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Mutation in Cytochrome B gene causes debility and adverse effects on health of sheep

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A B S T R A C T

Cytochrome B is the mitochondrial protein, which functions as part of the electron transport chain and is the main subunit of transmembrane cytochrome *bc*1 and b6f complexes affecting energy metabolism through oxidative phosphorylation. The present study was conducted to study the effect of mutation of Cytochrome B gene on the health condition of sheep, which the first report of association of mitochondrial gene with disease traits in livestock species. Non-synonymous substitutions (F33 L and D171N) and Indel mutations were observed for Cytochrome B gene, leading to a truncated protein, where anemia, malfunctioning of most of the vital organs as liver, kidney and mineral status was observed and debility with exercise intolerance and cardiomyopathy in extreme cases were depicted. These findings were confirmed by bioinformatics analysis, haematological and biochemical data analysis, and other phenotypical physiological data pertaining to different vital organs. The molecular mechanism of cytochrome B mutation was that the mutant variant interferes with the site of heme binding (iron containing) domain and calcium binding essential for electron transport chain. Mutation at amino acid site 33 is located within transmembrane helix A, a hydrophobic environment at the Qi site and close to heme binding domain, and mutation effects these domain and diseases occur. Thermodynamic stability was also observed to decrease in mutant variant. Sheep Cytochrome B being genetically more similar to the human, it may be used as a model for studying human diseases related to cytochrome B defects. Future prospect of the study includes the therapeutic application of recombinant protein, gene therapy and marker-assisted selection of disease-resistant livestock.

1. Introduction

Cytochrome B, located in the mitochondria of eukaryotic cells is the polypeptide of the complex encoded by a mitochondrial gene. Besides aiding in the synthesis of amino acid and heme group (Kim et al., 2012) it also leads to the uptake and release of calcium ions essential for triggering cellular activities and in the process of apoptosis (Brown et al., 1999; Parsons and Green, 2010). Cytochrome B functions as part of the electron transport chain and is the main subunit of transmembrane cytochrome *bc*1 and b6f complexes (Howell, 1989; Baniulis et al., 2008). Being an essential component in the respiratory chain complex III, it is also known as ubiquinol-cytochrome *c* reductase (Berry et al., 2000; Up and Crofts, 2004). Mammalian complex III is a dimer with each subunit complex consists of 11 protein subunits. It is also known as Q-cytochrome *c* oxidoreductase or simply Cytochrome C reductase. Along with Cytochrome B, Cytochrome C, an iron sulfur cluster (2Fe−2S) is present (Up et al., 1998) and these complexes involved in the electron transport, plays a vital role for the pumping of protons to create a proton-motive force (PMF) and this proton gradient is used for the generation of ATP. (Berry et al., 2018).

As there is electron transfer, complex III's heme group undergo the reduction state from ferrous (+2) and oxidized ferric (+3). Accordingly, there is oxidation of one molecule of ubiquinol and reduction of two molecules of cytochrome C. Cytochrome c transfers a single electron, whereas coenzyme Q transfers two electrons. The mechanism of complex III is more elaborate compared to other respiratory complexes, occurs in two steps, called as Q cycle. In this process, only one electron can be transferred from QH2 donor to a cytochrome *c* acceptor at a time (Up and Trumpower, 1990).

Cytochrome b/b6 comprises of approximately 400 amino acid residues and eight transmembrane segments (Howell, 1989; Blakely et al., 2005). It is an integral membrane protein that binds two heme groups, known as b562 and b566 in a non-covalent bonding. In these two heme groups, ligands of the iron atoms were postulated to be four histidine residues, which were observed to be conserved.

Deleterious mutations in the Cytochrome B gene leads to the production of defective protein and leads to disease conditions. Muscle and nervous system being at a higher risk, it equally affects vital organs like heart, kidney, and brain. Cytochrome B proteins are produced in the immune system, brain, liver, and other tissues throughout the body. The Cytochrome B gene family member expressed in the liver is important in the production of haemoglobin. (Gacon et al., 1980). Cytochrome B is believed to play a major role in iron metabolism in tissues of duodenum (Latunde-Dada et al., 2002) and is expressed in other tissues besides apical region of the epithelium. Cytochrome b proteins participate in various pathways that form novel molecules by

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transferring electrons from one molecule to another. The new molecules that are formed are essential for the pathway to continue.

Diseases caused by mutations in Cytochrome B genes usually occur due to the absence of a key molecule in an important pathway. The inability of the abnormal cytochrome B protein to transfer electrons leads to a shortage of the novel molecule which disrupts the pathway and prevents it from moving forward. The features of the disease depend on the tissue where the protein is active. For example, mutations in the Cytochrome B gene, which instructs the formation of cytochrome B protein in the immune cells, leads to an immune deficiency disorder called chronic granulomatous disease (Bolscher et al., 1991). This condition results in an increased risk of developing bacterial and fungal infections.

Heteroplasmy is the condition when cells contains both normal and mutant mitochondria (Wallace and Chalkia, 2013; Stewart and Chinnery, 2015). Heteroplasmic mitochondrial DNA mutations were reported to have effect on normal and tumor cells (0.18), aging (Keogh and Chinnery, 2013; Kennedy et al., 2013; Pinto and Moraes, 2015; Ross et al., 2013), brain and neurodegenerative disorder (Pinto and Moraes, 2014). It was detected from high-throughput sequencing of complete human mitochondrial DNA genomes (Li et al., 2010; Payne et al., 2013).

Mutations in cytochrome *b* causes exercise intolerance in human patients. In adverse cases, severe multi-system disorders have also been reported due to mitochondrial mutations (Vafai and Mootha, 2012). Recent mitochondrial DNA mutations cause a series of disorders (Hudson et al., 2014) for instance increasing the risk of developing common late-onset human diseases (Ju et al., 2014) and even cancer (Pal et al., 2017). Human cytochrome B mutations have been linked with several diseases. Nonsense or frameshift mutations that result in truncated Cytochrome B almost invariably abolish complex assembly. The precise effect of the missense mutations is more difficult to predict, and their characterization is often hampered by the limited amount of tissue available.

Till date reports are lacking in studies regarding diseases in sheep and its association with mitochondrial mutations. Considering the importance of Cytochrome B for the vital organs of the body, the present study aims at identification of SNPs in Cytochrome B gene and its association with the disease condition of sheep, to identify the effect of deleterious gene effects supplemented with bioinformatics analysis.

2. Results

2.1. Characterization of Cytochrome B gene in Garole sheep

Garole sheep Cytochrome B gene is characterized by 1140 bp nucleotide and 379 amino acids. The sequences submitted for diseased sheep with mutated Cytochrome B were KY110724, KU246231, KU 246228, while the wild types were KU246230 and KU 246229.

2.2. Mutation detection in Cyt B gene

Four SNPs (T99G SNP, C189T SNP, T309C SNP, G511A SNP) and one site for deletion had been detected in Garole sheep. Identified non-synonymous SNPs between healthy and debilitated sheep were F33 L and D171N (Supplementary Table 1).

A deletion mutation was observed in Garole sheep, which lead to truncated Cytochrome B protein of 289 amino acids, with reduced biological efficiency. INDELs were identified as the insertion at G at 921 nucleotides, G at 934 nucleotide positions of sheep cytochrome B protein.

The sheep were observed to have exercise intolerance, extremely debilitated, unable to stand, extreme muscle weakness. Cases of cardiomyopathy were primarily assessed by ECG (electrocardiogram).

2.3. In silico analysis pertaining to different variants of Cytochrome B gene

2.3.1. Determination of functional stability and deleterious nature of mutant protein

In the present study, three non-synonymous mutations were detected. Bioinformatics study with thermodynamic stability analysis was studied with I-mutant 3.0 software. It is presumed that the mutant form is less stable compared to the wild-type. Enthalpy and entropy were analyzed for mutant form compared to a wild variant. DDG was observed to be −1.04 and − 0.79 respectively for the mutations at amino acid positions 33 and 171 of sheep cytochrome B (Supplementary Table 1, Supplementary Fig. 1). These depict large decrease of stability of the mutated protein. Hence these three non-synonymous mutations were observed to lead the decreased stability of the protein.

The provean score was estimated to find out the deleterious nature of the mutated protein. The provean score was observed to be −3.937 and − 3.708 respectively for mutations at amino acid positions 33 and 171 of sheep cytochrome B. Hence all the mutations were observed to be deleterious as the value was more than the cut-off or default threshold level as −2.5 (Supplementary Table 1, Supplementary Fig. 2).

