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IDENTIFICATION OF AQUAPORIN 7 GENE POLYMORPHISM AND IN-VIVO FERTILITY OF EGYPTIAN BUFFALO BULLS

Identificación del polimorfismo aquaporin 7 Gene y fertilidad in vivo de búfalos en Egipto

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ABSTRACT

This work aimed to investigate the genetic polymorphism of Aquaporin 7 (AQP7) gene and its association with the in-vivo fertility of Egyptian buffalo bulls. A total of 188 fresh semen ejaculates were collected from 47 buffalo bulls and subjected to semen evaluation. The bulls were grouped according to in-vivo fertility relying on sire conception rate (SCR) into high (SCR>50%, n=41) and low (SCR<50%, n=6) fertile bulls. DNA was extracted from semen and a 200 bp fragment of the AQP7 gene was amplified by PCR. The genetic polymorphism of AQP7 gene was detected by restriction fragment length polymorphism, the single strand conformation polymorphism and the nucleotide sequencing. The results revealed that SCR was significantly ($p<0.001$) increased in high (71.4±1.3) than low (44.7±2.8) fertile bulls while the ejaculate volume, individual motility%, lives sperm%, sperm concentration and sperm abnormalities showed a non-significant difference in both groups. The AQP7 gene showed no genetic variation in all bulls. The alignment of the resultant sequence with buffalo AQP7 gene sequence (ID: XM_006066699.2) showed 100% identity (122/122), however, deletion of A49 was discovered when the sequence was aligned with ID: XM_006066700.2 of buffalo in GenBank. In conclusion, the sire conception rate is not associated with any genetic variation in Aquaporin 7gene. The Aquaporin 7 gene could be a highly conserved sequence in Egyptian buffalo bulls. Future research on large herd populations in different regions of AQP7 gene is required for evaluating the AQP7 gene polymorphism and its association with the fertility of Egyptian buffalo bulls.

Keywords: Buffalo bull- in vivo fertility- Aquaporin 7 gene- polymorphism

RESUMEN

Este trabajo tiene como objetivo investigar el polimorfismo genético del gen de la acuaporina 7 (AQP7) y su asociación con la fertilidad in vivo de búfalos egipcios. Se recolectaron 188 eyaculados de semen de 47 toros búfalos y se sometieron a evaluación de semen. Los toros se agruparon de acuerdo con la fertilidad in vivo basándose en la tasa de concepción del padre (SCR) en toros fértiles altos (SCR> 50%, n = 41) y bajos (SCR <50%, n = 6). Se extrajo ADN del semen y se amplificó mediante PCR un fragmento de 200 pb del gen AQP7. El polimorfismo genético del gen AQP7 se detectó mediante el polimorfismo de la longitud del fragmento de restricción, el polimorfismo de conformación monocatenario y la secuenciación de nucleótidos. Los resultados revelaron que la SCR se incrementó significativamente ($p<0.001$) en toros con alta fertilidad (71.4 ± 1.3) que en toros con baja fertilidad (44.7 ± 2.8) mientras que el volumen eyaculado, la motilidad individual, los espermatozoides vivos, la concentración de espermatozoides y las anormalidades de los espermatozoides no mostraron una diferencia significativa en ambos grupos. El gen AQP7 no mostró variación genética en todos los toros. La alineación de la secuencia resultante con la secuencia del gen AQP7 de búfalo (ID: XM_006066699.2) mostró una identidad del 100% (122/122), sin embargo, se descubrió la deleción de A49 cuando la secuencia se alineó con la ID: XM_006066700.2 de búfalo en GenBank. En conclusión, la tasa de concepción del padre no está asociada con ninguna variación genética en el gen Aquaporin 7. El gen de la acuaporina 7 podría ser una secuencia altamente conservada en búfalos egipcios. Se requieren investigaciones futuras sobre grandes poblaciones de rebaños en diferentes regiones del gen AQP7 para evaluar el polimorfismo del gen AQP7 y su asociación con la fertilidad de los búfalos egipcios.

