NUCLEOTIDE SEQUENCE POLYMORPHISM WITHIN EXON 8 OF HEAT SHOCK PROTEIN (HSP) 90AA1 GENE AND ITS ASSOCIATION WITH MILK PRODUCTION TRAITS IN DEONI COWS

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Received: 07-01-2013 Accepted: 18-04-2013

ABSTRACT

A group of proteins known as Heat Shock Proteins (HSPs) are synthesized during heat stress. HSP genes have been reported to be associated with heat tolerance and production performance in cattle. PCR-SSCP technique and direct sequencing was used to identify novel SNPs in Exon 8 of HSP90AA1 gene in Deoni cows. In Exon 8, three unique SSCP patterns corresponding to TT, GG and TG genotype with frequencies of 0.250, 0.639 and 0.111, respectively were observed. The sequence analysis of Exon 8 revealed T→G transversion at position 3650 of HSPAA1 gene (GenBank accession number NC-007319 as reference sequence). The observed polymorphism (T→G) at position 3650 results in substitution of an amino acid from Phenylalanine to Leucine. The cows having TG genotype had significantly higher lactation Milk Yield (Kg) as compared to cows with TT and GG genotypes (P≤0.01). There was no difference in Lactation Length (days) in cows with different genotypes.

Key words: Deoni cattle, Heat shock protein, Heat tolerance, Polymorphism

INTRODUCTION

Heat stress has adverse effects on reproduction and milk production of dairy cattle. A group of proteins are synthesized during heat stress called Heat Shock Proteins (HSPs). Which protect cells from biotic and abiotic stresses. Ninety-kilodalton heat shock proteins (HSP90) act as important molecular chaperones that are constitutively expressed as a consequence of heat or stress induction (Chen et al. 2006). Heat shock protein 90 is one of the most abundant members of the heat shock protein family. The HSP90 molecule serve as a molecular chaperone after an organism is exposed to pathological or environmental stress. The protective function of HSPs relies on their chaperone activity which consists of assisting the non-covalent assembly and/or disassembly of other macromolecular structures (Ellis, 2006). Single Nucleotide Polymorphisms (SNPs) in HSP90AA1 gene could potentially alter the gene expression and likely to be associated with differences in productive and reproductive performances. Many earlier studies have shown association between Single Nucleotide polymorphisms (SNPs) at certain HSP genes and stress resistance (Reddacliff et al., 2005; Li et al., 2011). Association of polymorphisms of the HSP90AA1 gene with production performance in cattle is yet to be known. Hence the present study was undertaken to determine the genetic polymorphism of Exon 8 of HSP90AA1 gene in n Deoni breed of cattle and to associate the observed genetic polymorphisms with milk production traits.

MATERIALS AND METHODS

Experimental animals and DNA extraction: About 10 ml of blood was collected in EDTA coated vacutainer tube from each of the 72 Deoni cows maintained at National Dairy Research Institute, Bangalore. The samples were stored at -20°C until DNA isolation. The genomic DNA was isolated from white blood cells utilizing high salt method (Miller et al., 1988). The quality and quantity of DNA were checked by UV- spectrophotometer. The DNA samples were diluted to 100ng/µl for utilizing as DNA
template in PCR. The production performance data viz was collected from history-cum-pedigree sheets.

**PCR primers and amplifications:** Primer was designed based on the 5332bp sequence for bovine HSP90AA1 gene (NCBI GenBank NC-007319) using Primer3 software and was procured from Chromous Biotech Bangalore, India. A 539 bp fragment of exon-8 of HSP90 gene was amplified by PCR using Forward: 5’-CCC ATG GGA ACA GTT GAG TG-3’ and Reverse: R-5’-GCT TTA AGC TCC TTT TAA GTT CG-3’ primers. The amplifications were carried out in 0.2 ml PCR reaction tubes using a programmable thermal cycler ((Eppendorf, Germany). The thermal cycling conditions involved an initial denaturation at 94°C for 5 min, followed by 30 cycles with initial denaturation at 94°C for 1 min, annealing temperature of 55°C for 1 min 30 sec, extension at 72°C for 1 min followed by a final extension at 72°C for 5 min. PCR products were detected by electrophoresed at 90 V in 2% agarose gels.

**Single Stranded Conformational Polymorphisms (SSCP):** The 12µl PCR product was mixed with 15µl of denaturing solution containing (95% formamide, 10 mM NaOH, 20 mM EDTA with pH 8.0) and 5 ml loading dye (0.05% xylene cyanol, and 0.05% bromophenol blue, 10% glycerol) and denatured at 95°C for 10 min, quickly chilled on ice and resolved on polyacrylamide gel. The SSCP study was carried out for all the samples using 6 per cent non-denaturing polyacrylamide gel. The denatured and chilled PCR products were loaded in to different wells and gel was run at 200 volts for 10 hrs. As SSCP was found to be very much temperature sensitive, the room temperature was maintained below 20°C. The electrophoresis was carried out in a (SCIE-PLAS, U.K) vertical electrophoresis unit using 1X TBE buffer for SSCP analysis. The gels were silver-stained (Sambrook and Russell, 2001) and examined using transilluminator and photographs were taken using Sony digital camera.