2.3.2. Three-dimensional structure prediction and Model quality assessment with respect to different variants of Cytochrome B of sheep

3D model of Variant A, B, C and D of sheep Cytochrome B has been visualized by Swiss homology-based model (Fig. 1a, b, c, and d) of which variant A was the wild-type. Cytochrome B structure identified from Variant A, B, C and D has been depicted (Fig. 1e, f, g, and h) respectively (pymol view). Variant D was observed to be a truncated protein, resulting due to deletion mutation. The surface view of Cytochrome B for the variants have been depicted in (Fig. 2a, b, c and d). In the figures generated by Pymol, secondary structure as helix, strand, and sheet have been depicted in cyan, red and pink respectively (Fig. 1e, f, g, h, 2a, b,c,d) and also confirmed with the Phyre structures for Variants A, B, C and D (Supplementary Fig. 3,4,5,6 respectively). It is the structure of the Cytochrome B protein that actually functions. Fig. 3 depict the sites for amino acid variations at position 33 and 171 in variant A represented respectively by green and red colour, which are the sites prone to mutation as SNP. Fig. 3b depict the sites for amino acid variations at position 33 and 171 in variant B represented respectively by green and red colour, which are the sites prone to mutation as SNP. The site for deletion mutation has been depicted in Fig. 3c. In order to analyze the structural differentiation for the two variants, TM align software analysis has revealed two different variants overlapped with two colours. Alignment of cytochrome B of Variant D with variant A, B and C had been depicted in Fig. 4, 4b, and 4c respectively, Green: Variant A/B/C and Blue: Variant D). Random mean square deviation (RMSD) was observed to be 0.91, and sequence identical as revealed by the ratio of nucleotide identical to nucleotide aligned was observed to be 0.997, 0.993 and 0.993 respectively for Variant D with that of Variant A, B and C. The alignment of wild type variant A with variant D(with INDEL mutation) had been depicted in Fig. 4d, with the sites for mutation depicted as blue for aa 33, hot pink for aa 171 and grey sphere for the site of truncation at amino acid position 290. Cytochrome B from the healthy animal as green and mutated Cytochrome B as red. The alignment view of cytochrome B surface view of Variant D with that of variant A, B and C had been depicted in Fig. 5, 5b, and 5c respectively, Green: Variant A/B/C and Blue: Variant D).

2.3.3. Protein-protein interaction network depiction and estimation of the biological function

In order to understand the network of Cytochrome B protein, we performed analysis with submitting FASTA sequences to STRING 9.1. Cytochrome B interacts with other proteins for the formation of bc1 complex, which is the ultimate functional unit for oxidative

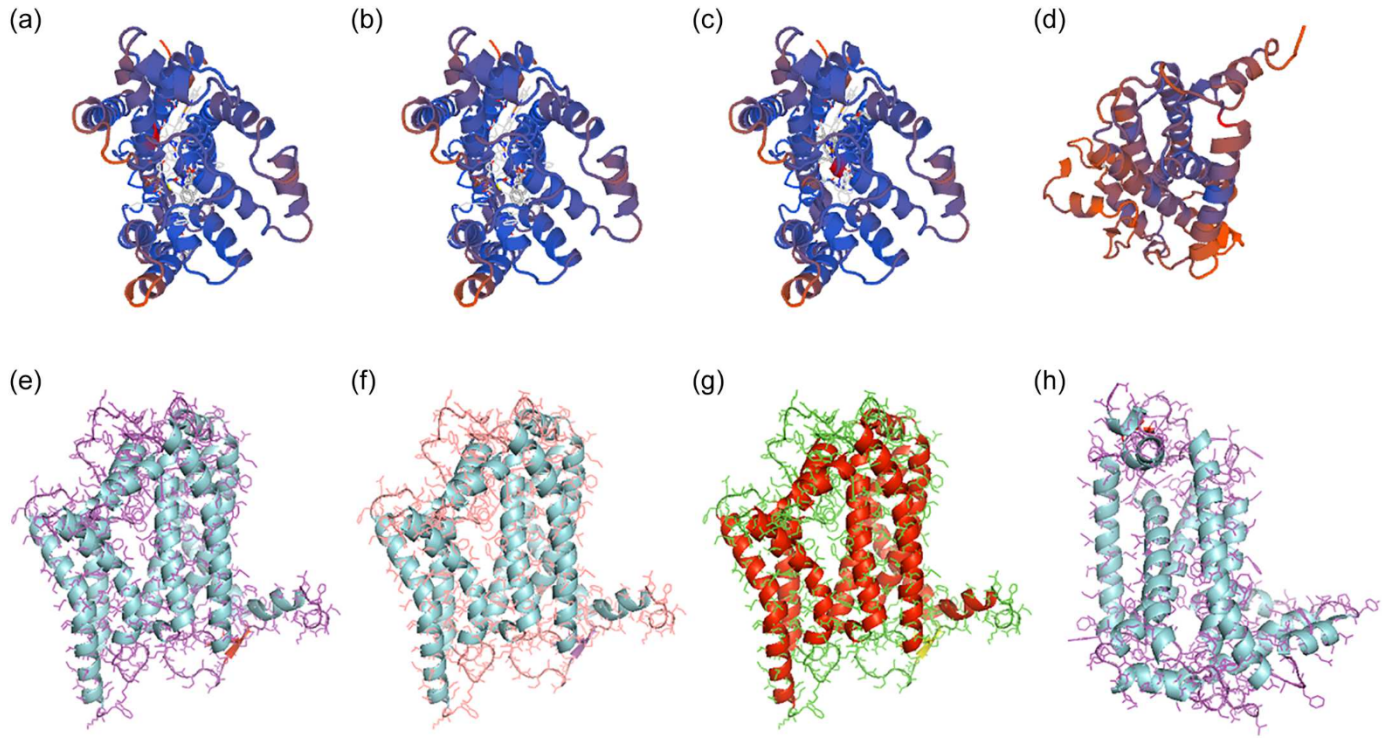

Fig. 1. *In-silico* study to assess of the stability of different forms of mutant variants of Cytochrome B of sheep thermodynamically by I-mutant software.

phosphorylation. The interaction of Cytochrome B protein with other functional partners had been studied by String analysis (Fig. 6). These proteins are Mt-Co-1, Mt-Co-2, Mt-Co-3, Mt-ND1, Mt-ND2, Mt-ND4, Ubiquitin-protein as UQCRB, UQCRFS1, UQCRQ. The functional partners were depicted in Table 1. The functional relevance of sheep Cytochrome B had been revealed by KEGG analysis as depicted in Fig. 7.

2.3.4. Assessment of post-translational modification analysis with respect to different variants of Cytochrome B

The sites for post-translational modification varies for different mutant forms from that of wild-type and has been tabulated in Table 2. It has been observed that the variant D of sheep cytochrome B had most of the depressed post-translational modification processes, in comparison to the wild-type Variant A.

The wild-type Variant A containing 9 transmembrane helix, leading to increased biological potentiality compared to Variant D with deleted mutation (Table 2). The wild-type Variant A containing 9 transmembrane helix, leading to increased biological potentiality compared to Variant D with deleted mutation (Table 2). The entropy of Variant B, C, D was observed to be more than that of Variant A, which indicated better stability of Variant A (Table 2). The sites for post-translational modification sites had been studied through various bioinformatics software. The secondary structure of Cytochrome B had been depicted for different variants. Gene 3D result analysis reveals the stereochemical quality of model and Procheck reveals the index of thermodynamic stability as ΔG being −14,747 kCal/mol. Ramachandran plot was depicted for both the wild-type and mutant variants of Cytochrome B for the determination of Stereochemistry of polypeptide chain configurations (Fig. 8).

2.4. Effect of mutation of Cytochrome B gene on health parameters (physiological, haematological and biochemical)

A deletion mutation was observed in Garole sheep number, which leads to truncated Cytochrome B protein, with reduced biological efficiency. The sheep were observed to be extremely debilitated, unable to stand, extreme muscle weakness (Table 3). The case of cardiomyopathy was primarily assessed by ECG (electrocardiogram). Respiratory disorders were detected with an examination by stethoscope.

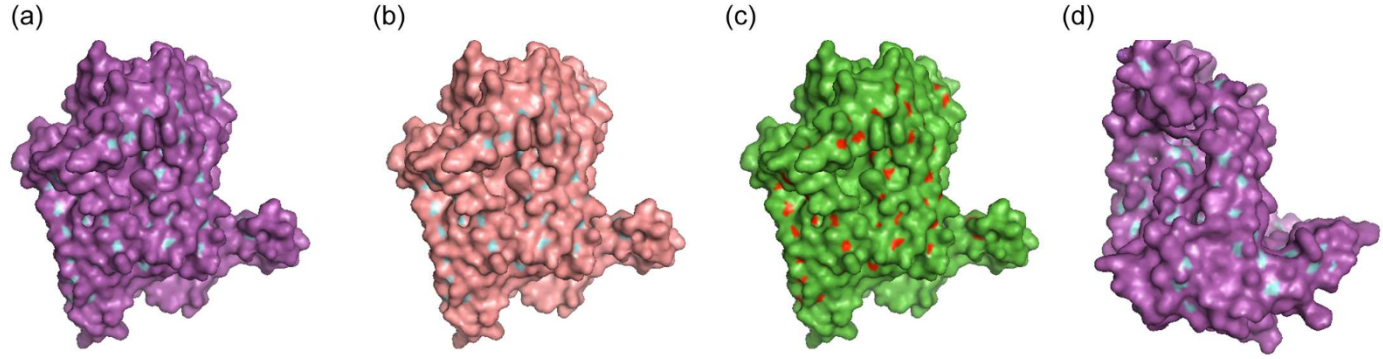

Fig. 2. In- silico analysis to assess the deleterious effect of different forms of mutant variants of Cytochrome B of sheep by Provean software.

