ASSOCIATION BETWEEN *ATP1A1* GENE POLYMORPHISMS WITH SEMEN CHARACTERISTICS IN HOLSTEIN BULLS

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ABSTRACT

This study was conducted to identify the polymorphism of *ATP1A1* gene, distribution ratios, and its association with semen characteristics of Holstein bulls. Twenty-five Holstein bulls were used currently. Blood samples were collected via venipuncture to separate the DNA and identify the polymorphism of the *ATP1A1* gene. Semen was collected using an artificial vagina for fresh evaluation. Results were showed the change of cytosine (C) nucleotide at site 15738 to adenine (A) at the exon 17 (SNP C15738A) of the *ATP1A1* gene. Two polymorphisms were obtained according to this change, the first is wild (CC; allele frequency 0.84), and the second is heterogeneous (CA; allele frequency 0.16). The CC polymorphism of the *ATP1A1* gene exhibited a highly significant ($p<0.01$) superiority for ejaculate volume, sperm concentration, and mass activity, and for sperm's cell individual motility ($p<0.05$), compared with the CA polymorphism of Holstein bulls. The other semen characteristics did not differ between the two mentioned polymorphisms. This polymorphism exhibited obvious semen characteristics despite stress conditions of feed shortage and poor management practices accompanied with the covid-19 pandemic. This gene can be used to predict the fertility and stress resistance of bulls before they reach sexual maturity and its reflection on the economic return of breeding these bulls at the artificial insemination centers.

Keywords: gene, sperms quality, bulls, covid-19 pandemic.

مجلة العلوم الزراعية العراقية -2023 :54(2):330-337 عيدان وخضير

العلاقة بين تعدد المظاهر الوراثية لجين *ATP1A1* وصفات السائل المنوي لثيران الهولشتاين

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المستخلص

أجريت هذه الدراسة للتعرف على تعدد المظاهر الوراثية لجين *ATP1A1* ونسب التوزيع وارتباطها بخصائص السائل المنوي لثيران هولشتاين. استخدام في الدراسة خمسة وعشرين ثورمن الهولشتاين وتم جمع الدم من الوريد الوداجي لها لفصل الحامض النووي وتحديد تعدد المظاهر الوراثية لجين *ATP1A1*. كما تم جمع السائل المنوي الطازج باستخدام المهبل الاصطناعي لتقييمه. أوضحت نتائج التجربة الاولى تغير قاعدة نيوكليوتيدية واحدة هي السايتوسين (C) في الموقع 15738 إلى الأدينين (A) في منطقة التشفير (17 Exon17, C15738A) من جين *ATP1A1* في الحصول على مظهرين احدهما البري (CC ؛ تكرار الأليل 0.84) ، والثاني هجين (CA ؛ تكرارالأليل 0.16). أظهر المظهر الوراثي CC للجين *ATP1A1* تفوقًا عالي المعنوية ($p <0.01$) لحجم السائل المنوي وتركيز النطف، والحركة الجماعية والفردية للنطف ($p <0.05$) مقارنة مع المظهر الوراثي CA لثيران هولشتاين. ولم تختلف صفات السائل المنوي الأخرى بين المظهرين المذكورين. أظهر المظهر الوراثي CC صفات سائل منوي متفوقة على الرغم من ظروف الإجهاد من نقص الأعلاف وقلة الرعاية خلال جائحة كورونا. ان ذلك يمكننا من استخدام الجين للتنبؤ بالخصوبة ومقاومة الإجهاد للثيران قبل بلوغها مرحلة النضج الجنسي وانعكاس ذلك على العائد الاقتصادي لتربية هذه الثيران في مراكز التلقيح الاصطناعي.

الكلمات المفتاحية: جين، نوعية النطف، ثيران، جائحة كورونا.

