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Comparative Sequence Analysis of PRKAG3 Gene Between Pakistani Buffalo and Cattle Breeds

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Abstract.- Fraudulent beef/meat labeling is an issue of concern in Pakistan because of the lack of an effective regulatory mechanism. DNA markers provide a diagnostic tool to detect fraudulent labeling. We therefore investigated species-specific variation in the PRKAG3 gene as a resource for diagnostic marker development. A total of 147 DNA samples from four cattle (Sahiwal, Lohani, Dhanni and Cholistani) and three buffalo (Nili, Ravi and Nili-Ravi) breeds were analyzed for sequence differences by direct sequencing of the genomic protion of the gene spanning exons 3 and 4. Inter and intra-generic variation was detected at 27 nucleotide sites, amongst which 15 distinguished cattle from buffalo. For further confirmation, additional 35 DNA samples from all breeds were sequenced, which further validated our initial findings. The sequence variability detected in the PRKAG3 gene are now available as a resource to develop diagnostic markers to detect fraudulent labeling or illegal mixing of the meat from buffalo and cattle.

Key words: PRKAG3 gene, genetic variation, Pakistan

INTRODUCTION

The 13 exons of the bovine gene AMP-activated protein kinase gamma 3 (AMPKγ3 or PRKAG3) spans approximately 6.8 kb on BTA2 and encodes a protein of about 465 amino acids (Yu *et al.,* 2005). The encoded protein is denoted as gamma 3 to differentiate it from other two isoforms (γ1 and γ2) of the same gamma regulatory subunit of AMPK family. The AMPK is a heterotrimeric complex made up of two more subunits, a catalytic subunit alpha and a regulatory subunit beta, in addition to the regulatory subunit γ (Carling, 2004). Each of the alpha and beta subunits consists of two isoforms (α1 and α2) and (β1 and β2), respectively. These seven α, β and γ isoforms are encoded by different genes and make assemblage in 12 different combinations to synthesize tissue-specific AMPK heterotrimers (Thornton *et al.,* 1998; Cheung *et al.,* 2000; Yu *et al.,* 2005; Birk and Wojtaszewski, 2006).

PRKAG3 is expressed abundantly in the tissues of skeletal muscles of different mammalian species including *Homo sapiens* (Cheung *et al.,* 2000; Mahlapuu *et al.,* 2004; Yu *et al.,* 2005; Costford *et al.,* 2007; Weyrich *et al.,* 2007) and plays an indispensable role in the regulation of AMPK complex. The AMPK is described as molecular switch that integrates various hormonal and nutritional signals to keep the energy demands of an organism in balance. Its activation in skeletal muscles in response to the stress condition stimulates cellular uptake and oxidation of fatty acids and glucose to restore energy supply, and inhibits the key enzymes of biosynthetic pathways such as cholesterol and fatty acid synthesis to conserve the energy input for life-sustaining physiological processes (Kahn *et al.,* 2005; Hardie and Sakamoto, 2006).

Based on the conservation of the AMPK complex across species ranging from yeast to mammals, we hypothesize that the interspecies nucleotide variation in genes encoding the AMPK complex could be robust source of diagnostic markers to detect mislabeled or contaminated meat. Therefore, we sequenced more than one third (186 out of the total 465 amino acids) of the protein encoding region of PRKAG3 avoiding the most

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conserved CBS domains of the PRKAG3 gene to identify maximum genetic variability at this locus in bovine family with a focus on finding variability that would distinguish beef/meat from buffalo and cattle.

