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Polymorphism Investigation of Sex Determining Factor gene (SRY) and the association of Semen Criteria with Field fertility in Egyptian Buffalo Bulls

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Abstract

The current study aimed to determine the genetic polymorphism of sex determining factor or sex determining region, Y-chromosome gene (SRY) and the association of semen parameters with field fertility of Egyptian buffalo bulls. Fresh semen ejaculates (n=264) from 66 bulls were collected and evaluated. The bulls (n= 56), with ejaculates ≥ 60% motility, were categorized according to pregnancy rate (PR) into high (PR≥50%, n=47) and low (PR<50%, n=7) fertile. SRY gene polymorphism was identified by the restriction fragment length polymorphism, the single strand conformation polymorphism, and the nucleotide sequencing. The results revealed a significant difference between high and low fertile bulls in PR (P< 0.001). A significant negative correlation (r=-.369, P< 0.01) was shown between sperm abnormalities and PR and a significant positive correlation (r=.273, P< 0.05) was obvious between live sperm and PR. The SRY gene showed no genetic variation among bulls. The Basic Local Alignment Search Tool (BLAST) of the sequence showed 100% identity with Pakistani buffaloes. In conclusion, sperm abnormalities and live sperm can be used as good predictors for fertility in buffalo. PR is more accurate than semen analysis for evaluating fertility. The SRY gene could be highly conserved in Egyptian buffalo. Further research is recommended by expanding SRY gene fragment and increasing number of samples.

Key words: Buffalo bull; SRY gene; polymorphism; semen; pregnancy rate

1. Introduction

The precise assessment of semen quality is highly important in order to predict bull's fertility especially in artificial insemination (AI) with cryopreserved semen, in which semen ejaculate may provide hundreds of straws [1]. The routine semen analysis tests like ejaculate volume, motility%, live sperm%, sperm abnormalities or morphology% and sperm concentration are traditionally evaluated immediately after semen collection. Motility, live sperm and morphological evaluation are the most common parameters used for predicting fertility potential in farm animals [2-4]. The sperm morphology appears to have a major role in the success of fertilization, early embryonic development and the pregnancy rate in reproduction practices [5]. These conventional evaluation assays cannot be used as confirmatory tests for bull fertility due to the difficulty to determine some functional sperm impairments, which are able to decrease fertility [6] but only give information about the spermatogenesis and health of the testes [7]. In general, combinations of parameters of semen quality for predicting in vivo fertility were better than single test [3, 8].

One of the major limitations to maximize the production of buffalo is the poor fertility rate due to the use of bulls with unknown fertility in service that in turn decrease the overall field fertility [9]. The use of low fertile bulls leads to delay the pregnancy, elongate the season of calving, reduce the weight of calf at weaning age and increase culling of dams [8]. In general, bull breeding soundness evaluation requires breeding many healthy fertile females and assessment of pregnancy rates [8, 10]. Bull fertility is a complex physiological process composed of sexual differentiation, maturation and determination.

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Each of these steps is controlled by a number of genes considered as genetic markers for bull fertility [11]. It was published that around 2000 genes are included in spermatogenesis and bull fertility; some of which are present on the Y chromosome considered as biomarkers for semen quality traits in bull [12]. One of these genes is SRY gene that is a male specific gene and considered the main functional region of Y chromosome [13]. SRY gene is an intronless gene [14] and its coding region consists of 690 nucleotides (from base pair 150 to 839) encoding a protein of 229 amino acids in river buffalo [15] which is highly similar to that of cattle with six amino acids different [16, 17]. The encoded protein has DNA binding domain like that in high-mobility-group box (HMG) proteins suggesting that SRY gene plays a role as a transcription factor [16].The SRY protein is expressed in Y chromosome carrying sperm and some other tissues like kidney, adrenal gland and brain of the adult males [17].