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INTRODUCTION

The reproductive efficiency of dairy cows has become one of the main challenges facing the dairy industry in the world (14). In the last century, the intensive selection of productive traits declined the fertility of both bulls and cows. The bull constitutes more than half of the herd due to the extensive semen used in cow's artificial insemination (32). Bulls' fertility is an important consideration in the dairy cows' productivity and selection programs. The low fertility of bulls constitutes a great economic loss for cow breeders. Bull fertility is more important than cow fertility. Bull can inseminate more than 40 and hundreds to thousands of cows by natural and artificial insemination respectively (10). The low bull fertility level can lead to a decrease in the fertility rate, weaning weight, calving interval prolongation, and increased culling females annually, which is reflected significantly negatively, in a decrease in the breeders' economic profit. The seminal plasma, molecular components of the sperm and extenders greatly affect bulls' fertility. These components play an important role in spermatogenesis, function, motility, capacitation, acrosomal reaction, the ability of fertilization, and the development of embryos as well as the protection of sperm from damages or stresses (28, 34,35). Recently, several studies have indicated deterioration in the quality of bull semen (1,3 , 4,18,19). There are many reasons behind this deterioration, including genetic mutations, age, diseases, stress, transmission, vaccination against diseases, nutrition, and management (1,5, 11, 12,13 ,26, 27, 34, 35). The coronavirus pandemic is starting to have a major negative impact on the local reality. The partial, complete roaming ban and the decrease in working hours for workers in all sectors of life (which did not exceed 50% during the past two years) have a great impact on the bulls stress as a result of the reproductive influence, management practices and feed shortage for artificial insemination bulls. These was negatively reflected on the semen produced from Holstein bulls in Iraq, and the consequent negative impact on the pregnancy rates of cows inseminated from these bulls. Economic losses of poor quality Holstein semen have been estimated at \$3–4/bull/day (17). Therefore, improving the quality of semen or selecting elite bulls by any is an important goal to maximize using of genetically superior bulls. Moura and Memili (28) indicated that the classical evaluation of semen (concentration and individual motility of sperm, abnormalities etc.) was considered a weak indicator for predicting fertility even though it showed significant differences between bulls (23), due to the culling of a good bulls with low semen attributes . These semen characteristics depend not only on genetics but also on the management, environment, and physiological condition of the animal. So that, many studies were focused on using molecular sperm biomarkers as a better tool in detecting any abnormality in sperm function. A large number of genes with a highly significant correlation with semen traits have been proposed that help in animals selecting based on their relationship with semen traits with greater accuracy (2, 34 35). The Na^+/K^+-ATPase genes are stress-sensitive genes responsible for the subunits of the Na^+/K^+-ATPase pump proteins. Na^+/K^+-ATPase proteins are present in the plasma membrane of many cells of the body. These proteins are among the functionally active types in the cell membrane. They have several roles due to their presence on the plasma membrane of many body cells, including sperm (19). It is expressed through the stages of spermatogenesis. The action of Na^+/K^+-ATPase proteins is concentrated in regulating Na+ and K^+ concentrations inside and outside the cells of the body. This lead to maintaining physiological or vital functions of organs and the body such as regulating blood pressure, the osmotic pressure of the cell, neurotransmitters, stress resistance, as well as its relationship to the delivery of neuromuscular signaling, tight junction, cell movement, energy metabolism, body temperature regulation, and other functions depending on the type of cells present in them (7, 24, 29, 30). The pump consists of alpha, beta, and gamma subunits. The alpha type exists in four forms (α1, 2, 3, and 4). The beta type has three forms (β1, 2, and 3). The ATP1A1 gene encodes for the α1 form and the ATP1B2 gene for the β2 form. Some studies have indicated that the genetic features of the ATP1A1 gene had a role in maintaining the balance of Na$^+$ and K$^+$ salts of cells and in resistance to heat stress in cows (20, 24), in addition to its role in the feed intake of cows (7). The *ATP1A1* gene polymorphism and relationship with fresh semen attributes of Holstein bulls under Covid-19 pandemic situation was not previously investigated in Iraq and in the world. Thus, the current study was designed to explore the *ATP1A1* gene polymorphisms and their relationship with the semen characteristics of Holstein bulls in Iraq.

MATERIALS AND METHODS

This study was conducted at the Department of Artificial Insemination pertaining to the Directorate of Animal Resource, Ministry of Agriculture, Iraq. Twenty five Holstein bulls, 2-7.5 years old and 650-1000 kg live body weight were trained for semen collection using artificial vagina. The experimental bulls have good health and free of disease, being under the veterinarian supervision permanently.