Palabras clave: Toro de búfalo, fertilidad in vivo, gen de la acuaporina 7, polimorfismo

INTRODUCTION

Bull fertility is an important factor influencing animal reproductive and productive efficiency (Peddinti et al., 2008). Semen quality can be used as a predictor for bull fertility (Hossein-Zadeh, 2012). Determining the bull fertility traits during sire selection is difficult, so it has been considered that the only measure of bull fertility truly is the conception rate (Butler et al., 2019).

Artificial insemination (AI) is a major essential technique for the genetic improvement of breeding efficiency of the herds (Pan et al., 2013). Commercial artificial insemination companies develop methods continuously to improve herds using frozen semen from genetically superior bulls (Parisi et al., 2014). The successful artificial insemination depends to mainly on good semen quality which is influenced by the management, environment, physiological status and the genetic factors of the animals (Mathevon et al., 1998).

The traditional selection of the sires for semen quality traits is not accurate because of their low heritability and late expression in life (Rothschild and Ruvinsky, 1998). Hence, the molecular DNA markers can be utilized for the genetic improvement of bull reproduction, through marker-assisted selection. The association of the genetic markers with bull fertility is valuable to identify the genomic regions that inherit good fertility characters resulting in economic benefits (Blaschek et al., 2011). In this respect, different studies were performed on the genetic basis of semen quality traits as molecular markers for bull fertility (Dilbar et al., 2019; Iannuzzi et al., 2020; Pardede et al., 2020; Wang et al., 2020).

Aquaporins (AQPs) are membrane proteins that allow water to pass through the cell membrane (King and Agre, 1996; Verkman, 2002). In mammals, there is at least 13 AQPs have been identified (Morishita et al., 2004). Among them, AQP3, AQP7, and AQP9 have been identified as aquaglyceroporins, which transport glycerol and water through the cell membrane, while the other aquaporins transport water only (Agre et al., 2002; Ishibashi et al., 2002). AQP1, AQP7, AQP8, and AQP9 are expressed in testis, and they play an important role in water transportation across the cell membranes in the testis (Calamita et al., 2001; Badran and Hermo, 2002). The AQP7 protein maintains sperm quality during sperm maturation by transporting glycerol, urea and other small non-electrolytes from epididymal fluid and also acts as a valuable cryoprotectant which suggests its main role in glycerol permeability of sperm during cryopreservation (Agre et al., 2002; Sales et al., 2013).

AQP7 gene is located on chromosome 8 in bovines but on chromosome 3 in buffaloes and it is composed of 8 exons in both (7 coding and 1 non-coding) beside the included introns (Ishibashi et al., 1997). AQP7 gene has been composed of 16.25 kilobase pairs and encodes a protein of 269 amino acids (Ishibashi et al., 1997). AQP7 is a candidate antifreeze gene (Ma et al., 2011) that expressed in a variety of tissues including male reproductive system (Moretti et al., 2011).

Studies in cattle confirmed AQP7 gene is a genetic marker for early selection in the bull industry that was associated with sperm motility, post-thaw motility, live sperm percentages and acrosome integrity ratio (Rui-feng et al., 2009; Ma et al., 2011; Kumar et al., 2015). In buffaloes, few studies were conducted in Indian buffalo bulls (Kumari et al., 2017; Kumari et al., 2018a; Kumari et al., 2018b; Kumari et al., 2018c). However, reports on AQP7 gene polymorphism and its relationship with semen quality and in-vivo fertility of Egyptian buffalo bulls are lacked. Keeping in view the importance of AQP7 gene, the current investigation aimed to identify the genetic polymorphism in exon 3 of AQP7 gene in Egyptian buffalo bulls using the restriction fragment length polymorphism (RFLP), the single strand conformation polymorphism (SSCP) and the nucleotide sequencing in a trial to find any association with in-vivo fertility of the sires.