This gene is responsible for sex determination in mammals [18] as it is required to initiate testis formation from the urogenital ridge [19]. SRY gene was confirmed as a suitable marker for sex determination in cattle [20] and so for diagnosing freemartin in Holstein cattle [21]. In this regard, SRY gene has been well recommended for embryo sexing based on PCR method with 100% accuracy as described in cattle [22] and ovine [23]. Moreover, Awan et al [24] recommended an effective method for buffalo sperm sexing by modified swim up validated by real time PCR for SRY gene.

On the other hand, the deletion of the SRY region on the Y chromosome was associated with sex reversal with gonadal hypoplasia in cattle [25] and buffaloes [26]. In addition, some SRY gene mutations leads to male sexually reversed to female (XY female) with gonadal hypoplasia in rabbits [27].

In general, mutations in Y-chromosomal genes are very rare and the sequence of the SRY gene is highly stable [28]. In polymorphism studies of SRY gene, the single nucleotide polymorphisms (SNPs) identified at the 202 and 470 bp position of the SRY gene coding region can be used as marker for differentiation between swamp and river domestic buffaloes [29, 30]. Besides, Ramesha et al [31] stated that, single strand conformation polymorphism (SSCP) assay of SRY gene could be used to identify male mediated introgression in bovines. There are few reports regarding the association of SRY gene variants with semen production traits in buffaloes [32] and cattle [11, 32].

Considering the role of the SRY gene in bull fertility, the objective of this investigation was to determine the genetic polymorphism of SRY gene and the association of fresh semen parameters with field fertility of Egyptian buffalo bulls.

2. Experimental

2.1. Animals and samples

A total of 66 mature Egyptian buffalo bulls (Bubalus bubalis) with the same ages (ranged from 3-4 years old) belonged to a breeding station of bulls at Sakha, Kafr el-Sheikh Governorate, Egypt, were used in the present work. They were clinically healthy and subjected to the same management and nutrition programs. Semen samples were collected by artificial vagina twice a week in early morning for evaluation of raw semen before it is used in insemination programs. The collected ejaculates were kept immediately after collection in 37 ºC water bath during the initial semen evaluation.

2.2. Semen evaluation

Fresh semen was evaluated according to Almadaly et al [33] for volume of the ejaculate (estimated to nearest 0.1 ml), individual motility%, sperm cell concentration (calculated by billion per ml of whole semen), live sperm and sperm abnormalities %s (estimated using eosin-nigrosin stained smears). Four ejaculates were evaluated from each bull during the same winter season so a total of 264 ejaculates were evaluated. The bulls producing ejaculates of lower than 60% progressive motility were excluded from insemination.

2.3. Assessment of buffalo bull fertility

The bulls producing semen with ≥ 60% progressive motility (n = 56) were used in natural insemination under the same location, year and season of insemination. A total of 1680 healthy buffaloes free from reproductive diseases or anomalies showing signs of true estrus were inseminated approximately 24 hrs. after onset of heat. The pregnancy was recorded by ultrasonography after two months of insemination. The bulls were categorized into high and low fertile based on pregnancy rate (the number of pregnant females to the total number of inseminated females). The bulls that had pregnancy rate ≥ 50% were considered highly fertile (n = 49) and which had pregnancy rate < 50% were categorized as low fertile bulls (n = 7) according to previous study [10].

2.4. DNA extraction from sperm cells

Semen samples from all buffalo bulls (n = 66) were transported to the reproductive genetics laboratory and preserved at -20 until used in DNA extraction. Genomic DNA was extracted from semen of buffalo bulls according to Hasanain et al [34]. The

excessive drying of DNA pellets was avoided that may fragment DNA. The extracted DNA pellets were then dissolved in 50 µl nuclease-free water. After that DNA concentration was measured by NanoDrop1000 Thermo Scientific spectrophotometer. Then, a working concentration (50 ng/µl) was prepared for each sample which is more suitable for the polymerase chain reaction.