DNA genotyping

Blood (10 ml) was collected from all bulls via jugular venipuncture and kept under -20ºC for detection of *ATP1A1* gene polymorphism. Genomic DNA of the all Holstein bulls was extracted from frozen blood using genomic DNA kit supplied by Geneaid, Korea. *ATP1A1* gene was amplified using specific foreword primer, 5'…ACAAACAAAAGGGTCACAACAT…3' and reverse primer, 5'...CTTACCCTAGATCCTGGCTCAT...3' amplified the target fragment 310bp(19). All PCR reactions were amplified in a thermal cycler (Bioneer, Korea). AccuPower® PCR PreMix that contained DNA polymerase, dNTPs, a tracking dye and reaction buffer in a premixed format, freeze-dried into a pellet, different concentration of each used primer (10pmol), 75-100 ng of DNA template and sterile distilled water was added to achieve a total volume. Thermo-cycling of samples was done (Table 1). The detection of nucleotide sequencing for *ATP1A1* was carried out in Microgene Company, Korea, using Geneious program (Geneious 10.13. 2017).

Semen evaluation: Semen was collected from bulls using artificial vagina at one ejaculated / bull / week for 21 weeks. Semen was evaluated in terms of ejaculate volume, sperms concentration, mass activity, sperm's cell individual motility, live sperm, abnormal sperms percentage sperm's plasma membrane and acrosome integrity, DNA damage percentage, total antioxidant, malondialdehyde concentration(1), Ca, K and, Na concentrations (Automatic Electrolyte Analyzer) were also determined.

Statistical analyses

Statistical computations were carried out using General Linear Model (GLM) procedure in the SAS program, using CRD to examine the effect of *ATP1A1*gene on semen characteristics. Means with significant differences were compared using Duncan multiple range test. The statistical model for analysis of variance was as follows:

$Yij = \mu + Ci + e_{ij}$

Y_{ij} = dependent variable (semen characteristics)

μ= Overall mean

C_i = Effect of the *ATP1A1* gene polymorphism (i = CC and CA).

e_{ij}= Error term

Table 1. The program of *ATP1A1* gene primers used in PCR reaction

Steps	Temperature(ºC)	Time(min.)	Cycle No.
Initial denaturation	**95**	**5**	**1**
Denaturation	**95**	**1**	**30**
Annealing temperature	**60**	**0.45**	
Extension	**72**	**1**	
Final extension	**72**	**10**	**1**

Chi-square test was used for comparing the significant differences among the percentage of *ATP1A1* gene polymorphism. Allele frequency was determined by using Falconer and Mackay equation.

RESULTS AND DISCUSSION

Amplification and polymorphism of *ATP1A1* gene by PCR: The amplification of the *ATP1A1* gene (exon 17) target fragment for the bulls' genome was 301bp (Fig. 1). The nucleotide sequencing and analysis of these polymorphisms revealed that an SNP(C>A) was located at exon 17(Site 15738) of the *ATP1A1* gene. The CC polymorphism (%) was higher ($p<0.01$) than the CA of *ATP1A1* gene, being 68 and 32% respectively for the Holstein bulls. The allele frequency was 0.84 and 0.16 for C and A allele respectively (Table2). These results were in agreement with Liu *et al* (24), who indicated that the allelic frequency of the C allele (0.86) was significantly (P≤0.01) higher than the A allele (0.14) in Chinese Holstein cows. In contrast, Kashyap *et al* (20) did not find significant differences for the frequency of the C allele in Vrindavani (0.49) and Tharparker cows (0.48) as compared with the frequency of the A allele (0.56 and 0.52 respectively). The highly significant differences of the CC polymorphism as compared with the CA of the *ATP1A1* gene may attribute to the fact that the CC polymorphism is related to heat stress resistance in bulls'. The ATP synthesis and hydrolysis are the main sources of heat production in the body. The Na+/K+-ATPase pump is one source of heat production in animals. Clarke *et al* (9) found that the main source of thermogenesis is associated Na+/K+-ATPase pump and the active transport of sodium in the cell. The Na$^+$/K$^+$-ATPase activity increases the body temperature. Liu *et al* (24) and Kashyap *et al* (20) indicated that the CC polymorphism in cows plays a role in heat stress resistance and considered as one of the beneficial alleles in cows living under heat stress conditions. So, Holstein bulls were born in Iraqi has adapted to resist the heat stress danger in the summer period. Iraq recorded(above 40 ° C) the highest temperatures in the world, and temperatures reached above 30 ° C until November fifth of the year 2020 (Iraqi Meteorological Department, 2020).

