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# Association of milk production and udder type traits with polymorphism of phosphorylase kinase regulatory subunit alpha-2 gene in Sahiwal cattle

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ABSTRACT

The study was conducted in Sahiwal cattle in tropical region in the Indian sub-continent where the production of dairy animals is yet to be optimised. The present investigation was executed to identify SNPs in *PHKA2* gene and to explore its effect on udder type and milk production traits. The study was based on the hypothesis that the *PHKA2* gene has highly variable exons that could be related with udder traits and eventually milk production. PHKA2 gene regulates glycogen phosphorylase a, a catalyst in breakdown of glycogen. Milk production traits were recorded; 9 udder type, 5 teat type and 8 visual traits were measured for 100 animals. Five highly variable targeted regions of *PHKA2* gene were amplified using PCR and sequenced. The association analysis was carried out using general linear model (SAS) to study the fixed effect of genotype on studied traits. The synonymous type SNP g.124556852C>T was found for the first time in Sahiwal cattle and possibly associated with udder type traits. The genotypic frequencies with respect to targeted loci g.124556852C>T indicated that homozygote CC (0.58) were highest in our resource population. The chi-square $^2$ test showed an agreement to Hardy–Weinberg equilibrium. The association analysis revealed significant association of genotypes with udder width, udder balance and 305 days milk yield. The attempt to find significant association with the visual udder traits was also done, however no significant alliance was observed. Homozygote CC animal were desirable as they favoured the selection of animal with superior udder width, udder balance and 305 day's Milk Yield values.

**Keywords**: Alliance, Milk production traits, *PHKA2*, Polymorphism, Sahiwal, Udder type traits

The world has been seeking the means to increase the average lactation milk yield since years. For profitable dairy farming life time milk production and longevity is the key factor of economic efficiency of high-yielding cows (Heins *et al*. 2012, Martens and Bange 2013, Novakoviæ *et al*. 2014). There are the indicators of technical performance at the farm accounting to complex interplay between pathogens, management practices and other factors such as genetic and climatic (Magotra *et al*. 2020). Lifetime productivity/ efficiency depends upon various production and reproduction traits in cattle. Sahiwal (*Bos indicus*) breed of cattle is the dominant milch breed of Indian origin having native tract in North-Western region (Punjab region alongside India–Pakistan border) but a much broader breeding tract in the country (Ratwan *et al*. 2019b, Chopra *et al*. 2020). It is popular for higher milk production, remarkable power of endurance for hot climate of subtropics, comparatively resistant to diseases and low maintenance cost (Ratwan *et al*. 2019a). Despite being genetically superior, cattle has limited production, especially in smallholder management systems.

However, production potential of animal also depends upon on various anatomical traits like udder shape, udder size, teat shape and size (Getu and Misganaw 2015). Udder confirmation is very important to be considered in selection criterion as udder contributes 40% while other categories which are dairy structure, feet and legs, frame and body capacity contribute 20, 15, 15 and 10% in an ideal dairy conformation that can be used to predict production performance (Vukasinovic *et al*.1995). In dairy cattle, udder anatomy and its attachment with fine texture had significant effect on lifetime production potential. Thus, it becomes imperative to develop breeding strategies implementing selection of animals for desirable teat and udder traits.

Bovine phosphorylase kinase regulatory subunit alpha-2 (*PHKA2*) gene present on X-chromosome was found to be associated with udder traits. Cytogenetic and molecular location of *PHKA2* is Xp22.13. PHK is one of the largest and most structurally complex protein kinases known made up of 16 subunits- 4 each of alpha, beta, gamma and delta. Alpha subunit is part of that version of enzyme present in

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liver. Basic functioning of *PHKA2* is to activate the inactive glycogen phosphorylase b by converting it to more active form, glycogen phosphorylase a, which leads to breakdown of glycogen. It has been reported in mutations in *PHKA2* gene cause X-linked recessive liver-specific *PHK* deficiency and glycogen piles up in cell with no energy source left resulting in hepatomegaly and sometimes even cirrhosis (Hendrickx *et al*. 1999).