2.5. Polymerase Chain Reaction (PCR) for SRY gene

The DNA fragment (228 bp) of SRY gene was amplified for all bulls (n= 66) through 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 ul of each primer (10pM/ul), 2.0 µl of DNA (50 ng/µl) and nuclease-free water up to 25 µl. The PCR primers (Metabion Inc., Germany) for SRY gene were designed using the online tool ("Primer3" input version 0.4.0) based on the *Bubalus bubalis* gene sequence available in the GenBank (ID: AY341337.1). The sequence of the primers used F: AGAGACATTGCACCCCTTCA R: GCACAAGAAAGTCCAGGCTC**.** The PCR program for SRY gene was initial denaturation at 95 °C for 5 min. then 33 cycles of denaturation at 95 °C for 45 s., annealing at 57 °C for 1 min., then an extension at 72°C for 1 min. and a final extension at 72°C for 5 min. PCR products were detected on 1.5% agarose gel via electrophoresis and stained with red safe to be visualized on UV transilluminator.

2.6. PCR-Restriction fragment length polymorphism (PCR-RFLP)

The amplified PCR fragment (228 bp) of SRY gene was genotyped using PCR-RFLP technique [35]. The *MseI* restriction enzyme (New England Biolabs Inc, U.K.) was identified according to Gopinathan et al [11] and confirmed using the online tool NEB cutter (*http:/ tools,neb.com/NEBuffer2*). The restriction mixture for each sample was 2.0µl of 10 × restriction buffers, 1.0 µl of the restriction enzyme and the volume was completed to 20 µl by DNase free water then 10 µl PCR products was added. Fast digest type of restriction enzyme was used. This mixture was incubated at 37°C for 10 min. The technique was repeated using *MseI* restriction enzyme from another supplier (Thermoscietific Inc., Lithuania) with same conditions but incubated at 65°C for 5 min. The digested PCR products were showed on 2% agarose gel electrophoresis and stained with red safe to be visualized using UV transilluminator.

2.7. PCR-single strand conformation polymorphism (PCR-SSCP)

The PCR fragment of SRY gene was further analyzed using the PCR-SSCP technique [36]. A reaction of 3 µl of PCR products,17 µl distilled water and 10 µl denaturing solution (95% formamide, 10 mM NaOH, 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 immediately on an ice block for 15 minutes. After that the samples were run on 12% non-denaturing polyacrylamide gel (acrylamide and bis-acrylamide ratio, 29:1) containing TEMED (N, N, N´, N´-Tetramethylethylene-diamine) and Ammonium per sulphate (APS). The electrophoresis then performed using 0.7X TBE buffer in a vertical electrophoresis unit (Cleaver, UK) for 20 hours (25 mA and 180 V). The gels were stained with half µg/ml ethidium bromide to be visualized easily on UV transilluminator.

2.8. Nucleotide Sequencing

The PCR products have been well purified using Thermo Scientific GeneJET PCR Purification Kit (product #K0701) following the manufacturer's guidelines. The purified PCR products for 8 samples (4 forward and 4 reverse) were subjected to sequencing by an automated DNA Sequencer (Macrogen Inc., Seoul, South Korea). After sequencing, BioEdit software has been used for multiple sequence alignment for comparing the sequence data among the different samples to search for polymorphisms. The sequence data were further aligned using NCBI/BLAST/blastn suite for comparing the observed sequences in the study with the reference sequences of SRY gene in the GenBank**.**

2.9. Statistical analysis

The statistical analysis was performed using SPSS version 25 software. The obtained data of semen and pregnancy rate were expressed as mean± standard error means (SE). The pregnancy rate in high and low fertile buffalo bulls were compared by t-test. The obtained data of semen and pregnancy rate of bulls used in insemination were further analyzed for association using Bivatiate correlations.