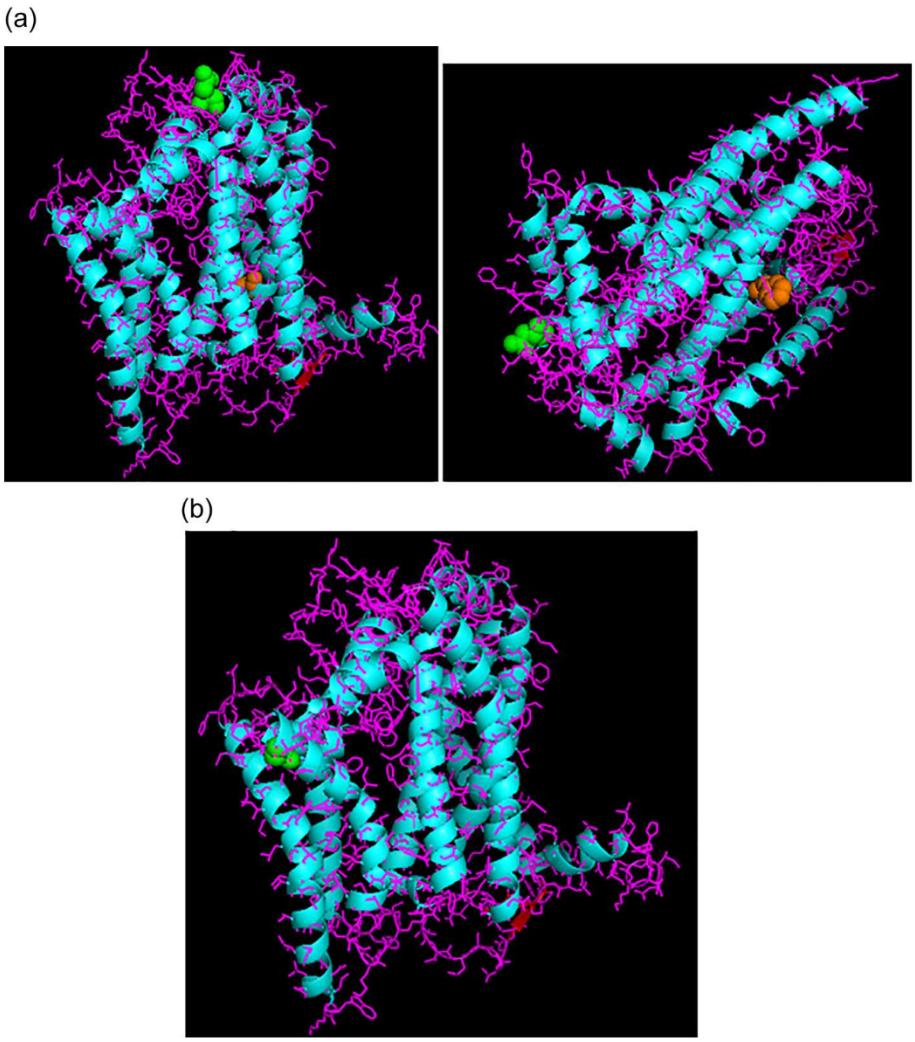

Fig. 3. a: Assessment of protein structure by 3D model of variant A of Cytochrome B by Swiss homology modelling.
Fig. 3b: Three-dimensional structure prediction and Model quality assessment with respect to variant B of Cytochrome B of sheep. 3D model of variant B of Cytochrome B by Swiss homology modelling.
Fig. 3c: Three-dimensional structure prediction and Model quality assessment with respect to variant B of Cytochrome B of sheep. 3D model of variant C of Cytochrome B by Swiss homology modelling.
Fig. 3d: Three-dimensional structure prediction and Model quality assessment with respect to variant B of Cytochrome B of sheep. 3D model of variant D of Cytochrome B by Swiss homology modelling.
Fig. 3e: 3D model of variant A of Cytochrome B by Pymol view. In the figures generated by Pymol, secondary structure as helix, strand, and sheet have been depicted in cyan, red and pink respectively.
Fig. 3f: 3D model of variant B of Cytochrome B by Pymol view. Secondary structure of CytB Variant B as helix, strand, and sheet have been depicted in cyan, red and pink respectively.
Fig. 3g: 3D model of variant C of Cytochrome B by Pymol view. Secondary structure of CytB Variant B as helix, strand, and sheet have been depicted in cyan, red and pink respectively.
Fig. 3h: 3D model of variant D of Cytochrome B by Pymol view. Secondary structure of CytB Variant B as helix, strand, and sheet have been depicted in cyan, red and pink respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

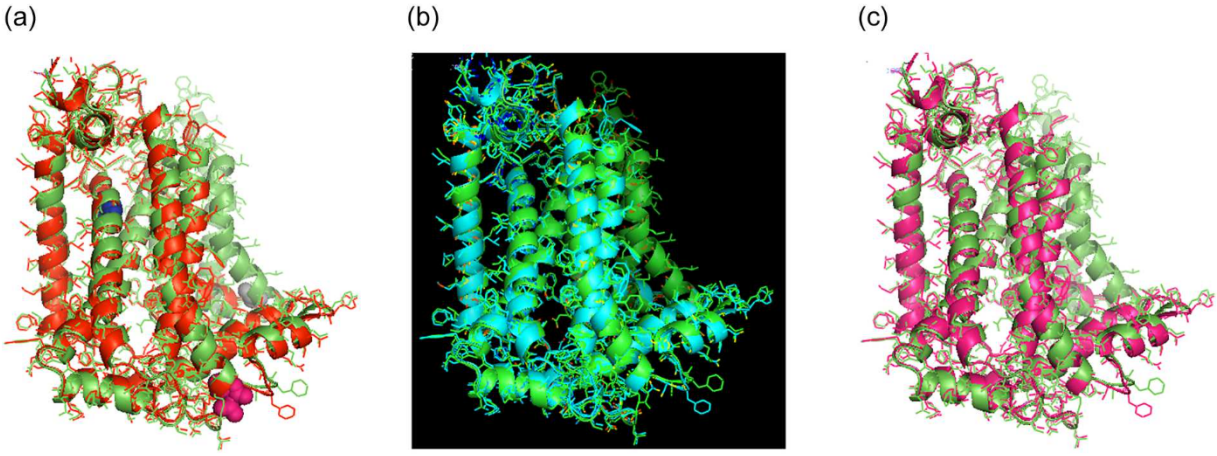

Fig. 4. a: 3D model of surface view of variant A of Cytochrome B by Pymol view. Secondary structure of CytB Variant B as helix, strand, and sheet have been depicted in cyan, red and pink respectively.
Fig. 4b: 3D model of surface view of variant B of Cytochrome B by Pymol view. Secondary structure of CytB Variant B as helix, strand, and sheet have been depicted in cyan, red and pink respectively.
Fig. 4c: 3D model of surface view of variant C of Cytochrome B by Pymol view. Secondary structure of CytB Variant B as helix, strand, and sheet have been depicted in cyan, red and pink respectively.
Fig. 4d: 3D model of surface view of variant D of Cytochrome B by Pymol view. Secondary structure of CytB Variant B as helix, strand, and sheet have been depicted in cyan, red and pink respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

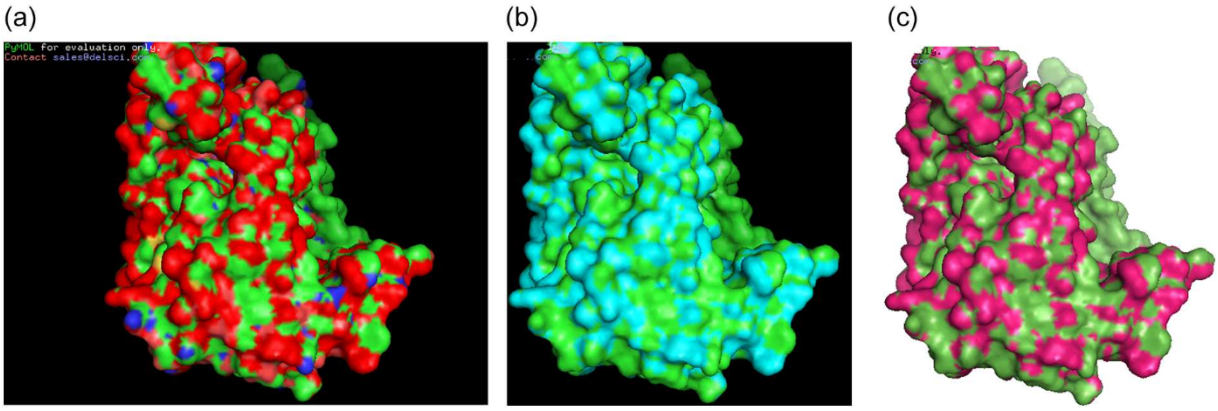

Fig. 5. a: 3D structure of wild type variant A with SNP at different amino acid position. The sites for amino acid variations at position 33 and 171 in variant A represented respectively by green and red colour, which are the sites prone to mutation as SNP.
Fig. 5b: 3D structure of wild type variant B with SNP at different amino acid position.
The sites for amino acid variations at position 33 and 171 in variant B represented respectively by green and red colour, which are the sites prone to mutation as SNP.
Fig. 5c: 3D structure of wild type variantA with deletion mutation depicted by green spheres. Secondary structure of CytB Variant A as helix, strand, and sheet have been depicted in cyan, red and pink respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