The information on genetic polymorphisms of *PHKA2* and their association with glycogen disorder has been reported in *Homo sapiens* (Tsilianidis *et al*. 2013), but so far, no research has been carried out to check the genetic effect of *PHKA2* gene in *Bos indicus*. Thus, current study was designed to identify SNPs in the genomic sequence of *PHKA2* gene and to detect its effect on udder and milk production traits in Sahiwal cattle.

## MATERIALS AND METHODS

*Animal and DNA isolation:* The present study was conducted on randomly selected Sahiwal cows maintained at the Livestock Research centre of ICAR-National Dairy Research Institute (NDRI), Karnal. The experiment and plan of study was duly approved by Institutional Animal Ethics Committee (IAEC) of ICAR–NDRI, Karnal, India.

About 10 ml of venous blood was collected from jugular vein of 100 Sahiwal cows under sterile condition into a BD vacutainer coated with EDTA and tubes were kept in deep freeze until the isolation of DNA. Isolation was done using Wizard Genomic DNA Purification Kit (Promega, cat no # 1620A, USA) as per the manufacturer's instructions. Integrity of isolated DNA was checked by 0.8% agarose gel electrophoresis and purity of DNA was assessed by UV–vis spectrophotometer (Biophotometer Plus, Eppendorf) immediately after extraction. The ratio between OD260 and OD280 was observed using UV-visible range spectrophotometer. Samples having 1.7–2.0 ratio of absorbance at 260 and 280 nm were considered as pure DNA and were aliquoted to 50 ng/µL before PCR amplification.

*Primer designing and SNP identification:* The bovine *PHKA2* gene sequence was retrieved from NCBI (NCBI Ref Seq: NC_037357.1) for primer designing. PCR primers were designed using online software Primer 3, i.e. F-5'AGCCAGCTGGTCTTTCTGAC3' and R-5'CAACCTG GACCGCCACTTTA3' encompassing exon 30 and its flanking intronic regions of *PHKA2* gene. The specificity of the primer sequence was checked through 'BLAST' program. 25 ul PCR reaction mixture was prepared with 100 ng DNA template, 1×PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 20 pmol of each primer, and 1 unit of Taq DNA polymerase for PCR amplification. Standard process was executed in following steps—initial denaturation at 92°C for 5 min, followed by 35 cycles of 94°C for 30 sec, annealing at 63.5°C for 30 sec, extension at 72°C for 30 sec, and a final extension at 72°C for 5 min. Aliquots of 10 µl of the PCR amplicons were electrophoresed using 1.5% agarose gel including 0.5 µg/ml of ethidium bromide and photographed under UV light.

All of the amplified PCR products were sent to first base sequencing INT (Singapore) for purification and custom sequencing from both ends (Forward and reverse). The sequences were analysed using Bioedit software for the confirmation of variants. For determining SNPs, reference sequence (NC_037357.1 for *Bos taurus PHKA2* gene) was aligned with sequencing results for each animal and each target region using Clustal W multiple alignment programme.

*Milk production and udder traits:* Data comprised of milk production traits collected from the daily milk yield register in Livestock Record Unit of Animal Genetics and Breeding Division (AG&B), NDRI, Karnal along with Udder and teat type traits that were measured for each animal, spanning over period from August 2018 to July 2019. The traits comprised of 305 day's milk yield (MY), Lifetime MY and nine udder type traits, viz. fore udder attachment (FUA), rear udder height (RUH), udder depth (UD), udder balance (UB), rear udder width (RUW), central ligament (CL), udder length (UL), udder width (UW), udder circumference (UC), 5 teat type traits, viz. teat thickness (TT), teat length (TL), teat circumference (TC), average distance between teats (DBT), average shortest distance from teat ends to floor (DFF) and eight visual traits, viz. udder shape (US), udder suspension (USus), teat end shape (TES), teat end, fore teat placement, rear teat placement, skin condition (SC), long term changes in teat-end condition (LTCTEC). Measurement and scoring of traits were done according to procedure followed by International committee of animal recording (2012). All traits were measured 2 h before evening milk, on centimetre scale except FUA, which was measured in degrees. Teat measurements were done with help of Vernier callipers and Metal tape (200 cm) was used for udder traits. Data were already analysed to examine the effects of non-genetic factors, i.e. season, period and stage of lactation done for standardizing the variations of traits and minimizing error.