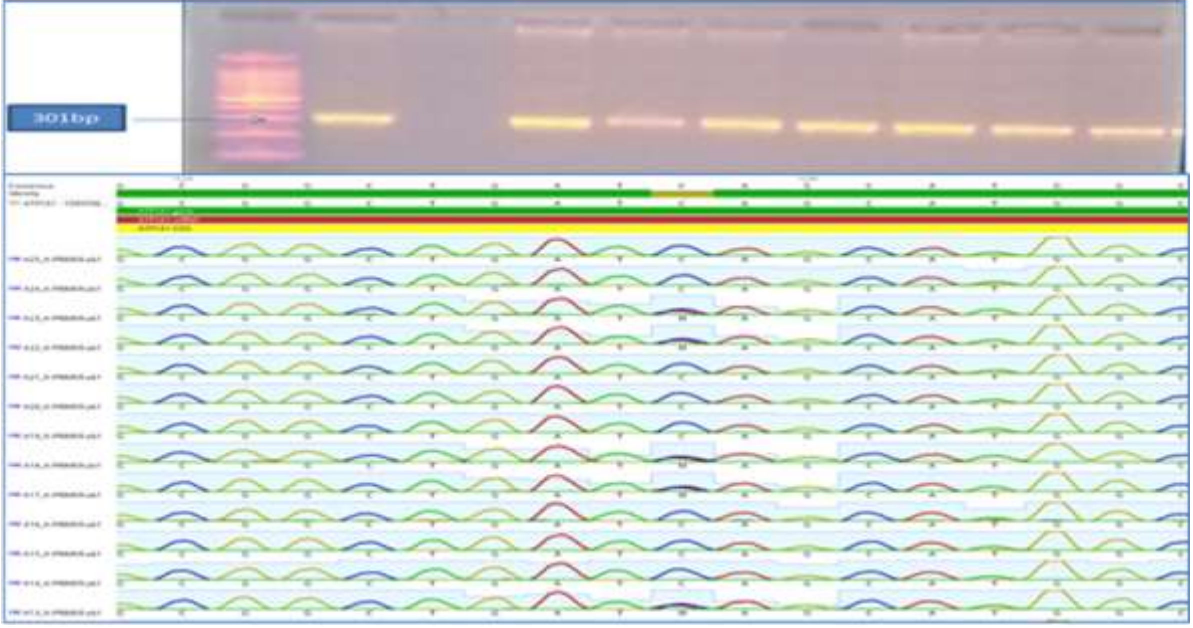

Figure 1. Up. The PCR products of *ATP1A1* gene in Holstein bulls with molecular size of 301bp, the product was electrophoresis on 1.5% Agarose gel., 85v/90mint, Ethidium bromide stain. Down: The nucleotide sequencing detection by using the Geneious program. M: C<A

Table 2. *ATP1A1* gene polymorphism (%) distribution and allele frequency of Holstein bulls

Polymorphism of *ATP1A1* gene	No.	Percentage	Allele type	Allele frequency
CC	17	68	C	0.84
CA	8	32		
Total			A	0.16
Chi-Square (χ^2)	---	26.36 **		