3. Results

3.1. Fresh semen analysis and fertility evaluation

A total of 264 ejaculates were evaluated from 66 buffalo bulls. There were 10 bulls producing ejaculates with lower than 60% progressive motility excluded from insemination. The rest of bulls (n = 56) used in natural insemination had fresh semen

analysis values (mean± SE) as following, volume of the ejaculate by ml (2.9±0.1), individual motility% (74.9±0.8), sperm cell concentration by billion/ml (1.42±0.06) , live sperm% (82.5±0.9) and sperm abnormalities % (18.3±0.6). These inseminating bulls were divided according to pregnancy rate results into high fertile (pregnancy rate ≥ 50%, n = 49) and low fertile (pregnancy rate < 50%, n = 7) with a significant difference (P< 0.001) between the two groups (table 1).

Table 1: The difference of pregnancy rate (Mean ± SE) in high and low fertile buffalo bulls

Bull fertility	No. of bulls	Pregnancy rate (%)
High fertile	49	72.6±1.2***
Low fertile	7	44.4±0.8

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

Table 2 showed the correlations between different semen criteria and pregnancy rate in the inseminating bulls (n=56). A significant negative correlation (r= -.369, P< 0.01) was shown between sperm abnormalities % and pregnancy rate. Moreover a significant positive correlation (r= .273, P< 0.05) was obvious between live sperm % and pregnancy rate. There were no significant correlations between each of ejaculate volume, individual motility %, sperm concentration and the pregnancy rate.

Table 2: Correlations coefficient (r) between semen criteria and pregnancy rate in fertile buffalo bulls

Correlation		Sperm abnormal-ities %	Live sperm %	Ejaculate volume	Individual motility %	Sperm concentra-tion
Pregnancy rate	r value	-.369	.273	.049	-.074	.188
	P value	.005	.042	.722	.588	.165

3.2. Detection of genetic polymorphism of SRY gene

All the tested buffalo bulls (n= 66) gave the specific PCR fagment at the expected size (228 bp) of SRY1 gene (Fig.1). By performing PCR-RFLP technique, all the amplified PCR fragments of 228 bp were digested with *MseI* restriction enzyme into two fragments (154 and 74bp), so, all the studied Egyptian buffalo bulls were genotyped as BB with the predominance of B allele (Fig.2). After PCR-SSCP application on all PCR samples, all the tested buffalo bulls revealed the same SSCP pattern with no genetic differences (Fig.3).

After sequencing of 8 purified PCR samples, ClustalW Multiple alignment was applied for all the sequences using BioEdit software. No genetic difference was observed among the resulted sequences of bulls (Fig.4). These results proved the monomorphic pattern of SRY gene fragment in all the studied Egyptian buffalo bulls without genetic variation. When the sequence alignment was performed with the help of NCBI/BLAST/blastn suite; the sequences of SRY gene in our Egyptian buffaloes were 100 % similar to SRY gene sequence of Pakistani buffalo (Nili-Ravi and Kundi breeds) in the GenBank (Sequence ID: JX667998.1, JX667993.1, and JX667992.1) as shown in Fig.5. The sequence of SRY gene of Egyptian buffalo bulls was submitted to GenBank with accession No. (MZ964599).

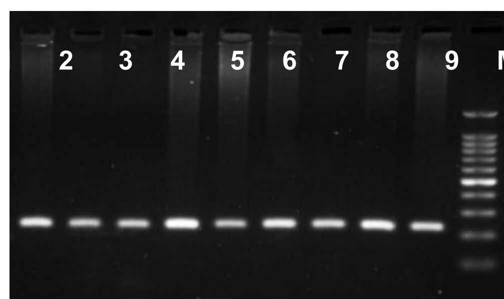

Fig.1: PCR product of SRY gene (228 bp) visualized on 1.5% agarose gel. M= 100 bp.Molecular marker Lanes 1-9 resemble PCR products.