*Statistical analysis:* Gene frequency, genotype frequency and Hardy–Weinberg equilibrium (HWE) were calculated through $\chi^2$ test via POPGENE 1.32 software. Association of genotypes with visual traits was done with $\chi^2$ test. Association among genotypes and measurable traits under study were tested using General linear model (GLM) procedure of SAS (Statistical Analysis System 9.3) with a model including genotype as fixed effect. The model used in the study was:

$$Y_{ij} = \mu \pm G_i \pm A_i \pm e_{ijk}$$

where, $Y_{ij}$, observed value of targeted traits; µ, overall mean; $G_i$, fixed effect of $i^{th}$ genotype and $e_{ijk}$, random residual error NID (0, $\sigma^2_e$). Data were presented as mean±SE, and significance was declared at $P< 0.05$.

## RESULTS AND DISCUSSION

*Genotyping of the PHKA2 gene:* The PCR amplified product of 405 bp (Fig.1) in our targeted population after sequencing exhibits g.124556852C>T synonymous

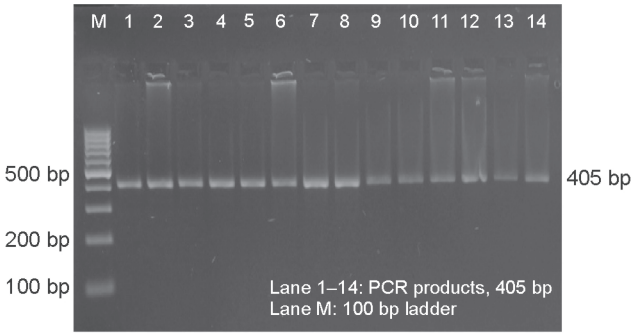


Fig. 1. PCR amplified product of target region of *PHKA2* gene of Sahiwal cattle.

mutation in exon 30 of *PHKA2* gene. Chromatogram changes in *PHKA2* gene in targeted animals are shown in Fig. 2. Genetic architecture of targeted locus in our resource population exhibits 0.76 and 0.24 frequency of C and T allele. The frequency of CC, CT and TT genotypes were observed as 0.58, 0.36 and 0.06 respectively (Table 1). Chi-square test showed that *g*.124556852C>T SNP met with the Hardy–Weinberg equilibrium (P< 0.01).

Production traits are significant in dairy cattle in particular. First and foremost, high milk production is conditioned by a good and healthy udder. Udder morphology is closely linked to sustainable milk production (Tribout *et al*. 2020). Patel *et al*. (2016) mentioned the positive and significant correlations between milk yields and various udder measurements, viz. UL (0.499), UW (0.413) and UD (0.178) and thus, the selection of these traits would result in selection of animal with high milk yield. Also, conformation (type) traits are related to functionality of the cow's body and thus increase the value of cow as a show animal (Kshatriya *et al*. 2009). Thus, there are great benefits to consider these traits in the same study.

A number of studies has been accomplished regarding *PHKA2* gene in case of humans but informative reports are limited in animals. Guo *et al*. (2019) attempted to screen candidate genes affecting skeletal muscle growth and development in pigs where 16 candidate genes were identified, including PHKA1, *PHKA2*, PHKG1, PHKG2. Though there are few mutational studies and their effects

Table 1. Distribution of genotype and allelic frequency of SNP g.124556852C>T of *PHKA2* gene in Sahiwal cattle

| Genotype frequency | | | Total | Allele frequency | | $\chi^2$ |
|---|---|---|---|---|---|---|
| CC | CT | TT | | C | T | 0.895* |
| 0.58 (58) | 0.36 (36) | 0.06 (6) | 100 | 0.76 | 0.24 | |

*, P<0.05; figure in parentheses indicates number of animals; $\chi^2$ = chi-square test for Hardy-Weinberg equilibrium.