MATERIALS AND METHODS

Bulls and samples

The current study was performed on 47 mature buffalo bulls bred in a station at Sakha, Kafr el-Sheikh Governorate, Egypt. All bulls were healthy and had the same environment, management and nutrition programs. Semen samples were collected by sterile artificial vaginae and immediately kept in a warm water bath at 37oC for fresh semen evaluation directly. All bulls were subjected to semen evaluation, in-vivo fertility assessment and genetic polymorphism investigation for AQP7 gene.

Semen evaluation

Using the traditional techniques, fresh semen was directly evaluated as described by Dilbar et al., (2019) for ejaculate volume, individual motility %, live sperm %, sperm concentration, and sperm abnormalities. Four ejaculates were evaluated from each bull during a rainy winter season and the means of values were calculated.

In-vivo fertility assessment

The bulls were then used for natural insemination under the same locality, year and season of insemination. All the inseminated buffaloes were healthy with genitalia free of diseases or abnormalities according to the inspection before insemination. The conception rate was evaluated by ultrasonography after two months of insemination. Those bulls were grouped into high and low-fertile based on sire conception rate (SCR) that was calculated for each bull relying on the number of pregnant females on the total number of inseminations for each bull. According to Kumar et al., (2017), the bulls that had SCR over 50% were classified as high-fertile bulls (n = 41), and that had SCR under 50% were classified as low-fertile bulls (n = 6).

DNA extraction from sperm cells

DNA was extracted from sperm of buffalo bulls according to Hasanain et al., (2016). After extraction, the DNA concentration was measured by NanoDrop1000 spectrophotometer (Thermo Scientific Inc. USA). After that, a working concentration of DNA (50 ng/µl) was prepared for polymerase chain reaction.

Polymerase chain reaction (PCR) and DNA amplification

The DNA fragment (200 bp) of exon 3 for AQP7 gene was amplified by PCR technique. The PCR mixture was 12.5 µl of PCR master mix (composed of 0.1 U/µlTaq polymerase, 500 µM of dNTP each, 20 µM of Tris-Hcl (pH 8.3), 100 mM of Kcl and 3 mM of MgCl), 2.0 µl of forward primer (10 pM/µl), 2.0

µl of reverse primer (10 pM/µl), 2.0 µl of DNA (50 ng/µl) and nuclease-free water up to 25 µl.

The primers (Metabion lnc., Germany) were selected according to Ma et al., (2011) with that sequences; Forward: TCACCTGATCTCATTTCTGCC and Reverse: AGTCTGCTCACCCTGTACC. The PCR reaction was cycled as, initial denaturation at 95 °C for period of 5 minutes and35 cycles of denaturation at 95 °C for 45 sec, annealing at 57 °C for 45 sec, then an extension at 72°C for one minute. After that a final extension at 72°C for five min. The PCR products were electrophoresed along with a molecular size marker in 1X TBE buffer at 100 V in 1.5percentagarose gel containing 0.5 µg/mL of red safe to stain DNA. The gels were then visualized using a UV transilluminator for determining the specific PCR product.

PCR-Restriction fragment length polymorphism (PCR-RFLP)
The PCR fragment of AQP7 gene was genotyped using the PCR-RFLP technique (Ota et al., 2007). TaaI (HPyCH4III) restriction enzyme (New England Biolabslnc, U.K.) was designed according to Kumar et al., (2015) for identifying polymorphism in the G371C locus. The reaction was carried out following the manufacturer's guidelines (Product R0618S). For more confirmation, the test was repeated by using TaaI restriction enzyme (Thermoscietific lnc., Lithuania) and the reaction was performed according to directions of the manufacturer's (FD1364). The PCR-RFLP products were detected on 2% agarose gel stained with 0.5 µg/mL of ethidium bromide electrophoresed at 100 V. The gels were visualized on UV transilluminator.

