or heat stress (Norup et al., 2008), however, they did not study the *cMBL* gene expression. Therefore, it seems that chickens, like human and fruit fly, have some sites on *cMBL* promoter for heat shock proteins that enhance the gene expression (Laursen and Nielsen, 2000). Mutation in the promoter region, especially in CCAAT and TATA boxes, can change gene expression. However, in this study, no mutation was found in these sites of the prompter sequence.

Transcription factors of −296-bp region did not significantly differ between CC or TT genotypes. However, in −185-bp region two important transcription factors were detected in mutant birds. The first transcription factor was myogenin, which is essential for muscu

Figure 2. Sequence of a part of the *cMBL* promoter including mutations. Number 1 to 4 are sequences of WANF, 4 to 8 sequences of ACS, 9 is mixed DNA of 10 WANF birds, 10 is mixed DNA of 10 ACS birds and 11 is sequence of NCBI (Accession Number: NC_006093.3).

lar development across genome. The relationship of myogenin with immunity is not clear. Another transcription factor was *c-Jun* which is a protein encoded by JUN gene in human and some other vertebrates. This is an intron-less gene and is one of the most important factors in expression of immune system genes (Hartl et al., 2001). The structure of *c-Jun* is very similar to Avian Sarcoma Virus (ASV) product. Mutation in the promoter of *cMBL* gene (−185-bp) leads to generate the structure of the binding site of *c-Jun* in mutant WANF birds which facilitate joining c-Jan and competes with virulence effect of ASV (Wisdom et al., 1999). Consequently, mutant WANF birds are likely more resistant to ASV than ACS birds. In the current study, offspring were not challenged with pathogens, therefore, only baseline *cMBL* gene expression was detected. Moreover, baseline mannose-binding lectin gene expression was very low (Figure 3). In addition, semi-quantitative RT-PCR products showed no differences in baseline gene expression in ACS, WANF, ACS × WANF and WANF × ACS (Table 1).

Expression of *cMBL* gene in both crossbreds were significantly greater than in purebreds, which may be due to heterosis in crossbreds. Norup et al., (2009) reported differences between serum *cMBL* protein concentrations of inbred and outbred lines of chicken, although, in another study there was no significant differences between protein concentration of two types of commercial and native strains (Schou et al., 2010). After heat stress or infectious occurrence, outbred chickens have shown higher levels of serum *cMBL* concentrations than inbred commercial strains (Nourp et al. 2009). This result can be due to higher levels of *cMBL* gene expression in crossbred chickens.

In conclusion, in the current study a valuable SNP was detected in WANF birds. Consequently, it seems that native strain such as Western Azerbaijan Native Fowls are more successful to perform under free range conditions and may transmit their immune potential to their offspring. Furthermore, while gene expression between crossbreds and purebreds was not statistically significant, crossbreds showed higher levels of expressed gene than did the purebreds. It is recommended that *cMBL* gene expression repeat be compared after challenging the offspring with antigens to detect *cMBL* gene expression levels. In addition, it is recommended that