conducted on the lab animals (Maichele *et al*.1996) but *PHKA2* polymorphism association studies in cattle is almost nil. However, Cole *et al*. (2011) suggested that this candidate gene is related to udder and other body measurements traits in US Holstein cattle. Due to differences in ancestry between *B. taurus* and *B. indicus*, it was thought whether markers identified in taurine could be used in zebu cattle (Casas *et al*. 2005). Moreover, this gene is present on X-chromosome and few evident studies on association studies among X-chromosome and udder traits have been conducted. De Camargo *et al*. (2015) has mentioned the identified QTL regions in the X chromosome responsible for percentage of normal sperm and scrotal circumference in Brahman and Tropical Composite cattle. Generally, these traits are indicators of male fertility and are correlated with female sexual precocity, productive and reproductive longevity. Yan *et al*. (2020) also found in a GWAS conducted in Chinese Holstein cattle population, that X-chromosome harbors the region (BovineHD3000037672) significantly associated with mammary system of the cattle. Genetic improvement for bull fertility is possible through genomic selection, which is likely more accurate if the QTL on chromosome X are considered in the predictions. Polymorphisms associated with male fertility accumulate on this chromosome in cattle, as in humans and mice (Fortes *et al*. 2020).

*Association of genotypes with udder and milk production traits:* Nowier *et al*. (2020) determined associations of SNP with udder support scores, teat length, and teat diameter using a commercially available array in half *B. indicus* (Nellore), half *Bos taurus* (Angus) cows. They found out BTA 5 is important and suggestive of a few genes /

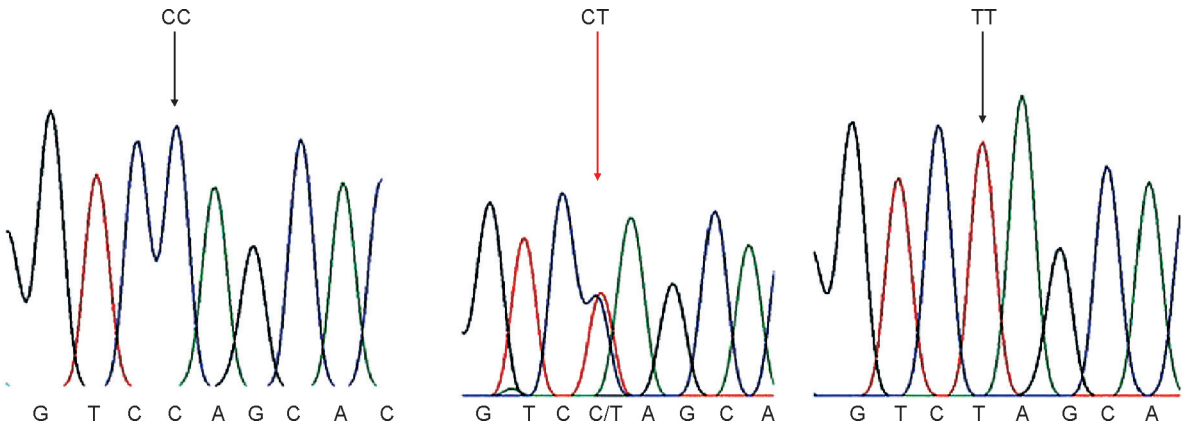


Fig. 2. Chromatograph showing 3 genotypes at target g.124556852C>T in Sahiwal cattle.

regulatory elements in the region for udder traits. In another genome-wide association analyses in goats by Luigi-Sierra *et al*. (2020), they found 12 chromosome-wide significant associations for udder traits using the GoatSNP50 BeadChip (Illumina Inc., San Diego, CA) in resource population.

In order to disentangle the biological relationship between these complex traits and proposed candidate causative variant, the objective of this study was to identify the polymorphisms, that are responsible for the genetic variation in traits related to milk production and udder morphology in the Sahiwal cattle.