PCR-single strand conformation polymorphism (PCR-SSCP)
The PCR fragment of AQP7 gene was further analyzed for polymorphism using the PCR-SSCP technique (Sheffield et al., 1993). A reaction of 4µl of PCR products,16 µl distilled water and 20 µl denaturing solution (95% formamide, 10 mMNaOH, 0.05% bromophenol blue, 0.05% xylene cyanol and 20 mM EDTA) was prepared then heat denatured at 95°C for 10 minutes followed by rapid chilling on an ice block for 15 minutes. After that the cocktail was run on 12% non-denaturing polyacrylamide gel (acrylamide and bis-acrylamide ratio, 29:1) using 0.7X TBE buffer in a vertical electrophoresis unit (Cleaver, UK) for 16 hrs (28mA and 180 V). The gels were stained with half µg/mL ethidium bromide to be visualized clearly on UV transilluminator.

Nucleotide Sequencing
The PCR products have been purified using Thermo Scientific GeneJET PCR Purification Kit (product #K0701) according to the manufacturer's guidelines. The purified PCR products for 12 samples (6 forward and 6 reverse) were sequenced using an automated DNA Sequencer (Macrogen lnc., Seoul, South Korea). BioEdit software has been used for multiple sequence alignment and comparing the sequence data among the different samples to detect polymorphisms. Also, the sequence data were further aligned using NCBI/BLAST/blastn suite for comparing the observed sequences in the study with the reference sequences of AQP7 gene of buffalo in the GenBank.

Statistical analysis
The statistical analysis was established by using SPSS version 25 software. The obtained data of semen and conception rate were expressed as mean± SE. The semen parameters and sire conception rate in high and low fertile buffalo bulls were compared by t-test.

RESULTS

Semen and in-vivo fertility evaluation
The ejaculate volume, individual motility %, lives sperm %, sperm concentration, sperm abnormalities showed non-significant differences in high and low fertile buffalo bulls (Table 1). Meanwhile the sire conception rate was significantly ($p<0.001$) increased in high (71.4±1.3) than low (44.7±2.8) fertile bulls (Table 1).

Table 1. Semen parameters and sire conception rate (Mean ± SE) in high and low fertile buffalo bulls.

Comparative items	High fertile bulls	Low fertile bulls
No. of bulls	41	6
Ejaculate volume (ml)	2.9±0.2	2.7±0.2
Individual motility (%)	75.1±1.1	75.3±2.3
Live sperm (%)	81.3±1.2	80.0±.9
Sperm concentration (10^9/ml)	1.47±0.08	1.10±0.07
Sperm abnormalities (%)	17.8±0.7	21.2±1.5
Sire conception rate (SCR)	71.4±1.3***	44.7±2.8

***= P< 0.001(t-Test)

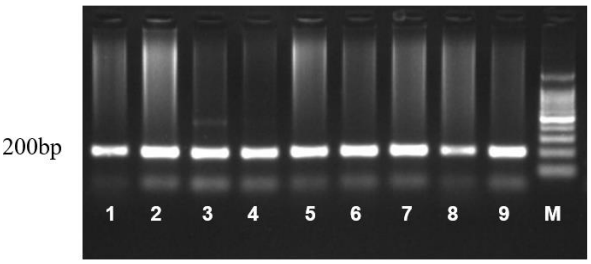

Fig.1. PCR product of exon 3 of AQP7 gene (200bp) visualized on 1.5% agarose gel. M= 100 bp. Molecular marker Lanes 1-9 resemble PCR products

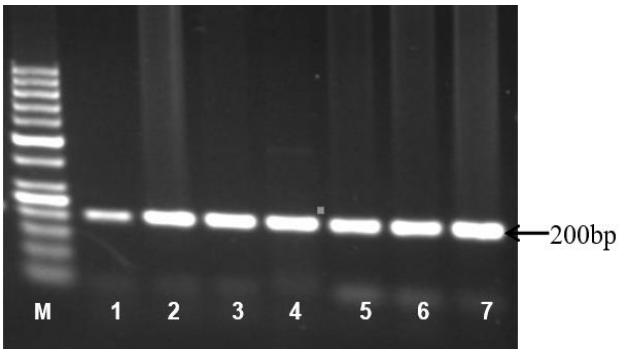

Fig.2. TaaI PCR-RFLP patterns of AQP7 locus in 2% agarose gel. M= 50 bp Molecular marker. Lanes 1-7 resemble CC Genotype (200bp).