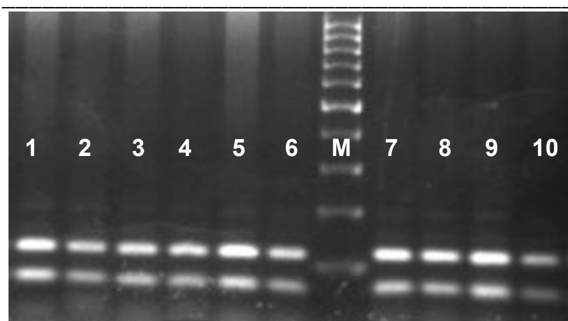

Fig.2: MseI PCR-RFLP Genotypes of SRY gene in 2% agarose gel. M= 100 bp Molecular marker. Lanes 1-10 resemble BB Genotype (74, 154 bp).

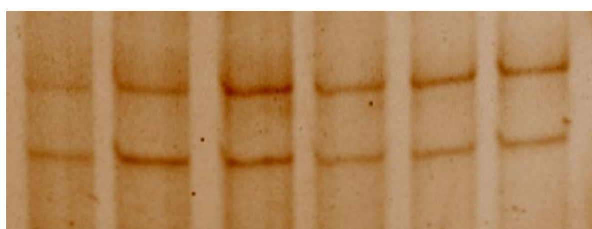

Fig.3: PCR-SSCP patterns of SRY gene of buffalo bulls using 12% PAGE. Samples 1-6 are the same

4. Discussion

The current study aimed to determine the genetic polymorphisms of SRY gene and the association of fresh semen traits with the field fertility of Egyptian buffalo bulls represented by the pregnancy rate of the buffalo cows inseminated by these bulls. The progressive forward motility was used as a traditional indicator for ejaculate quality after semen collection as previously used in routine work [33] because motility act as a reliable predictor for fertility [2, 3].

The semen analysis values in our study were within the acceptable range of good buffalo semen.

This was in accordance with some previous reports [33, 37, 38]. There was a significant sharp increase in the pregnancy rates of the buffalo cows inseminated by high than low fertile buffalo bulls meaning that pregnancy rate is more reliable predictor than semen parameters as an indicator of bull fertility.

By observing the correlations coefficient (r) between semen criteria and pregnancy rate in all the fertile buffalo bulls, we recorded a significant negative correlation between sperm abnormalities % and pregnancy rate. This was in agreement with Gillan et al [39] but others reported no significant correlation between sperm abnormalities and pregnancy rate in buffalo bull [2, 3, 40]. Besides, a significant positive correlation was obvious between live sperm % and pregnancy rate.

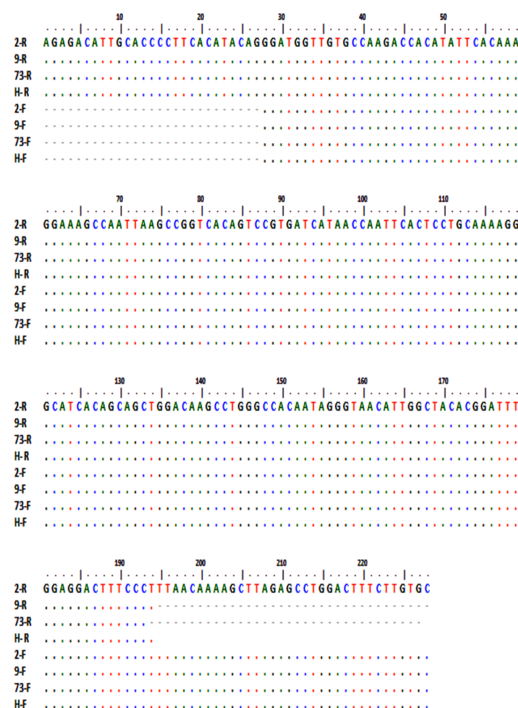

Fig.4: Multiple sequence alignment in 228 bp PCR fragment of SRY gene in 8 Egyptian buffalo bulls using BioEdit software showing 100% identity among the bulls without variation.