All the 3 possible genotypes at g.124556852C>T SNP loci were observed in our resource population. Sahiwal cattle showed significant association with 305 days MY, UB (P<0.05) and with UW (P<0.01) (Table 2). CC homozygote animals were higher milk yielders with higher UW values, which is desirable. TT homozygote revealed lowest values of UW and 305 days MY, being significantly different from CT heterozygotes. Similarly, the lower negative value of UB showed that CC homozygotes were superior than the rest of the animals and the values of different genotypes were significantly different from each other. Although, the associations of genotypes with the rest traits were nonsignificant, however, the probability of association with few traits like UL, TD and DBT was a possibility, if study was conducted on larger population. CC homozygote animals had longest udder length while it was lowest for the TT homozygote and heterozygote being the intermediate one. Similarly, CC homozygotes were also having the thickest teats and TT homozygotes had the lowest values for TD. These findings indicated the superiority of CC homozygotes over the heterozygote and TT homozygotes. On the contrary, the highest values of DBT were for CC homozygotes which is not a desirable as the DBT should be as small as possible which was seen in TT homozygotes. The association of other traits revealed almost the similar average mean values for all the genotypes. Similar association was observed in the results of Li *et al*. (2020) who found a significant relationship between SNP c.1571 G > A (*FADS2*) gene and 305-day milk yield where genotype GG was linked to the highest milk yield in Chinese Holstein cows. Udder is found to be more balanced and symmetrical in CC homozygote as compared to other genotypes which is a very desirable character since udder asymmetry was identified as a potential risk factor for clinical mastitis (Slettbakk *et al.* 1990). It has been observed that when rear halves are heavier and larger than front halves, then more chances of infection are there. Since no contrasting study of the gene has yet been done, therefore no more comparison could be done. The association of different genotypes was also studied with the visual traits as shown in Table 3, however no significant alliance was observed. The frequency of trough shaped udders was the highest in all genotypic groups. In case of udder suspension, almost 78% TT homozygotes had the intermediate suspension while in heterozygotes udders with tight suspension were observed with a higher frequency. For teat end shape, round shape was the most frequent followed by conical shape in all the genotypic groups. Funnel type teat shape was the most frequent one in all the genotypic groups. Fore teats most frequently were placed intermediately in CC homozygotes and CT heterozygotes whereas in TT homozygotes, the frequency of outward placed teats was the highest. In case of rear teat placement, CC homozygotes had most frequent inward placed teats, CT heterozygotes had most frequent outward placed teats and TT homozygote had most frequent intermediately placed teats. Mostly animals showed normal skin conditions in all genotypic groups. The most frequent long-term change in teat-end condition in homozygotes animals was rough rings on the teats while heterozygote animals most frequently showed no rings at all.

Table 2. Association of SNP g.124556852C>T of *PHKA2* gene with milk production and udder type (measurable) traits in Sahiwal cattle

| Trait | Genotype (LSM±SE) | | |
|---|---|---|---|
| | CC | CT | TT |
| Rear udder height (cm) | 26.56±0.38 | 26.01±0.46 | 26.24±1.33 |
| Rear udder width (cm) | 10.47±0.45 | 9.89±0.80 | 10.02±1.32 |
| Udder width (cm) | 85.6±3.03$^{ac}$ | 63.5±2.73$^{bc}$ | 46.7±0.98$^{c}$ |
| Fore udder attachment (°) | 117.45±1.80 | 119.00±2.40 | 116.46±4.74 |
| Udder circumference (cm) | 126.40±1.30 | 127.56±3.06 | 126.18±5.08 |
| Udder balance (cm) | –0.34±0.67$^{a}$ | –0.59±0.78$^{b}$ | –0.92±1.99$^{c}$ |
| Central ligament (cm) | 4.08±0.40 | 4.13±0.87 | 5.11±1.24 |
| Udder depth (cm) | 35.60±0.87 | 34.37±0.98 | 31.34±2.77 |
| Udder length (cm) | 75.54±1.56 | 73.45±0.40 | 72.89±3.42 |
| DBT (cm) | 2.09±0.29 | 1.98±0.44 | 1.76±0.87 |
| DFF (cm) | 48.82±2.22 | 47.62±1.09 | 48.52±0.17 |
| Teat circumference (cm) | 6.53±0.10 | 5.04±0.30 | 5.56±0.15 |
| Teat diameter (cm) | 2.24±0.05 | 2.02±0.07 | 1.99±0.34 |
| Teat length (cm) | 5.22±0.50 | 4.53±0.21 | 5.56±0.30 |
| 305 days milk yield (kg) | 2220.25± 156.76$^{a}$ | 1908.63± 147.30$^{ab}$ | 1416.45± 153.19$^{c}$ |
| Life time milk yield (kg) | 5725.98± 372.12 | 5497.56± 420.32 | 5210.34± 847.10 |

$^{a,b,c}$Means within the same row with different superscripts are significantly different (*P<0.05).