MATERIALS AND METHODS

Twenty-one blood samples from each of three buffalo (Nili, Ravi and Nili-Ravi) and 4 cattle breeds (Sahiwal, Lohani, Dhanni and Cholistani) were randomly collected from Livestock Production Research Institute Bahadarnagar, Okara; Rakh Dera Chahl, Lahore; Barani Livestock Production Research Institute (BLPRI) Kherimurat, Attock; and Research Centre for Conservation of Sahiwal Cattle (RCCSC) Jahangirabad, Khanewal. Amongst these cattle breeds Sahiwal and Cholistani are milk breeds, while Lohani and Dhanni are medium draught breeds. The buffalo breeds are well-recognized for milk production. These breeds may be contributing to beef production in Pakistan. Five animals of each breed from village herds were also sampled for blood. Genomic DNA was extracted from these 172 blood samples by using the protocol of Grimberg *et al.* (1989). The concentration of extracted DNA was adjusted to 50 ng/µl in TE buffer (pH 8.0), with the use spectrophotometer and agarose gel electrophoresis.

An 885 bp fragment including intron 3 and exons 3&4 (GeneBank accession number AY692035) of the AMP-activated protein kinase gamma subunit (PRKAG3) was PCR-amplified by using a forward primer U1A (5´-919GAGCAAGGAGACAGCACTTCA939-3´) and a reverse primer U2B (5´-1782ACCTGTAGCATGGTGTCGAAGA1803-3´). In addition, forward UP SEQ1 (5´-1295GACCTCAGCATCCAGGCT1312-3') and reverse UP SEQ2 (5´-1362GACAGTAACTCCATCTTCCA1381-3´) primers were used in sequencing PCR reactions. The PCR reactions contained 50 ng genomic DNA, 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 µM dNTPs, 10 pM each primer, 1.5 units Ampli Taq Gold (Applied Biosystems, Foster City, CA) in 25 µl volume. The amplification was performed at following temperature conditions: an initial step of denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, extension at 72 °C for 1 min. A step of final extension was programmed at 72 °C for 10 min. The PCR products were resolved on 1.5% agarose gel mixed with DNA-intercalator ethidium bromide to visualize their presence under UV light.

The PCR products were purified by Exo SAP-IT (Amersham) according to manufacturer's instructions and were used in sequencing PCR reactions for bidirectional sequencing to analyze and confirm polymorphism. After purification, the sequencing PCR products were resolved on an ABI 3130 capillary DNA analyzer (Applied Biosystems, Inc., Foster City, CA). Sequence analyses were performed using the Chromas Lite (2.01) software and the 2-sequence alignment program available at NCBI(www.ncbi.nlm.nih.gov.library.vu.edu.au/BLAST/) .

RESULTS AND DISCUSSION

The comparison of the PRRKAG3 gene in Pakistani buffalo and cattle showed a total of 12 intraspecies polymorphic sites (4 in buffalo and 8 in cattle), amongst which 8 sites were present in intron 3 (3 in buffalo and 5 in cattle) and 4 in exon 4 (Table I). No variation was detected in the third exon of the gene of either species. Of the four polymorphisms detected in exon, the gAT/aAT1688 at codon 149 of the gene in cattle induces an amino acid change D→N. In addition to polymorphic sites, interspecies variations were also detected at 15 sites of the gene (7 in intron 3 and 8 in exon 4) (Table I). Two of these sites, (cCA/gCA1412 [P/A57] and CtG/CaG1575 [L/Q111]) varied in amino acid translation. As the non-synonymous polymorphisms in the PRKAG3 gene have been shown to influence the contents of glycogen, lactate and intramuscular triglycerides (IMTG) in skeletal muscle tissues of pig and human (Milan et al. 2000; Ciobanu et al. 2001; Costford et al. 2007), the D/N149 in cattle should be investigated for association with beef quality as well as other production traits like milk yield and quality, and weight gain. The presence of P57, D149 and L111 in buffalo and A57 and Q111 in cattle could also have influence on

cholesterol/fat-contents in lipoproteins (Weyrich *et al.,* 2007) and in milk as there is a significant

Table I.- Genotypic frequencies (%) of variations found in the PRKAG3 gene of three buffalo and four cattle breeds.