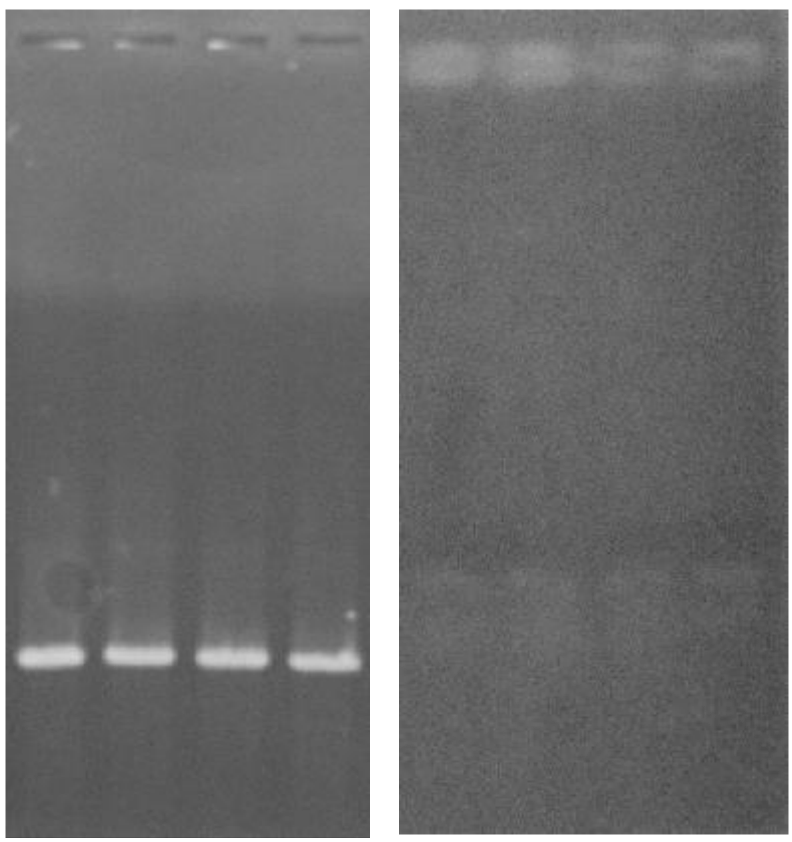

Figure 3. *GAPDH* (Left) and *cMBL* gene (Right) expression in 4 genotypes. From right to left for each figure, ACS: Arian Commercial Strain (purebred); WANF: Western Azerbaijan native fowls (purebred); ACS × WANF (crossbred) and WANF × ACS (crossbred), respectively.

Table 1. Least squares means ± SE of gene expression in 4 genotypes of the offspring of the different crosses of the birds

Strain/Crossbred	*cMBL* gene expression
ACS*	126.7 ± 6.06
ACS × WANF	133.0 ± 6.06
WANF × ACS	141.8 ± 5.25
WANF	131.2 ± 6.06

*ACS: Arian Commercial Strain (purebred); WANF: Western Azerbaijan native fowls (purebred); ACS × WANF (crossbred) and WANF × ACS (crossbred).

crossbred chickens may be useful for rearing in free range production systems.

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تجزیه و تحلیل چندشکلی ژنتیکی و بیان ژن *cMBL* در پرندگان بومی و جوجه‌های تجاری ایران

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چکیده هدف این مطالعه مقایسه توالی ناحیه پروموتر ژن لکتین متصل شونده به مانوز در سویه‌های مرغ بومی و تجاری ایران و همچنین مقایسه بیان ژن *cMBL* در جوجه‌های آمیخته و خالص بود. در مجموع تعداد ۷۹ قطعه (پرندگان آذربایجان غربی، WANF) و ۴۶ قطعه پرنده تجاری (سویه تجاری آرین، ACS) به عنوان والدین تحت شرایط مدیریتی یکسان پرورش داده شدند. سپس چهار ژنوتیپ نتاج نسل اول (خالص آرین و بومی و آمیخته حاصل از خروس‌های آرین × مرغ‌های بومی و خروس‌های بومی × مرغ‌های آرین) با استفاده از تلقیح مصنوعی تولید شدند. تجزیه و تحلیل توالی پروموتر و اگزون ۱ ژن *cMBL* در والدین آرین و بومی انجام شد. سپس، بیان ژن در ۴ ژنوتیپ نتاج تجزیه و تحلیل شد. یک SNP ارزشمند (T>C) در موقعیت ۱۸۵- پروموتر *cMBL* در پرندگان بومی یافت شد. تغییر ایجاد شده در پروموتر موجب تسهیل اتصال فاکتور رونویسی *c-Jun* در پرندگانی شد که دارای جهش مورد نظر بودند. به دلیل شباهت *c-Jun* با پروتئین تولیدی توسط ویروس سرطان‌زای پرندگان، به نظر می‌رسد که پرندگان بومی از نظر عملکرد سیستم ایمنی مقاوم‌تر هستند. تجزیه و تحلیل بیان ژن نشان داد که تفاوت معنی‌داری بین بیان ژن *cMBL* در ۴ ژنوتیپ مختلف نتاج وجود ندارد؛ به هر جهت، بیان ژن در پرندگان آمیخته اندکی نسبت به پرندگان خالص بالاتر بود. نتایج نشان داد که توالی پروموتر ژن *cMBL* در پرندگان بومی و تجاری ایران دارای تنوع است و بررسی‌های بیشتر در مطالعات آتی ضروری است.