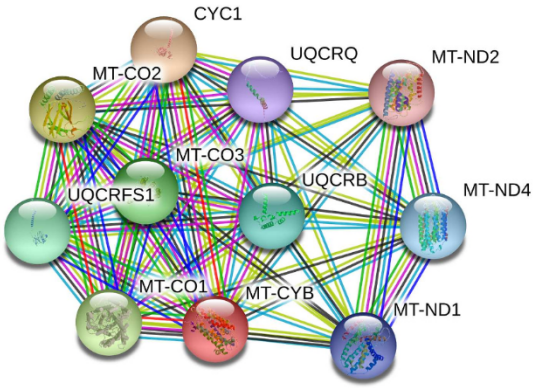

Fig. 6. a: Alignment of cytochrome B of Variant D (mutant variant with deletion) with variant A (wild) as depicted by TM align software. Green: Variant A and Blue: Variant D.
Fig. 6b: Alignment of cytochrome B of Variant D (mutant variant with deletion) with variant B as depicted by TM align software. Green: Variant B and Blue: Variant D.
Fig. 6c: Alignment of cytochrome B of Variant D (mutant variant with deletion) with variant C as depicted by TM align software. Green: Variant C and Blue: Variant D.
Fig. 6d: The alignment of wild type variant A with variant D (with INDEL mutation) as depicted by TM align software.
The sites for mutation depicted as blue for aa 33, hot pink for aa 171 and grey sphere for the site of truncation at amino acid position 290. Cytochrome B from healthy animal as green (variant A) and mutated Cytochrome B as red (variant D).

2.4.1. Association of SNP of cytochrome B with the general health condition and exercise intolerance

The sheep with the mutated form of cytochrome B (Variant B, C, and D) were observed to show exercise intolerance (Table 3). The individuals with Variant B, C and D exhibited sub-normal body temperature, tachycardia, weak and feeble pulse, tachypnoea and animals were extremely debilitated with muscle weakness (Table 3). The sheep were unable to rise and stand on its own. Cases of cardiomyopathy for the sheep with Variant D was confirmed by ECG.

2.4.2. Association of SNP of cytochrome B with blood and haematological properties

The sheep containing the mutated variant of Cytochrome B (Variant B, C, and D) were observed to possess decreased haemoglobin concentration, PCV and total erythrocytic count (Table 4). Similarly, Variant B, C, and D also possess reduced MCV, MCH, MCHC compared to that of the wild-type Variant A.

Total leucocytic count and the differential leucocytic count was found to be lowered in variant B, C, and D (Table 4). Platelet count was also found to be depressed (Table 4).

2.4.3. Association of Cytochrome B polymorphism with kidney function

In extreme cases of few animals, multi-organ failure was observed. Few individuals for variant B, C and D were observed to have depressed kidney function as revealed by biochemical tests of kidney function, revealed through Urea level, BUN, creatinine level of serum (Table 4).

2.4.4. Association of Cyt B SNP with liver function test (LFT)

Few individuals for variant B, C and D were observed to have a depressed liver function as revealed by biochemical tests of liver

Table 1
Predicted functional partners for Cytochrome B: (refer String, expassy) with score.

T1	CYC1	cytochrome *c*-1; This is the heme-containing component of the cytochrome *b*-c1 complex, which acc […] (325 aa)
T2	MT-CO2	mitochondrially encoded cytochrome *c* oxidase II; Cytochrome c oxidase is the component of the r […] (227 aa)
T3	MT-CO1	mitochondrially encoded cytochrome *c* oxidase I; Cytochrome c oxidase is the component of the re […] (513 aa)
T4	MT-CO3	mitochondrially encoded cytochrome *c* oxidase III; Subunits I, II and III form the functional co […] (261 aa)
T5	UQCRFS1	ubiquinol-cytochrome *c* reductase, Rieske iron-sulfur polypeptide 1; Component of the ubiquinol- […] (274 aa)
T6	UQCRB	ubiquinol-cytochrome *c* reductase binding protein; This is a component of the ubiquinol-cytochro […] (111 aa)
T7	MT-ND4	mitochondrially encoded NADH dehydrogenase 4; Core subunit of the mitochondrial membrane respire […] (459 aa)
T8	MT-ND1	mitochondrially encoded NADH dehydrogenase 1; Core subunit of the mitochondrial membrane respire […] (318 aa)
T9	UQCRQ	ubiquinol-cytochrome *c* reductase, complex III subunit VII, 9.5 kDa; This is a component of the u […] (82 aa)
T10	MT-ND2	mitochondrially encoded NADH dehydrogenase 2; Core subunit of the mitochondrial membrane respire […] (347 aa)

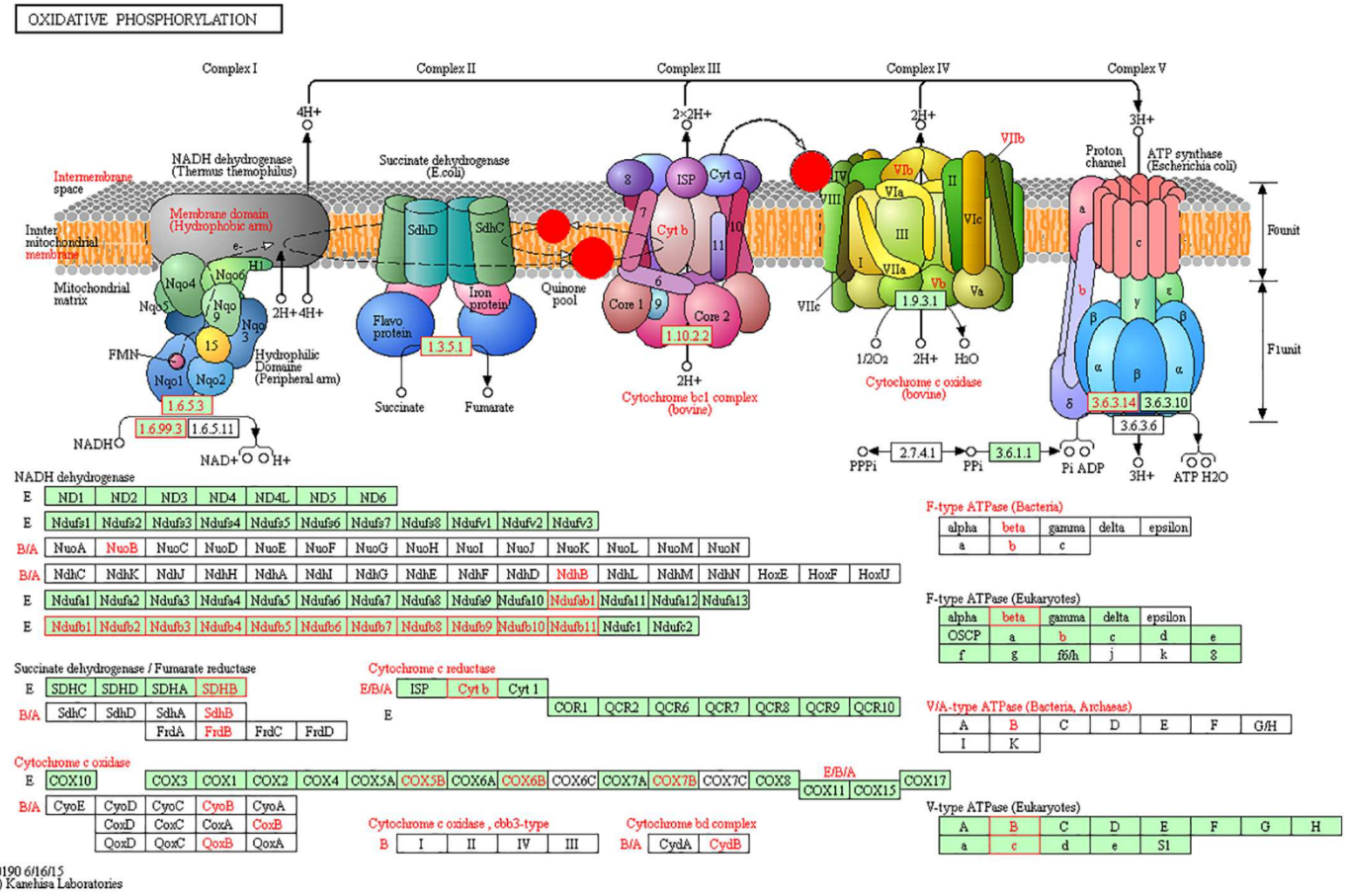

Fig. 7. a: The alignment of cytochrome B surface view of Variant D (mutant variant with deletion) with that of variant A by TM align software Green: Variant A and Blue: Variant D.
Fig. 7b: The alignment of cytochrome B surface view of Variant D (mutant variant with deletion) with that of variant B had been depicted by TM align software. Green: Variant B and Blue: Variant D.
Fig. 7c: The alignment of cytochrome B surface view of Variant D(mutant variant with deletion) with that of variant C had been depicted by TM align software, Green: Variant C and Blue: Variant D. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

function, revealed through serum level of total protein, bilirubin, SGOT, SGPT and alkaline phosphatase (Table 4).