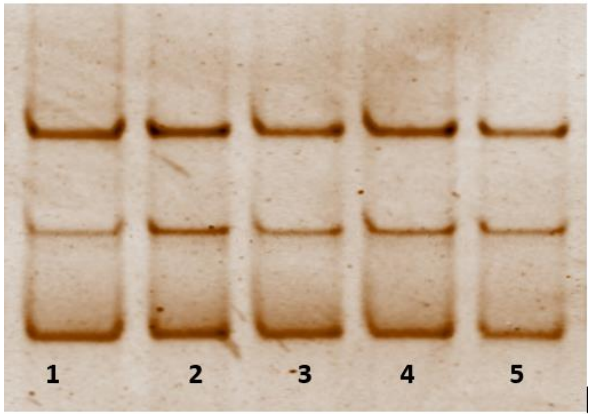

Fig.3. PCR-SSCP patterns (P) in exon 3 of AQP7 gene among Egyptian buffalo bulls showing the same pattern

Fig.4. AQP7gene multiple sequence alignment in Egyptian buffalo bulls in 12 samples using BioEdit software showing 100% similarity among bulls in the 200 bp fragment without variation.

Genetic polymorphism of Aquaporin 7 gene

All the tested buffalo bulls gave the specific PCR product at the expected size (200bp) targeted in AQP7 gene (Fig.1). By carrying out RFLP technique on the 200 bp PCR fragment, no genetic variation was observed with the dominant of CC genotype for G371C locus as the PCR products were not digested by TaaI restriction enzyme in all bulls (Fig.2). That was then confirmed by sequencing. When SSCP was applied on the PCR product of AQP7 gene, no genetic variation was determined as all the samples showed the same pattern (Fig.3). A total of 12 samples were successfully sequenced for the 200 bp fragment of AQP7 gene. By multiple sequence alignment of the samples using BioEdit software, no genetic variation was detected showing 100% similarity among the resulted sequences (Fig.4). There was 100 % identity when the resulted sequence was aligned, using NCBI/BLAST/blastn suite, with the reference sequence with ID: XM_006066699.2 of AQP7 gene for Bubalus bubalis in the GenBank (Fig.5). While our sequence had one nucleotide deletion (A49) showing 99 % identity when aligned with another buffalo sequence (ID: XM_0060666700.2) as shown in Fig.6. The sequence of our Egyptian buffalo bulls were then submitted for the GenBank that provided accession number (MZ513607). The sequencing results confirmed the monomorphic pattern of that fragment of AQP7 gene with no genetic variation in the studied population.

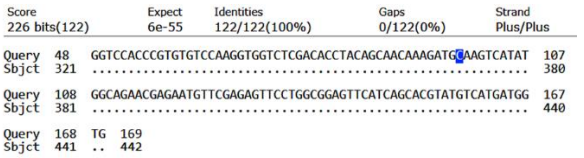

Fig.5. The alignment of AQP7 gene sequence in Egyptian buffalo bulls, by NCBI/BLAST/blastn suite, withBubalusbubalisAQP7 gene sequence ID: XM_006066699.2 representing 100% identity (122/122) and confirming the dominant of CC genotype for C371 locus.

Fig.6. Alignment of AQP7 gene sequence in Egyptian buffalo bulls, by NCBI/BLAST/blastn suite, with sequence ID:XM_0060666700.2 of buffalo AQP7 gene showing 99% identity (124/125) due to absence of A49 in Egyptian buffalo bulls.