Positions of species-specific and polymorphic nucleotides* (% frequency)	Breed**						
	N (21)	R (21)	NR (21)	S (21)	L (21)	D (21)	C (21)
1070*	CC (72) CT (14) TT (14)	CC (62) CT (38)	CC (52) CT (48)	CC***	CC	CC	CC
1093	AA	AA	AA	CC	CC	CC	CC
1122	GG	GG	GG	AA	AA	AA	AA (86) AG (14)
1172	GG	GG	GG	GG	GG (57) GA (43)	GG (72) GA (28)	GG (28) GA (72)
1186	TT	TT	TT	CC	CC	CC	CC
1195	GG	GG	GG	GG (14) CC (86)	GG (38) GC (38) CC (24)	GG (14) GC (43) CC (43)	GG (14) GC (86)
1197	GG	GG	GG	GG	GG	GG	GG (86) GA (14)
1218	GG	GG	GG	AA	AA	AA	AA
1240	AA	AA	AA	AA	AA (72) AT (28)	AA (86) AT (14)	AA (57) AT (43)
1264	GG	GG	GG	AA	AA	AA	AA
1267	CC	CC	CC	TT	TT	TT	TT
1284	TT	TT	TT	CC	CC	CC	CC
1319	GG	GG	GG	AA	AA	AA	AA
1344	AA (72 AG (28))	AA	AA (86) AG (14)	AA	AA	AA	AA
1382	CC (72) CT (14) TT (14)	CC (62) CT (38)	CC (52) CT (48)	CC	CC	CC	CC
1412!	CC	CC	CC	GG	GG	GG	GG
1420	CC	CC	CC	TT	TT	TT	TT
1489	GG (72) GA (14) AA (14)	GG (62) GA (38)	GG (52) GA (48)	GG	GG	GG	GG
1510	GG	GG	GG	AA	AA	AA	AA
1531	TT	TT	TT	CC	CC	CC	CC
1575!	TT	TT	TT	AA	AA	AA	AA
1603	GG	GG	GG	GG (14) GA (14) AA (72)	GG (38) GA (38) AA (24)	GG (14) GA (43) AA (43)	GG (14) GA (86)
1609	GG	GG	GG	GG (86) GA (14)	GG (57) GA (43)	GG (72) GA (28)	GG (28) GA (72)
1618	TT	TT	TT	CC	CC	CC	CC
1648	TT	TT	TT	CC	CC	CC	CC
1688!	GG	GG	GG	GG (72) GA (28)	GG (86) GA (14)	GG (57) GA (43)	GG (90.5) GA (9.5)
1711	CC	CC	CC	TT	TT	TT	TT

* Nucleotide positions are comparable with PRKAG3 gene sequence provided in GeneBank accession number AY692035

** Names of buffalo breeds are abbreviated as N (Nili), R (Ravi) and NR (Nili-Ravi) and of cattle breeds as S (Sahiwal), L (Lohani), D (Dhanni) and C (Cholistani)

*** Refers to 100% frequency

! Indicates single-nucleotide polymorphisms causing change in amino acids
@ The underlined nucleotide positions exist in intron 3, while others exist in exon 4 of the gene

Table II.- The targets of restriction enzymes in 14 bp sequences with detected species-specific polymorphisms.