**Sequencing and data analysis:** PCR products giving unique SSCP patterns were given for direct sequencing (Chromous Biotech Pvt Ltd., Bangalore, India). Sequence data were analyzed using, Bioedit software (Hall, 1999) CLUSTAL W multiple alignments for detecting single nucleotide polymorphisms.

**Statistical analysis:** Statistical procedures were carried out as described by Snedecor and Cochran (1994) and tests were performed using of SAS Version 9.2 to find out the significant difference (SAS Inc., 2003). The gene and genotype frequencies, the heterozygosity and x² test of significance were estimated using the POPGENE software (Yeh et al., 1999). The effect of the observed novel SNP in HSP90AA1 gene on production traits were analyzed using the General Linear model (GLM) procedure of SAS Version 9.2, by using the model:

\[ Y_{ij} = \mu + P_i + e_{ij} \]

Where,
- \( Y_{ij} \) = production trait (LMY, and LL) of \( j^{th} \) animal belonging to \( i^{th} \) pattern
- \( \mu \) = Overall population mean
- \( P_i \) = Effect of \( i^{th} \) pattern
- \( e_{ij} \) = random error associated with \( Y_{ij} \) observations

**RESULTS AND DISCUSSION**

**PCR-SSCP analysis:** Total 72 Deoni cows were studied for PCR-SSCP patterns in exon 8 of HSP90AA1 gene. The SSCP analysis of exon-8 of HSP90 gene resulted in three different patterns with the following frequencies: PI= 25%, PII= 63.9% and PIII= 11.1% respectively. The pattern with maximum frequency of 63.9% and lowest frequency of 11.1% was observed in exon 8 of HSP90AA1 gene. SSCP of PCR products was run on neutral polyacrylamide gel. Optimised PCR conditions with annealing temperature of 55°C amplified a fragment of 539 bp of Exon 8 (Fig.1). The electrophoresis was carried out in a vertical electrophoresis chamber (SCIE-PLAS, U.K) in 1× TBE buffer using 6% concentration of non-denaturing polyacrylamide gels. The gels were silver

**FIG 1:** PCR amplification of exon 8 of HSP90AA1 gene in Deoni cattle

LANES 1-4:539BP PCR products; lane 5= marker 100 bp
stained (0.1%) for 30 minutes, dried and documented for detecting mobility shifts. Three different SSCP patterns were observed: PI, PII and PIII (Fig. 2).

**FIG 2: Silver stained PAGE representing PCR-SSCP band patterns of Exon 8 of HSP90AA1 gene**

Lane-3, 4, 6, 7 and 8 shows allelic pattern P-I (TT genotype)

Lanes 2, 9 and 10 shows allelic pattern P-II (GG genotype)

Lanes 1, 5 and 11 shows allelic pattern P-III (TG genotype)

**Sequencing and analysis:** DNA samples representing different SSCP variants were sequenced in order to precisely identify the location and the nature of mutations underlying the SSCP polymorphism. Sequence analysis revealed single nucleotide substitution between individual SSCP patterns. PCR product representing SSCP pattern PI and PII were shown to be opposite homozygote: TT, GG and the SSCP pattern PIII was shown to be heterozygote: TG at position 3650. The position of the individual was calculated according to the reference sequence available in the GenBank accession No. NC-007319 of HSP90 AA1 gene.

**CLUSTAL-W multiple sequence analysis of Exon 8** revealed T→G transversion at position 3650 of HSPAA1 gene. The nucleotide sequences of pattern I and pattern II in exon 8 were found to be two unique homozygotic sequences viz. TT and GG respectively, while the pattern III was observed to be TG. The Results of direct Sequencing is indicated in chromatogram (Fig. 3). The homozygote, consistent with the sequence of GenBank accession No. NC-007319 was named the TT genotype, another homozygote was named the GG genotype, and the heterozygote was named the TG genotype. Based on transeq predict database of EMBL-EBI the observed polymorphism (T→G) at position 3650 results in substitution of an amino acid from Phenylalanine to Leucine which could potentially modify HSPAA1 expression.

**Genotypic and allelic frequencies:** The PCR-SSCP analysis of HSP90AA1 Exon8 resulted in three genotypic combinations viz. TT, TG and GG in the frequencies of 0.250, 0.111 and 0.639, respectively (Table 1). Thus the resulting allelic frequencies were 0.3056 for T allele and 0.6944 for G allele. The high frequency of the G allele suggests that this allele might have been favoured by selection in the Deoni population. The population genetic indices viz. gene homozygosity, gene heterozygosity (He), effective allele number (Ne) and fixation index (Fis) were calculated by x² test and presented in Table 1. The x² revealed highly significant differences between the allele frequencies. The obtained results showed that TG heterozygote frequency is low. The Deoni population was observed to be away from Hardy Weinberg equilibrium and heterozygote deficiency was observed. No reports are available to compare or contrast the present findings.