2.4.5. Association of CytB SNP with serum mineral status

In Garole sheep, Variant B, C and D were observed to have reduced serum calcium level (Table 4).

2.5. Phylogenetic analysis of Cytochrome B gene and derived peptide among different mammalian species

Sheep Cytochrome B was observed to be genetically more related to mammalian species. It is evident from the genetic distance study and phylogenetic analysis that human is closely related to sheep. Human Cytochrome B was observed to be 77.3% identical to sheep cytochrome B (Table 5). Hence sheep may be considered as an excellent animal for studying human as an animal model.

3. Discussion

Cytochrome B is an important mitochondrial gene, which takes an active part in electron transport chain. The fact was also confirmed by KEGG analysis. Since each and every organ needs energy for its normal functioning, any defect in oxidative phosphorylation process in electron transport chain leads to energy deficiency in respective vital organs leading to defect.

Four SNPs were identified for Cytochrome B of sheep, out of which three were non-synonymous and even deleterious. Non-synonymous substitutions were observed as F33 L, D171N. Hence it is evident that non-synonymous substitution did not exceed that of synonymous substitution, which indicates that there is no positive selection. So the mutations were tested for their effect on protein stability and deleterious nature. Mutation at amino acid position 33 was previously reported in human. Indels were also identified for Cytochrome B of sheep. Deletion mutations were identified leading to a truncated protein. Similar studies have been reported as non-synonymous. SNPs identified as G511A leading to D171N has been observed with extreme debility of the animal. Some of the preliminary studies were reported earlier (Brown et al., 1992). This mutation changing aspartate to asparagines altered a highly conserved amino acid in an extramembrane domain of cytochrome B that is associated with the ligation of the low potential b566 heme (Bender et al., 2006). While analyzing the samples based on biochemical parameters, it was found that the individuals had subnormal haemoglobin percentage.

To study the effect if the mutations were having a deleterious effect on physiological body parameters, bioinformatics analysis was conducted to analyze the protein stability and deleterious nature of the mutation. It was observed through bioinformatics analysis based on the principle of thermodynamics that these three mutations were disadvantageously leading to decreased stability of the cytochrome B protein. DDG or difference in Gibb's free energy was calculated and it was observed that it is negative which indicates difference in Gibb's free energy (Δ G) for mutant is less than that of wild protein.

Table 2
Sites for post translational modification of sheep Cytochrome B.

Post transcriptional modifications	Variant A	Variant B	Variant C	Variant D
TMHMM	No. of helix-9	No. of helix- 9	No. of helix-9	No. of helix-6
	33–55	33–55	33–55	35–57
	76–98	76–98	76–98	77–99
	113–135	113–135	113–135	112–134
	140–158	140–158	140–158	144–166
	178–200	178–200	178–200	178–200
	229–251	229–251	229–251	229–251
	288–310	288–310	288–310	
	323–340	323–340	323–340	
	350–372	350–372	350–372	
HMMTOP	Total entropy of the model-17.0085	Total entropy of the model-17.0083	Total entropy of the model-17.0085	Total entropy of the model-17.0129
	Best path- 17.0122	Best path- 17.0121	Best path- 17.0123	Best path- 17.0165
NetNes	9 sites	11 sites	9 sites	10 sites
NetGlycate	4 sites	4 sites	4 sites	4 sites
NetCGlyc	12 sites- 30,31, 77,113,135,141,163,165,272,326,337,379	12 sites- 30,31, 77,113,135,141,163,165,272,326,337,379	12 sites- 30,31, 77,113,135,141,163,165,272,326,337 ,379	12 sites- 30,31,77,113,135,141,163,165,272,336, 347,395

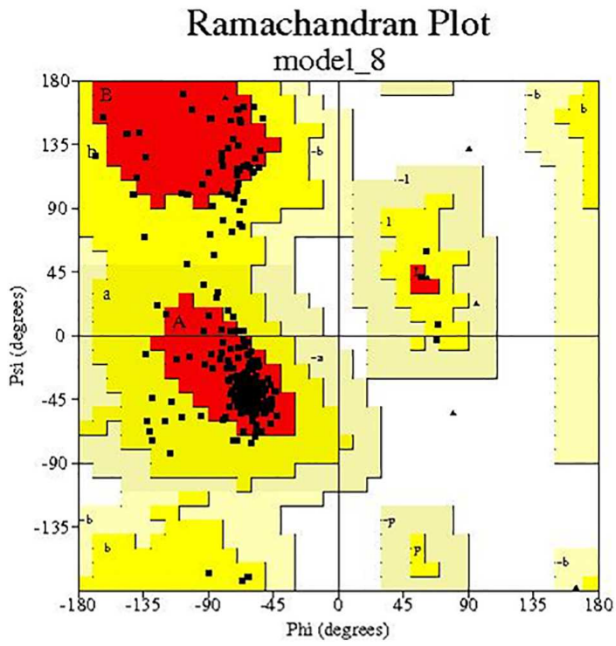

Fig. 8. Interaction of Cytochrome B protein with other functional partners had been studied by String analysis.

$$\Delta\Delta G = \Delta Gmutant - \Delta Gwild$$

$\Delta G = \Delta H - T\Delta S$, Gibb's free energy was calculated by the difference in enthalpy and difference in entropy multiplied with temperature in Kelvin. ΔG was observed for mutant type to be less may be because of increased entropy of Cytochrome B, arising due to new protein conformation or alignment of moieties in amino acids. Variation in amino acid position 33 was observed to be least stable. Entropy of Variant B, C, D was observed to be more than that of Variant A, which indicated better stability of Variant A. Thus it is evident that mutation causes more entropy, leading to decreased stability. Thermodynamic principle had been reported in other instances in biological science in disease studies (Rietman et al., 2016).

3 D structural analysis of Cytochrome B and its alignment revealed definite structural differences of variant D with other variants as Variant A, B or C. It is the actually the 3 D structure of the protein that is functionally active. Protein interaction of Cytochrome B with functionally related protein have been observed. These are involved in oxidative phosphorylation process. Mitochondria is an important organ involved in a variety of functions. The major functions are electron carrier activity, metal ion binding and ubiquinol-cytochrome-c reductase activity. Mitochondrial Cytochrome B interacts with varieties of protein to be functionally active and form the complex and participate in oxidative phosphorylation. These proteins are Mt-Co-1, Mt-Co-2, Mt-Co-3, Mt-ND1, Mt-ND2, Mt-ND4, Ubiquitin protein as UQCRB, UQCRFS1, UQCRQ. The effect of Cytochrome B on other biological processes are animal organ regeneration, hyperosmotic salinity response, mitochondrial electron transport, ubiquinol to cytochrome *c*, response to cadmium ion, response to calcium ion, response to cobalamin, response to copper ion, response to drug, response to ethanol, response to glucagon, response to heat, response to hypoxia, response to mercury ion and response to toxic substance (UniprotKB, P00156, CyB_Human, 2016). These are the vital functions of the cell. Any defect or abnormality in these functions will lead to adverse effect on vital functions of the body, causing diseases. In the truncated Cytochrome B protein due to Indel mutation, metal binding site for iron to heme b562 and 566 axial ligand at amino acid position 83, 97, 182 and 196 of Cytochrome B was lacking. Lack of Ubiquinone binding site at amino acid position 201 was detected. The b-c1 complex of Cytochrome B leads to electron transfer from ubiquinol to cytochrome *c*. Cytochrome

Table 3
: Association of SNPs of Cytochrome B gene with respect to physiological parameters in sheep.