Polymorphism position	Target sequence*	Restriction enzymes	Recognition sequences/sites**
1093	tggga**c/a**tgggggca	*Bse*1I, *Bsm*FI	actgg, gggac
1186	ttccc**c/t**acatgaga	ND (not detected)	ND
1218	gatcc**a/g**gggggccca	*Msp*I	c/cgg
1264	gtgtg**a/g**ctttctca	*Mae*III, *Tsp*45I, *Cvi*JI	/gtnac, /gtsac, rg/cy
1267	gtgtg**t/c**ctctctca	*Bsm*AI, *Mn*lI	gtctc, cctc
1284	tcact**c/t**gcaaaatg	ND	ND
1319	cccac**a/g**ctgctctg	*Bbv*I, *Ita*I	gcagc, gc/ngc
1412	ccagg**g/c**cagctgct	*Bsa*JI, *Bso*FI, *Bst*71I, *Cac*8I, *Cvi*JI	c/cnngg, gc/ngc, gcagc, gcn/ngc, rg/cy
1420	gctgc**t/c**gagtccacc	*Dde*I	c/tnag
1510	acaga**a/g**cgagacag	ND	ND
1531	tctga**c/t**tgtacagc	ND	ND
1575	ggatc**a/t**gggcatag	*Xho*II	r/gatcy
1618	gagct**c/t**ggcctggt	*Ban*II, *Ecl*136II, *Psp*124BI	grgcy/c, gag/ctc, gagct/c
1648	cagtg**c/t**ccgtcccc	ND	ND
1411	ggggc**t/c**caggtcta	*Dde*I, *Apa*I, *Asp*S9I, *Bse*DI, *Bsp*120I *Cvi*JI, *Dra*II, *Hae*III, *Msp*R9I, *Nla*IV	c/tnag, gggcc/c, g/gncc, c/cnngg, g/ggccc rg/cy, rg/gnccy, gg/cc, cc/ngg, ggn/ncc

*The concerned polymorphisms are shown in bold letters in 14 bp target sequences.
**Recognition sequences/sites of respective restriction enzymes are given in row-wise descending order *i.e.*, 1st enzyme corresponds to 1st recognition sequence.
Note: More restriction enzymes with same recognition sequences/sites can be found by Google search using keywords (restriction enzyme + name of the enzyme) *i.e.*, Key word restriction enzyme *Hae*III will result in finding of *Bsh*FI, *Bsp*ANI, *Bsu*RI and *Pho*I enzymes which also recognize gg/cc like *Hae*III does.

difference in milk fat contents (6.5%) and (4.5%) of buffalo and cattle, respectively (Khan *et al.*, 2005).

To the best of our knowledge, this is the first study of PRKAG3 gene in buffalo (DQ646431). While the comparison of polymorphisms detected in the present study with those previously-reported for cattle showed that G/C1195 and G/A1197 have also been found in Brahman (Mckay *et al.*, 2003) indicating the specificity of these polymorphisms for Asian cattle breeds. The G/T1172, A/T1240 and G/A1609 polymorphisms have also been reported for various cattle breeds (Mckay *et al.*, 2003; Yu *et al.*, 2005; Roux *et al.*, 2006), while the remaining A/G1122, G/A1603 and G/A1688 polymorphisms have to-date been found in Pakistani cattle only.

This study provides 15 interspecies nucleotide differences in the PRKA3 gene that provides clear differentiation between buffalo and cattle. These include 1093, 1186, 1218, 1246, 1267, 1284, 1319, 1412, 1420, 1510, 1531, 1575, 1618, 1648 and 1711. The corresponding haplotypes for buffalo and cattle for these positions are ATGGCTGCCGTTTTC and CCAATCAGTACACCT, respectively (Table I). These differences may serve as a resource for development of diagnostic DNA marker to detect meat content for buffalo and cattle. To test whether the specific variability detected in the PRKAG3 gene could be considered as one of the markers to assess fraudulent labeling or illegal mixing of beef/meat, 35 additional DNA samples were sequenced to ascertain the findings of this study. No deviation was found from the previous findings.

CONCLUSION

The results of this study suggest that specific variability detected in the PRKAG3 gene of buffalo and cattle could be targeted for further confirmation by RFLP to be used for testing fraudulent beef/meat labeling or mixing. According to Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/) based search, most of the target sequences with detected polymorphisms harbor recognition sites for various

restriction enzymes (Table II). Furthermore, the detected amino acid variability if associated with various body tissues/fluids biochemistry could help improve the animals' health, and quality and quantity of food derived from genus *Bos* of the Bovidae family.

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