**Effect of different SSCP patterns on production parameters:** In order to unravel the effect of observed polymorphisms in exon 8 of HSP90AA1 gene on milk production parameters viz. lactation Milk Yield (Kg) and Lactation Length (days) the data were analyzed for association studies using general linear

**FIG 3: Result of direct Sequencing of exon 8 of HSP90AA1 gene showing polymorphic sites(*)position 3650**

Chromatograms P-I of exon 8 of the HSP90 gene

Chromatograms P-II of exon 8 of the HSP90 gene

Chromatograms P-III of exon 8 of the HSP90 gene

A. DNA sequence Homozygous thymine (TT) genotype

B. DNA sequence homozygous GG Genotype

C. DNA Sequence of the heterozygous TG genotype
TABLE 1: The population genetic indices for the novel SNP in Exon 8 of HSP90AA1 in Deoni cattle

<table>
<thead>
<tr>
<th>Locus</th>
<th>Exon 8 of HSP 90 AA1 gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position</td>
<td>3650</td>
</tr>
<tr>
<td>Sample size (n)</td>
<td>72</td>
</tr>
<tr>
<td>Genotypes</td>
<td></td>
</tr>
<tr>
<td>Genotypic frequencies</td>
<td>TT</td>
</tr>
<tr>
<td></td>
<td>TG</td>
</tr>
<tr>
<td></td>
<td>GG</td>
</tr>
<tr>
<td>Genotypic frequencies</td>
<td>0.2500</td>
</tr>
<tr>
<td></td>
<td>0.111</td>
</tr>
<tr>
<td></td>
<td>0.639</td>
</tr>
<tr>
<td>Gene frequencies</td>
<td>T - 0.3056</td>
</tr>
<tr>
<td></td>
<td>G - 0.6944</td>
</tr>
<tr>
<td>Observed Homozygosity</td>
<td>0.8889</td>
</tr>
<tr>
<td>Observed Heterozygosity</td>
<td>0.111</td>
</tr>
<tr>
<td>Expected Homozygosity</td>
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<tr>
<td>Expected Heterozygosity</td>
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<tr>
<td>Effective allele number</td>
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<td>fixation index</td>
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<tr>
<td>fiximation index</td>
<td>0.7382</td>
</tr>
<tr>
<td>( \chi^2 )</td>
<td>40.00</td>
</tr>
</tbody>
</table>

TABLE 2: Association between different SSCP patterns and Milk production traits

<table>
<thead>
<tr>
<th>Pattern/ Genotype</th>
<th>N</th>
<th>Milk Yield (Kg)</th>
<th>Lactation Length(days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI (TT)</td>
<td>18</td>
<td>946.9 ± 135.564a</td>
<td>202.200 ± 15.386a</td>
</tr>
<tr>
<td>PII (GG)</td>
<td>46</td>
<td>914.116 ± 33.123a</td>
<td>200.300 ± 12.288a</td>
</tr>
<tr>
<td>PIII (GT)</td>
<td>8</td>
<td>1020.354 ± 87.432b</td>
<td>206.650 ± 15.583b</td>
</tr>
</tbody>
</table>

Means followed by different superscript letters within columns differ significantly (P ≤ 0.01)

model (GLM) procedure of SAS System 9.2 VERSION (SAS Inc., 2003) and shown in Table 2. It was revealed that significant difference (P ≤ 0.01) of exon 8 of HSP90AA1 gene with pooled Lactation Milk Yield was found in Deoni cattle. The heterozygotic animals with TG genotype had higher Lactation Milk Yield (1020.354 ± 87.432 kg) as compared to both the homozygotic (TT and GG) cows. The Lactation Length was similar in cows with different genotypes. The genetic polymorphisms observed in HSP90AA1 exon 8 and their genetic association with milk production traits reveals the importance of heterozygotic TG genotype had higher Lactation Milk Yield can be useful for genetic improvement of Deoni cattle for milk production traits. The SSCP pattern PIII (GT) has higher least squares means for milk yield (1020.354 ± 87.432 kg) as compared to pattern PI and PII. SO pattern PIII can act as potential genetic marker for milk production traits and can be used in marker assisted selection. Umaiporn et al., 2006 reported association of HSP90AB1 gene polymorphism with heat tolerance traits in crossbred dairy cattle and Thai native cattle but they did not conduct association studies on production traits. The polymorphism of HSP90AB1 gene was reported by them in Thai native and crossbred cattle. Marcos-Carcavilla et al., (2010) reported a single nucleotide polymorphism (SNP) located at position “660 in the 52 flanking region of HSP90AA1 was associated with different thermal conditions in sheep. The results of the present study were first time reported, so no earlier reports are available to compare or contrast the present findings.

CONCLUSION

Single Strand Conformation Polymorphism (SSCP) is a simple and reliable technique used to detect nucleotide variations. SSCP is based on the assumption that changes in the nucleotide sequence of a polymerase chain reaction (PCR) product affect its single strand conformation. The association studies between genetic variants with production performances revealed higher lactation Milk Yield for heterozygotic cows with TG sequence. The genetic polymorphisms observed in HSP90AA1 exon 8 and their genetic association with milk production traits reveals their importance as a potential genetic marker for milk production traits in Deoni.
REFERENCES