I. Physiological parameters					
	Wild type/variant A	Variant B	Variant C	Variant D	Remarks
Body Temperature (°F)	102.5 ± 0.54 [a]	98.0 ± 0.57 [b]	100.00 ± 0.62 [a]	97.0 ± 0.65 [b]	Subnormal temp. For debilitated animals.
Heart rate (per minute)	75.5 ± 0.43 [a]	120 ± 0.56 [b]	110 ± 0.58 [b]	123 ± 0.62 [b]	Tachycardia was observed for diseased sheep
Pulse rate (per minute)	70 ± 0.45	65 ± 0.47	70 ± 0.56	64 ± 0.59	Weak and feeble pulse rate was observed
Respiration (per minute)	16.5 ± 0.12 [a]	40 ± 0.23 [b]	35 ± 0.28 [b]	45 ± 0.32 [b]	Tachypnoea was observed for debilitated sheep
Muscle weakness	Muscle weakness not detected	Increased muscle weakness	Moderate muscle weakness	Extreme muscle weakness-sheep is unable to rise	The debilitated sheep was not able to stand of its own, General weakness of muscle
Exercise intolerance	Not detected	Detected	Detected	Detected	

Superscript [a] and [b] represents significant differences $P \leq .05$ level of significance.

B contributes to the generation of a proton gradient across the mitochondrial membrane that is then used for ATP synthesis. Thus Cytochrome B is a part of mitochondrial respiratory chain (complex III or cytochrome *b*-c1 complex). High levels of mitochondrial DNA deletions had been observed in substantia nigra neurons in aging, Parkinson disease (Corral-Debrinski et al., 1994) and Alzheimers disease (Payne et al., 2013). Reports were available for Oxidative stress and neurodegenerative diseases caused by mitochondrial damage (Guo et al., 2013) and defects in oxidative phophorylation in human caused by defects in mitochondria (Vizarra et al., 2009).

Once it was observed through bioinformatics analysis that the mutations were deleterious leading to decreased functional stability, it was studied if the mutations causes any alteration in important post translational modifications of cytochrome B protein. Any alteration in important post translational modification will lead to depressed function of Cytochrome B protein. Domains of Cytochrome B are folding of transmembrane helices and location of quinine and heme binding pockets. Any change of this domain leads to loss of function of the Cytochrome and diseases are caused. Transmembrane helix is present at the outer surface of the cytochrome B, which is responsible for electron transport chain. It is the site responsible for binding to different moieties involved in respiratory complex III. There are specific binding sites for heme group in trans membrane helix. The wild type Variant A containing 9 transmembrane helix, leading to increased biological

Table 4
: Association of SNPs of Cytochrome B gene with respect to physiological parameters in sheep.

I. Haematological properties					
Complete Hemogram Test					
Haemoglobin	11 ± 0.32[a]	06 ± 0.28 [b]	10 ± 0.34 [a]	05 ± 0.31 [b]	gm/dl
PCV (Haematocrit)	40.34 ± 0.35 [a]	25 ± 0.48 [b]	35 ± 0.44 [b]	24 ± 0.56 [b]	%
Total erythrocyte count (TEC)	5.4×10^{6} [a]	4.92×10^{6} [a]	4.67×10^{6} [a]	3.28×10^{6} [b]	Cu mm
Total leucocyte count (TLC)	6300 ± 23.34 [a]	4700 ± 34.56 [b]	6230 ± 23.34 [a]	4300 ± 28.56 [b]	Cu mm
Differential leucocyte count (DLC)					
Neutrophil	29.5 ± 0.034	29.5 ± 0.235	29.3 ± 0.034	28.5 ± 0.352	%
Eosinophil	9.4 ± 0.383	11.0 ± 0.435	10.5 ± 0.383	11.0 ± 0.523	%
Basophil	–	–	–	–	%
Lymphocyte	51.5 ± 0.234	50.0 ± 0.324	51.4 ± 0.234	51.0 ± 0.367	%
Monocyte	9.6 ± 0.23	9.5 ± 0.435	8.8 ± 0.23	9.5 ± 0.435	%
Platelet count	450,000 ± 34.67 [a]	135,000 ± 56.54 [b]	200,000 ± 40.34 [a]	97,000 ± 45.34 [b]	%
MCV	54 ± 0.45 [a]	40.34 ± 0.34 [b]	38. 34 ± 0.47 [b]	35.56 ± 0.54 [b]	Fl
MCH	10 ± 0.034 [a]	5.5 ± 0.034 [b]	9.5 ± 0.023 [a]	4.67 ± 0.078 [b]	Pg
MCHC	36 ± 0.53	35 ± 0.64	37 ± 0.34	38 ± 0.45	%/dl
ESR (1st hour)	02 ± 0.04	2.35 ± 0.05	2.3 ± 0.03	2.38 ± 0.06	Mm
II. Kidney function test					
Glucose (Fasting) mg/dL	52 ± 0.24 [a]	120 ± 0.45 [b]	100 ± 0.56 [b]	245 ± 0.68 [b]	mg/dL
Urea	39 ± 0.47 [a]	72 ± 0.59 [b]	44 ± 0.58 [a]	74 ± 0.59 [b]	mg /dL
BUN	18.5 ± 0.13 [a]	25.3 ± 0.21 [b]	22.89 ± 0.23 [b]	27.0 ± 0.32 [b]	mg/dL
Creatinine	1.5 ± 0.034 [a]	1.9 ± 0.045 [a]	1.9 ± 0.063 [a]	2.5 ± 0.072 [b]	mg/dL
III. Liver function test					
Total protein(TP)	7.2 ± 0.12 [a]	8.5 ± 0.14 [a]	8.3 ± 0.23 [a]	10.7 ± 0.34 [b]	gm/dL
Albumin (ALB)	3.0 ± 0.03 [a]	1.8 ± 0.05 [b]	2.2 ± 0.07 [c]	1.3 ± 0.09[b]	gm/dL
Globulin (GLB)	4.2 ± 0.16 [a]	5.1 ± 0.08 [b]	4.5 ± 0.13 [a]	5.5 ± 0.17 [b]	gm/dL
Total Bilirubin	0.4 ± 0.02 [a]	7.0 ± 0.08 [b]	5.0 ± 0.12 [b]	7.8 ± 0.15 [b]	mg/dL
Direct Bilirubin (Conjugated)	0.1 ± 0.01 [a]	0.5 ± 0.04 [a]	0.4 ± 0.06 [a]	1.0 ± 0.09 [b]	mg/dL
SGOT (ALT)	100 ± 2.45 [a]	352 ± 3.64 [b]	300 ± 3.56 [b]	382 ± 4.5 [b]	IU/L
SGPT (AST)	24 ± 0.16 [a]	35 ± 0.19 [b]	30 ± 0. 18 [b]	45 ± 0.20 [b]	IU/L
IV. Mineral status					
Serum Calcium (Ca)	11.75 ± 0.13 [a]	8.23 ± 0.23 [b]	7.86 ± 0.32 [b]	6.78 ± 0.34 [b]	mg/dL
Phosphorus(P)	7.1 ± 0.34	5.2 ± 0.45	6.3 ± 0.37	4.1 ± 0.47	mg/dL
Magnesium	2.6 ± 0.23	1.8 ± 0.32	2.0 ± 0.34	1.6 ± 0.37	Mmol/L

Table 5
Genetic distance estimated for sheep and other livestock species with Human.

Pair Distances of Untitled ClustalW (Slow/Accurate, Gonnet)							
	Percent Similarity in upper triangle						
	Percent Divergence in lower triangle						
	Buffalo	Cattle	Goat	Human	Mouse	Rabbit	Sheep
ctyb buffalo	***	95.0	92.3	78.1	85.5	85.0	93.1
cytB cattle	5.2	***	92.3	77.0	83.4	83.6	91.8
cytb goat	8.1	8.1	***	77.8	83.9	83.9	95.0
cytB human	26.0	27.5	26.3	***	76.6	79.4	77.3
cytb mouse	16.2	18.8	18.2	28.1	***	83.1	83.9
cytB rabbit	16.8	18.5	18.2	24.1	19.2	***	84.4
cytB sheep	7.2	8.7	5.2	27.1	18.2	17.5	***

potentiality compared to Variant D with deleted mutation.

Gene 3D result analysis reveals stereochemical quality of model and Procheck reveals the index of thermodynamic stability as ΔG being −14,747 kCal/mol. Ramachandran plot was depicted for both the wild type and mutant variants of Cytochrome B, to assess the functional stability of the protein. It reveals decreased stability of mutated form as Cytochrome B variants B, C and D.

The exact functional associations of variants of Cytochrome B (wild versus mutant form) were studied. Mitochondrial mutations and abnormalities were observed to contribute to all aspect of diseases involving the vital organs, as heart, muscle, liver, kidney etc. Mutations in Cytochrome B affected the normal physiological functions. Exercise intolerance had been observed for mutated form. Variability of cytochrome B gene was found to be associated with haematological parameters. Haemoglobin percentage was severely affected by mutation in Cytochrome B gene. Since there was a mutation at the heme binding site, haemoglobin percentage has been drastically reduced in sheep with mutated Cytochrome B gene.