DISCUSSION

All the Egyptian buffalo bulls under estimation were categorized as high quality semen producing sires with individual motility more than 70 %. Vale et al (2014) reported the same values of bulls classification for both natural and artificial insemination. All the recorded values for semen evaluation criteria were within the normal reference range of good buffalo semen as reported previously (Khawaskar et al., 2012; Vale et al., 2014; Mahmoud et al., 2021). The sire conception rate was significantly ($p<0.001$) increased in high than low fertile bulls although a non-significant difference was shown in the evaluated semen parameters in both groups of bulls. This means that, the conception rate is more accurate than semen analysis in the assessment of bull fertility. Similarly, it was recorded that in vivo fertility is important in buffalo fertility estimation (Kumar et al., (2017).

Identification of nucleotide variation in exon 3 of AQP7 gene in this study is the foremost in Egyptian buffalo bulls. Hence, our results could not be compared with the same Egyptian breed. Using RFLP, SSCP and sequencing techniques, no genetic polymorphism was detected in exon 3 of AQP7 gene among the studied Egyptian buffalo bulls. The current study indicated the high degree of genetic conservation in the amplified fragment of the AQP7 gene represented by the monomorphic pattern in all bulls. Similarly, Kumari et al., (2018c) reported the monomorphic patterns in exons 2, 3 and 6 in Surti Buffalo bulls in India. Moreover, Kumari et al., (2018b) investigated the polymorphisms in exons 2, 3, 4, 5 in AQP7 gene of Murrah buffalo bulls and observed monomorphic patterns in exons 2 and 3 while exon 4 revealed three and exon 5 revealed five SSCP patterns.

On the other hand, the genetic variations previously identified in AQP7 gene were served as potential genetic markers for semen quality traits in bulls. In this respect, Ma et al., (2011)

associated AQP7 gene polymorphisms in exon 3 with some semen parameters in Holstein Friesian bulls. They found in locus G371C, the CC genotype has significantly higher percentages of sperm viability, motility and acrosome integrity ratio in frozen semen than CD genotype. Besides, Kumar et al., (2015) investigated association between SNPs in exon 3 of AQP7 gene and certain semen quality criteria in Frieswal bovine bulls in India. They observed in G371C locus that, CC genotype has significantly higher ejaculate volume, sperm motility and post-thaw motility than CD genotype in bulls. In addition, Kumari et al., (2018c) observed that variants in exon 1 had a significant effect on Hypo-osmotic swelling reactivity in frozen semen, exons 5 has a significant effect on live sperm in fresh semen and exon 8 had a significant effect on post thaw motility in frozen semen of Surti Buffalo bulls. Moreover, Kumari et al., (2017) observed genetic polymorphism in exon 5 of AQP7 gene that was associated with live sperm in Surti buffalo bulls. Also, AQP7 gene polymorphism in exon 5 was linked to sperm concentration and live sperm while variants of exon 8 were linked to post thaw motility in Murrah buffalo bulls (Kumari et al., 2018a). Besides, Kumari et al., (2018d) studied the genetic polymorphism in exon 1 of AQP7 gene in Murrah Buffaloes that was associated with sperm concentration and hypo-osmotic swelling reactivity in frozen semen.

CONCLUSION

The sire conception rate is not associated with any genetic variation in Aquaporin 7 gene. The Aquaporin 7 gene could be a highly conserved sequence for that first investigation in Egyptian buffalo bulls. Further research is required on large herd populations to discover the association of AQP7 variants with fertility of Egyptian buffalo bulls before sire selection.

Conflicts of interest

None

Author contributions

M. H. Hasanain, Karima Gh. M. Mahmoud designed and carried out the study. A. M. Sakr analysed the data, S.T. Ismail aided in the interpretation of the results. M. H. Hasanain wrote the manuscript. A. A. EL-Menoufy revised the manuscript. All authors approved the final version of the paper.

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