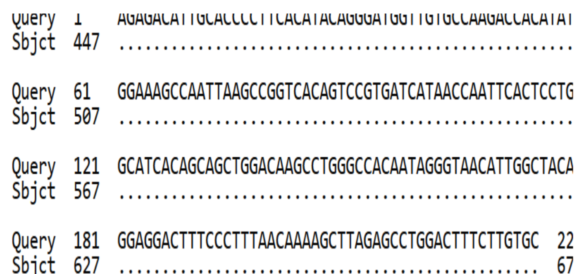

Fig.5: Sequence alignment of SRY gene in Egyptian buffalo bulls, by NCBI/BLAST/blastn suite, showing 100% identity with SRY gene sequence of Pakistani buffalo (Nili-Ravi and Kundi breeds) in the GenBank (Sequence ID: JX667998.1, JX667993.1 and JX667992.1).

Previously a positive correlation was also recorded between pregnancy rate and viability of buffalo bulls' semen [1-4]. However, Kumar et al [41] reported no significant difference between fertile and sub-fertile buffalo bulls in sperm viability of frozen thawed semen. Moreover Selvaraju et al [42] mentioned that sperm viability cannot predict bull fertility accurately.

The progressive motility of sperm was not significantly correlated with the buffalo bull fertility in our findings due to the fact that the bulls with low progressive motility were excluded from insemination of females. This finding was agreed with [1, 10, 40] in buffalo bulls. Other reports recorded a significant correlation between progressive motility and bull fertility in buffalo [2, 3, 43] and cattle [44]. Also, we recorded no significant correlation between sperm concentration and bull fertility that was agreed with Gillan et al [39] in dairy bulls.

Our results proved that no genetic variation in SRY gene in all the studied bulls. So that, SRY gene was highly conserved sequence in Egyptian buffalo bulls. This finding was supported previously by [16] in bovines, Fu et al [13] in swamp buffaloes, Mukhopadhyay et al [32] in Murrah buffaloes, Sahiwal and Karan Fries cattle (in SRY 3'-UTR region). Also, Hartatik et al [45] detected no genetic polymorphism in the coding region of SRY gene in bulls of Madura cattle. Besides, Hartatik et al [46] reported no variation in the promoter region of SRY gene in bulls of Wagyu-BX, BX, Limousin, Simmental, Madura, Ongole, and Hereford cattle.

On the other hand, Mukhopadhyay et al [32] observed polymorphisms in the SRY-HMG region in Sahiwal cattle and Murrah buffalo that were not associated with semen picture of the bulls. In addition, Jayakumar [47] studied polymorphism of SRY gene in Murrah buffalo bulls and reported three polymorphic sites with different three patterns. Also, Gopinathan et al [11] reported a SNP at SRY gene (G>T transversion in 641th position) that was related with sperm concentration and semen volume in crossbred Holstein Friesian and crossbred Jersey bulls. They considered this SNP as a candidate marker for the selection of bulls with good semen production. Besides, Hartatik et al [45] recorded a genetic difference in the crossbred hybrids of Madura cow with Limousin bulls using PCR-RFLP technique. In the same context, Hartatik et al [46] reported three polymorphisms in the promoter region of SRY gene in bulls of Bali cattle and two SNPs in Nellore cattle. In addition, Hartatik et al [48] reported 21 SNPs variations in SRY gene coding sequence in crossbred cattle by using Belgian Blue and Wagyu Bulls. They recommended that SRY can be a good marker for the paternal identification.

5. Conclusion

The sperm abnormalities and live sperm % can be used as good predictors for in vivo fertility in Egyptian buffalo bulls. The pregnancy rate is more accurate than semen analysis for evaluating sire field fertility. The amplified fragment of SRY gene is considered a highly conserved sequence in Egyptian buffalo bulls but further research is recommended for confirmation by expanding the studied fragment of that gene and increasing the number of samples obtained from different localities in Egypt.

6. Acknowledgments

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7. Conflicts of interest

None

8. References

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