**** = P<0.01**

Semen characteristics and biochemistry: The CC polymorphism of the *ATP1A1* gene exhibited higher ejaculate volume, sperm concentration, mass activity (P<0.01), and sperm motility (P<0.05) than CA polymorphism in Holstein bulls' semen (Table 3). Non-significant differences were observed between two polymorphisms (CC and CA) of the *ATP1A1* gene in other semen attributes (Table 3) and some of the semen biochemistry (Table 4). The higher semen volume and sperm concentration may be the bulls with CC polymorphism having a Na$^{+/}$K^{+} - ATPase pump more efficient than bulls having CA polymorphism. The pump efficiency was necessary for resisting various stress conditions. Which, it's maybe reflected in the performance maintenance of cells and organisms under these conditions. The active Na$^{+/}$K^{+} -ATPase pump plays a major role in cell differentiation and proliferation. The α and β units of the Na^{+} /K^{+} -ATPase enzyme correlated with gene expression of cell growth and motility. Another reason for the ejaculate volume and sperm concentration increases may be to high testosterone level in bulls having CC polymorphism. Testosterone plays a vital role in spermatogenesis and increases the accessory sex glands' secretion, which leads to an increased ejaculate volume and sperm concentration (21). The higher mass activity and sperms individual motility in the bull having CC polymorphism of *ATP1A1* gene within Exon 17, probably due to having a greater ability to regulate K and Na levels between sperm and semen, which leads to lower potassium levels in semen for these bulls. The Na$^{+/}$K^{+} -ATPase is a heterogeneous membrane protein. It consists of a catalytic α subunit (Na-K-α), a regulatory subunit (Na-K-β), and a helper γ subunit. The Na$^{+/}$K^{+} -ATPase α pump possesses Na^{+}, K^{+}, and ATP binding sites. The ATP hydrolysis leads to the influx of 3molecules of Na^{+} outside the cells and the entry of 2 molecules of K^{+} against the electrochemical gradient of the cell. This gradient leads to a decrease in the K concentration and a high concentration of Na outside the cells. Potassium is a cation, natural metabolic inhibitor and sperm motility (8). Our results revealed mathematically lower Ca (135.50 mg/dl) in bulls have CC polymorphism than the CA (148.59±9.12 mg/dl; Table 4). ==== The mass activity and individual motility were below the acceptable level for semen freezing (more than 70%) even live percentage (more than 80%) in all bulls. The reasons for these are due to the low seminal calcium level (Table 4) of bulls as compared with the normal level (167.4 mg/dl; 31). Several studies were indicated a clear relationship between a decrease in semen quality and calcium concentration in seminal plasma of men. Calcium is necessary for sperm movement. It depends on the energy rate of the glycolysis process. The tail sperm movement depends on the enzyme ATPase presence, which works in the presence of Ca $^{+2}$ as a cofactor for the release of energy from the ATP complex. Calcium is added to semen in the epididymis and enters the sperm after the capacitation. After capacitation the Ca influx in sperm and energy is released

Table 3. Effect of *ATP1A1* polymorphism on some semen characteristics of Holstein bulls semen (Mean ± SE).

ATP1A1 gene polymorphism Traits	**CC**	**CA**	**Level of significance**
Ejaculate volume (ml)	**5.8±0.16a**	**4.95±0.23b**	**P<0.01**
Sperm Concentration (10^6/ml)	**1324.11±59.1a**	**1112.96±67.7b**	**P<0.01**
Mass activity(%)	**28.28±1.8a**	**22.58±1.6b**	**P<0.01**
Sperm individual motility	**39.11±1.39a**	**33.38±2.34b**	**P<0.05**
Live sperm(%)	**73.1±0.8**	**69.80±1.5**	**NS**
Total sperm abnormalities	**9.33±1.3**	**10.57±0.6**	**NS**
Acrosome integrity	**84.6±0.6**	**82.8±1.2**	**NS**
plasma membrane Integrity(%)	**80.1±0.6**	**78.4±1.4**	**NS**
DNA damage(%)	**11.75±0.4**	**12.9±0.9**	**NS**

Means with small superscripts within each row indicated significant differences among polymorphism. NS: Non significant

Table 4.Effect of *ATP1A1* polymorphism on some of semen biochemistry in Holstein bulls (Mean ± SE).