The study reported first time possible association of phosphorylase kinase regulatory subunit alpha2 gene with udder and milk production traits in Sahiwal cattle. Any significant interaction of visual udder traits with polymorphism could not be seen in the resource population. The findings related to SNP g.124556852C>T in *PHKA2* gene can be used as a candidate marker in *Bos indicus* to select animals with desired udder confirmation and higher milk production. Results of present investigation can be considered as a preliminary foundation for further replication studies and implied that SNP g.124556852C>T, together with other gene polymorphisms, may be potentially used for genetic selection of udder type and milk production

Table 3. Association of SNP g.124556852C>T of *PHKA2* gene with udder type (visual) traits in Sahiwal cattle

| Trait | Coding | Genotype | | |
|---|---|---|---|---|
| | | CC (18) | CT (62) | TT (20) |
| Udder shape (US) | 1: Pendulous | 3 (16.67%) | 13 (20.96%) | 4 (20%) |
| | 2: Trough | 8 (44.45%) | 27 (43.54%) | 13 (65%) |
| | 3: Round | 7 (38.89%) | 12 (19.35%) | 3 (15%) |
| | | CC (16) | CT (65) | TT (19) |
| Udder suspension (USus) | 1: Very tight/ tight | 4 (25%) | 26 (40%) | 3 (15.78%) |
| | 2: Intermediate | 6 (37.5%) | 20 (30.76%) | 15 (78.94%) |
| | 3: Pendulous | 6 (37.5%) | 19 (29.23%) | 1 (5.20%) |
| | | CC (68) | CT (23) | TT (9) |
| Teat end shape | 1: Flat | 10 (14.70%) | 5 (21.73%) | 1 (11.12%) |
| | 2: Round | 37 (54.4%) | 8 (34.78%) | 5 (55.56%) |
| | 3: Pointed | 21 (30.88%) | 10 (43.47%) | 3 (33.34%) |
| | | CC (25) | CT (65) | TT (10) |
| Foreteat placement | 1: Inside (close) | 9 (36%) | 16 (24.61%) | 2 (20%) |
| | 5: Intermediate | 13 (52%) | 27 (41.53%) | 2 (20%) |
| | 9: Outside (wide) | 3 (12%) | 22 (33.84%) | 6 (60%) |
| | | CC (33) | CT (58) | TT (9) |
| Rear teat placement | 1: Inside (close) | 18 (54.54%) | 21 (36.2%) | 3 (33.34%) |
| | 5: Intermediate | 14 (42.42%) | 7 (12.06%) | 4 (44.45%) |
| | 9: Outside (wide) | 1 (3.03%) | 30 (51.72%) | 2 (22.23%) |
| | | CC (14) | CT (59) | TT (27) |
| Skin condition (SC) | 1: Normal | 13 (92.85%) | 47 (79.66%) | 15 (55.5%) |
| | 2: Dry | 1 (7.14%) | 3 (5.08%) | 12 (44.45%) |
| | | CC (70) | CT (23) | TT (7) |
| Long term changes in teat-end condition (LTCTEC) | 1: No ring | 17 (24.28%) | 8 (34.78%) | 1 (0.14%) |
| | 2: Rough ring | 37 (52.85%) | 4 (17.39%) | 4 (57.14%) |
| | 3: Smooth ring | 10 (14.28%) | 7 (30.43%) | 2 (28.57%) |
| | 4: Very rough ring | 6 (8.57%) | 4 (17.39%) | 0 |
| | | CC (72) | CT (22) | TT (6) |
| Teat shape (TS) | 1: Funnel shape | 43 (59.7%) | 10 (45.45%) | 3 (50%) |
| | 2: Cylindrical | 25 (34.7%) | 8 (36.37%) | 2 (33.34%) |
| | 3: Bottle shape | 4 (5.56%) | 4 (18.19%) | 1 (16.67%) |

traits in Sahiwal cattle.

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