Cytochrome b/b6 non-covalently binds two heme groups, known as b562 and b566. Four conserved histidine residues are postulated to be the ligands of the iron atoms of these two heme groups. Cytochrome B is an important protein for oxidative phosphorylation and supply of energy to each and every cell of the body. Indel identified leads to a frameshift mutation and hence protein truncation is very common. Frameshift mutation also causes functionally defective altered protein structure. Amino acid position 33 is responsible for heme binding, hence any alteration or mutation at this site leads to defect in haemoglobin synthesis and resulting in anemia. It is extremely essential for energy metabolism. G33S has been found in a patient with exercise intolerance. Gly-33 is located within transmembrane helix A, a hydrophobic environment at the Qi site and close to heme bh. Mutation at amino acid position 33 has been reported in human, as G33S in human (Vafai and Mootha, 2012). This residue has been observed in heme pocket (Yun et al., 1992; Bashford et al., 1987). This residue has been observed to be conserved for a wide range of species including mammals, avian, reptiles, fishes among eukaryotes and also among prokaryotes (Esposti et al., 1993). Other reported sites for mitochondrial mutations leading to pathogenesis in human were D171N, G251D, and V356 M (Uniprot,2017). Functional characterization of novel mutations in the human cytochrome *b* gene had been reported (Legros et al., 2001).

Mitochondria have a role in uptake and release of calcium ions. Ca ions are essential triggers for cellular activities and mitochondria (along with endoplasmic reticulum). It plays an important role in regulating calcium ions concentration rise to abnormally high levels in the cytosol. Calcium ion transporter in the inner mitochondrial membrane takes up excess calcium ion. Significant reduction in serum calcium level had been detected in the sheep containing mutated cytochrome B (Variant B, C, and D). The marked reduction had been observed in Variant D with truncated protein lacking heme binding site. Similar studies have also reported the role of mitochondria in Calcium uptake in a rat (Calderón-Cortés et al., 2008; Murugan et al., 2016).

Three-dimensional protein structure was determined for different variants of Cytochrome B with deletion identified, leading to the truncated protein lacking most of the moieties responsible for its effective function. Loop was absent with less number of transmembrane helix responsible for binding with other molecules in Complex III of oxidative phosphorylation. MT-CytB gene mutations impair the formation of complex III molecules. Thus the activity of complex III and oxidative phosphorylation are depressed. Researchers believe that impaired oxidative phosphorylation can lead to cell death by reducing the amount of energy available in the cell (Guo et al., 2013). It is thought that tissues and organs that require a lot of energy, such as the brain, liver, kidneys, and skeletal muscles, are most affected by a reduction in oxidative phosphorylation. Female reproductive organs as ovary, uterus etc. are also the vital organ and need energy which is supplied through oxidative phosphorylation process (Pal et al., 2016; Pradhan et al., 2017). In our earlier studies, mutations in Cytochrome b gene was observed affect female reproduction in *Sus scrofa* (Pradhan et al., 2018). Effect of Cytochrome B was observed in male reproduction in earlier studies (Pal et al., 2016).

From the haematological and blood biochemical studies, the marked reduction in haemoglobin concentration, TEC, TLC, DC, PCV, platelet count have been observed in sheep with mutated Cytochrome B.

In the current study, four parameters had been assessed for kidney function as glucose fasting, BUN, creatinine and blood urea which reflected marked reduction in kidney function of sheep under study. Similar findings were observed by other workers in human and animals. Researchers had revealed the significant impact of cytochrome B on renal function or kidney function. The cytochrome *b* content in mitochondria isolated from kidneys of 24-month-old animals was 23% reduced. It is known that the total cytochrome *b* content is distributed between complexes II and III within the mitochondria (Andreu et al., 2000). A pathogenic mutation in the mitochondrial cytochrome *b* gene (isolated from the liver) in a 4 wks aged girl patient with a multisystem disorder presenting as histiocytoid cardiomyopathy was detected arising due to a defect of ubiquinol cytochrome *c* oxidoreductase of the electron transport chain and also documented biochemically. The mutation, a G to A transition at nucleotide 15,498, resulted in the substitution of glycine with aspartic acid at amino acid position 251 resulting in acute tubular necrosis of kidney. The crystal structure of ubiquinol cytochrome *c* oxidoreductase was found to be affected by the mutation. The mutation was heteroplasmic and impaired the function of the holoenzyme of ubiquinol cytochrome *c* oxidoreductase, an important component of oxidative phosphorylation (Che et al., 2014).

Defects in mitochondria can lead to chronic kidney damage. It had been reported that reduction in mitochondrial DNA (mtDNA) copy

number, loss of mitochondrial membrane potential (Δψm), and drop of ATP production had adverse effect on kidney (Granata et al., 2009; Su et al., 2013; Yuan et al., 2012; Granata et al., 2015). It had been postulated that Mitochondria had active role in apoptosis and contribute to fibrogenic process due to epithelial to mesenchymal transition of renal tubular epithelial cells (Granata et al., 2015). Current studies reported that new therapeutic agents treat the defect in mitochondria resulting in case of chronic kidney disease (Finn et al., 2008).

The liver function had been assessed by seven parameters as Total protein(TP), Albumin (ALB), Globulin (GLB), Total Bilirubin, Direct Bilirubin (Conjugated), SGOT (ALT), SGPT (AST) in sheep in the current study. A mutation in cytochrome B (G to A transition at nucleotide 15,498, resulted in the substitution of glycine with aspartic acid at amino acid position 251) isolated from liver resulted in hepatic steatosis of the liver (Che et al., 2014). Two original deleterious mutations were found in the group of seven patients with overt complex III defect. Both mutations (G15150A (W135X) and T15197C (S151P)) were heteroplasmic and restricted to muscle. They had significant consequences on the complex III structure. In contrast, only two homoplasmic missense mutations with dubious clinical relevance were found in the patients without overt complex III defect (Brown et al., 1992). The role of Cytochrome b in the liver have been documented through the conditional hepatic deletion of microsomal Cytochrome B (Papadimitriou and Neustem, 1984).

Tachycardia was observed for the mutated sheep in the current study. Other researchers have also reported disorders in heart function. Biochemical studies of cardiac muscle showed isolated complex III deficiency and marked chain, suggesting that the disease was due to an inborn error of the electron transport chain (Kelley et al., 2015). A mutation in cytochrome B (G to A transition at nucleotide 15,498, resulted in the substitution of glycine with aspartic acid at amino acid position 251) resulted in Histiocytoid cardiomyopathy (Che et al., 2014).

Sheep Cytochrome B was observed to be closely related to that of the human. Hence, sheep may be considered as an animal model for studying human diseases, particularly for studying diseases related to defects in mitochondria as Cytochrome B.

A genome- wide and candidate gene study was conducted for mitochondrial DNA as recent advance and future scope of the study (Workalemahu et al., 2017). Association studies of mutation of mitochondrial genome with diseases as prostate cancer had been conducted in human (Giorgi et al., 2016). Hence mitochondrial gene have tremendous role in diseases.

So far our literature search, no studies were reported for identification and detection of mutations in the mitochondrial gene in relation to disease in livestock. This is the first report of disease association with livestock species. A recently identified technique as Mitochondrial replacement therapy or popularly known as the three-parent baby concept was observed to be successful in the correction of mitochondrial defects, thus the anomalies or defects arising can be easily corrected (Pal and Banerjee, 2018). It may be considered as the future scope of the study.

Gene editing technique as CRISPR/Cas9 technology exploits the principles of bacterial immune function to target and removes specific sequences of mutated DNA. This may have potential in treating individuals with disease caused by mutant mitochondrial DNA (Fogleman et al., 2016). It is the future potential of mitochondrial gene study.

4. Materials and method

4.1. Animals

The present study was conducted on 521 adult Garole sheep (1–2 years of age) reared at University LFC farm, West Bengal University of Animal & Fishery Sciences and Sheep Farm under Garole sheep Conservation unit, Animal Resource Development Department, Govt. of West Bengal. The animals were maintained under same agro-climatic region and reared under similar managemental conditions. They are raised under a semi-intensive system of rearing. They are allowed to graze for 9–10 h daily, supplemented with green fodder and adequate vitamin and mineral supplementation. They are housed in flocks.

The study involved drawing of ~5 ml blood from jugular vein aseptically. Tissue samples from different body organs under the study of sheep were collected during post-mortem examination after natural death. The ethical clearance and Biosafety approval have been obtained from University BioEthics and Biosafety Committee. All the methods were performed in accordance with the relevant guidelines and regulations.

4.2. Cytochrome B genotyping

Mitochondrial DNA was isolated from the blood and the tissues of interest of the sheep (Hair and Tissue extraction Kit, Promega). Cytochrome B gene was amplified with the following primers at annealing at 61C:

Forward: 5'CATTGATCTCCCAGCTCCA3'

Reverse: 5'GATGTAGGGGTGTTCAACTGG3'

The reaction conditions were optimized in 50 µl volume reaction comprising of 1× PCR buffer, 1.5 mM MgCl2, 100 µM dNTP mix, 1.25 U Taq DNA polymerase, 100 nM of each primer and 50 ng of DNA. The amplification conditions were as follows, initial denaturation 95C for 5 min (one cycle) followed by denaturation at 95C for 30 s, annealing at 61C for 30 s, extension at 72C for 45 s for 35 cycles with a final extension at 72C for 5 min. Purified PCR product was sequenced and amino acid was deduced.