***ATP1A1* gene polymorphism Traits**	**TAC (mM/ml)**	**MDA (µmol\ml)**	**Ca (mg/dl)**	**Na (mg/dl)**	**K (mg/dl)**
CC	**19.7±13.3**	**29.5±2.3**	**40.4±4.8**	**151.1±12.5**	**135.5±8.4**
CA	**18.1±2.5**	**28.6±3.6**	**30.2±2.3**	**163.4±3.8**	**148.6±9.1**
Level of significance	**NS**	**NS**	**NS**	**NS**	**NS**

NS: Non-significant

during the glycolysis (8). Calcium is necessary for the sperm acrosome reaction, which occurs before the fertilization process. The influx of calcium into the sperm does not depend on the amount of ATP, but rather on the amount of Na^+ in the sperm, since the influx of each Ca molecule corresponds to the efflux of a Na molecule. Calcium plays an important role in activating the movement of individual sperm, as it was observed that the entry or influx of Ca^{+2} and HCO_3^- into the intracellular activates enzyme Adenyl cyclase, which leads to increased production of cAMP as a sperm energy source, which in turn stimulates the phosphorylation of protein tyrosine, thus increasing the motility and mass activity of sperms. Our results revealed a lower calcium level than its normal level in two polymorphisms of Holstein bulls (Table 4). The main reason behind lower seminal calcium level may due to the decrease in the amount of feed provided to bulls throughout the study period, emergency circumstance of the Covid-19 pandemic. The pandemic caused a lack of working hours for workers at the Artificial Insemination Department belong to the Ministry of Agriculture and all Iraqi state employees (50% of the working hours), in addition to the negative economic effects of the Covid-19 pandemic on Iraq and all countries of the world, which greatly affected on the possibility of providing adequate feed for these bulls. It is known that the low level of calcium in blood or seminal plasma has a significant relationship with reduced feed intake and quality in men, cows, bulls, and horses (16, 22). Another reason for the low value of mass and individual motility in Holstein bulls for two polymorphisms of the *ATP1A1* may due to the environmental conditions. In terms of high temperatures (Iraq recorded a temperature above 40 ° C in the summer of 2020) and temperatures continued to be high even during the fall of the same year, Baghdad recorded temperatures above 30 ° C until the sixth day of November 2020 and above the appropriate temperature for sperm production until mid-November 2020 (Iraqi weather), and certainly, this heat stress on Holstein bulls continues for a long time on bulls until they regain their full activity in producing good quality sperm (1). The appropriate thermal range for milk cows and their bulls is between 4–24°C. So, when the air temperature rises above 24°C, heat stress begins on milk cows and bulls. The increase in temperature is one of the main factors affecting the productivity of animals. The effect of heat stress begins on the stages of sperm production in bulls from the beginning of the meiosis to sperm production (31). Heat stress causes productivity losses. And significant economic benefits for the dairy cow industry such as decreased milk production (6) and fertility, as well as a decrease in the quality of bull semen and an increase in the length of time required to obtain good quality semen samples after exposure bulls for heat stress (1). It is expected that milk production losses will reach more than 15 million tons in 2050. It can be concluded that Holstein bulls with the CC polymorphism of the ATP1A1 gene were more resistant to the stress conditions surrounding the bulls, these bulls showed significant superiority in the mass activity and individual motility of the sperm compared to the bulls with the CA polymorphism. The results of the current study indicated no significant differences in the concentrations of calcium, potassium, and sodium in the seminal plasma of Holstein bulls between the CC and CA polymorphisms of the *ATP1A1* gene (Table 4). Sodium, calcium, potassium, and phosphorous in seminal plasma have a role in regulating the osmotic pressure of sperm. The biochemical characteristics measurement in men, rams, bulls, horses, and pigs semen are important for assessing male fertility (14, 25). Bearden et al (8) indicated that there is a negative correlation between the concentration of potassium in the semen of Holstein bulls, ejaculate volume, individual motility, and sodium concentration in the seminal plasma, and this was confirmed by the current results. The reason might relate to the homogenous (CC) Holstein bulls possess a mathematically lower concentration of potassium compared to the bulls with the heterogonous appearance (CA). The researchers indicated the negative correlation between the potassium and sodium concentrations in the seminal plasma of Holstein bulls. The current results showed lower potassium than the sodium concentration in the seminal plasma within the same polymorphism (Table 4). It can be concluded that Holstein bulls with the CC polymorphism of the *ATP1A1* gene showed a better semen quality than the bulls with the CA polymorphism despite all the stress conditions that surrounded them, which were out of the control of the artificial insemination center management from the feed shortage, the workers lack provided to the animal due to the emergency situation of the Covid-19 pandemic.

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