4.3. SNP detection of Cytochrome B gene in Garole sheep

SNPs were identified for Cytochrome B gene and analyzed through MegAlign Programme of DNASTAR package. The SNPs present in the protein coding region may prone to deleterious effects on gene function. Thus, identified SNPs were evaluated and screened using two different computational tools/algorithms for improving the accuracy of predicted results. I-Mutant3.0 (http://folding.biofold.org/cgi-bin/i-mutant3.0). The SVM based server was used for the prediction of the protein stability change due to single mutations. The FASTA sequence of Cytochrome B protein along with a change in the respective residue with position was provided for the depiction of DDG value (Kcal/Mol). The DDG > 0 indicates that the protein with high stability and vice-versa. The PROVEAN (Protein Variation Effect Analyzer) is an algorithm which predicts the impact of single mutations via amino acid substitution on the protein function (http://provean.jcvi.org/index.php). The FASTA format of the Cytochrome B as a query sequence was submitted, while score prediction based on default threshold −2.5. The score < −2.5 designates mutation is deleterious, while, > − 2.5 deliberated as a neutral.

4.4. Three dimensional structure prediction and model quality assessment

The templates which possessed highest sequence identity with our target template were identified by using PSI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast). The homology modelling was used to build Cytochrome B native 3D structure based on homologous template structures using PHYRE2 server (45.). Predicts the 3D structure of a protein sequence based on HMM-HMM alignment techniques. For a given sequence, it detects known homologs based on PSI-Blast, constructs a hidden Markov model (HMM) of the sequence based on the detected homologs and scans this HMM against a database of HMMs of known protein structures. The 3D structures were visualized by PyMOL (http://www.pymol.org/) which is an open source molecular visualization tool. Subsequently, the mutant model was generated using

PyMoL tool. The Swiss PDB Viewer was employed for controlling energy minimization. SAVES (Structural Analysis and Verification Server), an integrated server (http://nihserver.mbi.ucla.edu/SAVES/) is used for the structural evaluation of predicted 3D model of derived polypeptide. It is also used for assessing the stereochemical quality assessment of 3D predicted model. The ProSA (Protein Structure Analysis) web server (https://prosa.services.came.sbg.ac.at/prosa) was used for refinement and validation of protein structure. The ProSA was used for checking model structural quality with potential errors and the program shows a plot of its residue energies and *Z*-scores which determine the overall quality of a model. The solvent accessibility surface area of the Cytochrome B was generated by using NetSurfP server (http://www.cbs.dtu.dk/services/NetSurfP/). TM align software was utilized for alignment of the 3 D structures of different variants of Cytochrome B protein. Ramachandran plot was employed for the assessment of the stability of protein structure. The basic principle is to visualize dihedral angles ψ against φ of amino acid residues in protein structure. It calculates relative surface accessibility, Z-fit score, the probability for Alpha-Helix, the probability for beta-strand and coil score, etc.

4.5. Protein-protein interaction network depiction

In order to understand the network of Cytochrome B protein, we performed analysis with submitting FASTA sequences to STRING 9.1. In STRING, the functional interaction was analyzed by using confidence score. Interactions with score < 0.3 are considered as low confidence, scores ranging from 0.3 to 0.7 are classified as medium confidence and scores > 0.7 yield high confidence (Von Mering et al., 2003; Szklarczyk et al., 2011) The functional partners were depicted.

4.6. Prediction of post-translational modification sites in mutant Cytochrome B

The post-translational modification of proteins is a vital event for the functioning and protein dynamics. We have used computational approaches for investigating modification sites in the mutant protein of Cytochrome B.

The protein phosphorylation was analyzed using the tool, specifically, Ser, Thr and Tyr residues which required for catalyzing its role. The NetGlycate 1.0 server (http://www.cbs.dtu.dk/services/NetGlycate/) were used for the prediction of glycation site. The serine (Ser), threonine (Thr) and tyrosine (Tyr) residues with a score of > 0.5 were depicted as to be glycated. The phosphorylation sites in mutant proteins were predicted by the Netphos2.0 server (http://www.cbs.dtu.dk/services/NetPhos/). The UbPred server (http://www.ubpred.org/) was used for prediction of ubiquitylation sites, where lysine (Lys) residues with the score of > 0.62 were considered as ubiquitylated.

Other analysis conducted for post-translational modifications are Das TM filter, DisEMBL, ExPASY Peptide cutter, ExPASY_get pl_MW, GENO 3D, HCA, HHpred-homology detection and structure prediction, LipoP, Net Acet 1.0 server, Net C GlyC 1, Net NES 1, Net Phos 3, Net Turn P-prediction of Beta-turns, NPS @-GOR4 secondary structure prediction results, Phobius prediction, Interpro (Cytochrome B protein family relationship), HMMTOP, SAPS, TMHMM and UbPrediction.

2.6. Health examination of the animals:

The animals were examined for health status as body temperature, pulse rate, heart rate and respiration rate. Accordingly, sheep were grouped as debilitated or healthy. Haematology and Blood Biochemical tests were conducted for general screening of the internal body organs as liver, heart, kidney and immune system. ECG was conducted for confirmation of cardiomyopathy cases.

The sheep were examined for exercise intolerance and extreme debility. Cases of exercise intolerance were observed by chasing the sheep for 5–7 min and sheep with exercise intolerance were unable to rise. Cases of sheep with extreme debility manifested through inability to rise were recorded. To confirm it, further investigations were conducted for biochemical parameters.

In the present study, apart from general health examination, the functional assessment of different body organs was conducted through biochemical assessment. The physiology of heart functioning was assessed by lipid profile estimation and assessment of heart rate, pulse rate and final confirmation of disorder through ECG (electrocardiogram). Lipid profile estimation includes estimation of serum level of Total Cholesterol, Triglyceride (TG), HDL Cholesterol, LDL Cholesterol, VLDL Cholesterol, Total Cholesterol: HDL and HDL: LDL. The physiology of muscle functioning was assessed through electrolyte imbalance study as the assessment of serum Calcium, magnesium, Phosphorus, Sodium (Na), Potassium (K) and Chloride (Cl).

The physiology of liver functioning was assessed through estimation of serum level of Total protein(TP), Albumin (ALB), Globulin (GLB), Albumin: Globulin (A:G) ratio, Total Bilirubin, Direct Bilirubin (Conjugated), Indirect Bilirubin (Unconjugated), SGPT (ALT), SGPT (AST), Alkaline Phosphatase (ALP).

Kidney functioning was assessed through estimation of serum level of Glucose (Fasting/Random), Urea, blood urea nitrogen (BUN), creatinine and uric acid.

Haematological parameters were assessed through Complete Hemogram Test (Haemoglobin percentage), Packed cell volume PCV (Haematocrit), Total erythrocyte count (TEC), Total leucocyte count (TLC). Differential leucocyte count (DLC) were estimated as Neutrophil, Eosinophil, Basophil, Lymphocyte, Monocyte percentage estimation. Platelet count, MCV, MCH, MCHC and ESR (1st hour).

4.7. Phylogenetic relationship

Phylogenetic relationship of sheep Cytochrome B was assessed with other livestock species and human. (Mega 6).

4.8. Statistical analysis

Identified SNPs were associated with the phenotypic traits in terms of haematological, biochemical parameters, general health parameters as heart rate, pulse rate, body temperature, respiration rate and occurrence of cardiac myopathy cases. ANOVA was used for finding the effect of genotype on above traits related to disease resistance traits. The model used for analysis was:

$$Y_{eijkl} = \mu + G_i + A_j + F_k + e_{ijkl}$$

where $Y_{eijkl} = l^{th}$ observation of the target trait, μ = Overall mean, G_i = Fixed effect of i^{th} genotype, A_j = Fixed effect of A^{th} animals, F_k = Fixed effect of k^{th} farm, e_{ijkl} = Random error.

Farm effect was considered since the sheep were sampled from two different farms. Effect of the season had not been considered since the observations were collected in the same season. The BLAST search was made using MegAlign Programme of Lasergene Software, DNASTAR and Predicted peptide sequence of GH gene was derived by Edit sequence Programme of Lasergene Software, DNASTAR (DNASTAR Version 4.0, Inc., USA).

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Author contribution

Conceived and designed the experiments: Aruna Pal, Abantika Pal. Performed the experiments: Aruna Pal, Samiddha Banerjee, S. Batobyal. Analyzed the data: Aruna Pal, Abantika Pal, S. Banerjee, P.N. Chatterjee. Contributed reagents/materials/analysis tools: Aruna Pal, Abantika Pal, S. Batobyal. Wrote the paper: Aruna Pal, Abantika Pal, S. Banerjee.

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