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Mutational and Evolutionary Analysis of Interleukin-2 Gene in Pakistani Goat Breeds

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Authors' Contribution
TH and AW did experimental work. JK collected samples and wrote the manuscript. AN and MH performed statistical and bioinformatics analysis. QUA helped in sample collection and data analysis. MB designed the project and supervised it.

Key words
Interleukin-2(IL-2), Goat, Phylogenetic analysis, Polymorphism

ABSTRACT

Interleukin 2 (IL-2) is produced by activated T cells and play important role in immune response against antigen. It acts in both autocrine and paracrine manner. It can stimulate B cells and various other phagocytic cells like monocytes, lymphokine-activated killer cells and natural killer cells. Acting in autocrine fashion, IL-2 protein plays a crucial role in proliferation of T cells. IL-2 triggers the release of pro and anti- inflammatory cytokines by activating several pathways. In present study, exon 1 of IL-2 gene of four local Pakistani breeds (Dera Din Panah, Beetal, Nachi and Kamori) was amplified by using reported ovine IL-2 primers. Amplified products of 4 breeds of goat were bidirectionally sequenced to decipher polymorphisms. Only a single substitution (T→A) was found in non-coding region of *IL-2* gene. Comparison of *IL-2* gene sequence of all four breeds with other goat breeds showed high similarity in sequence. Phylogenetic analysis of our local breeds with other mammals showed that IL-2 was highly variable. This high substitution rate could be due to changed selective pressure. These rapid changes may also lead to the changes in the functions of immune system.

ITRODUCTION

Cytokines are regulatory glycoproteins of approximately 20kD molecular weight and play vital role in defense system of an organism by controlling growth, differentiation and survival of several cells (Zelus *et al*., 2000). These cytokines are released by cells and affect other cells or the cell itself (Zelus *et al*., 2000; Saito, 2001). Cytokines are not only involved in immune system but they also have pleotropic regulatory effect on endocrine, nervous and hematopoietic system (Saito, 2001). Different cytokines have extraordinary immunologic and therapeutic promises in treatment (Perera *et al*., 2012; Sedger *et al*., 2014). Interleukin-2 (IL-2) is the prototype member of cytokines, which regulate and induce immune response (Zelus *et al*., 2000). In immune system, IL-2 is mainly secreted by T-lymphocytes, activated by exposure to antigen (Li and Li, 2020). It works in pleotropic manner and induce T cell growth, activation induced cellular apoptosis, differentiation of Regulatory T cells, differentiation of cytotoxic T cells into effector and memory T cells (Liao *et al*., 2011; Liu *et al*., 2021). By activating or inhibiting the cytokine cascade reactions, IL-2 control the differentiation of T helper cell 1 and T helper cell 2 (Liao *et al*., 2011; Liu *et al*., 2021). For humoral and cell mediated immunity, production of IL-2 is very necessary. Immune suppression and stimulation can also be favored by IL-2 (Boyman and Sprent, 2012; Yang *et al*., 2020). *IL-2* gene has been identified in many species since 1983, when human IL-2 was first cloned and expressed to produce 153 amino acids long polypeptide with signal peptide consisting on almost 20 amino acids, which are cleaved off during the secretion of mature IL-2 (Taniguchi *et al*., 1983). Mature IL-2 protein has three biologically active domains 1- NH3 terminus, 2- COOH terminus and 3- two of three cysteine residues. Cleavage or mutation in 1-20, 121-133 amino acids result in the 99% reduced biological activity of this polypeptide (Ju *et al*., 1987). Since then, many mammalian species have been characterized for *IL-2* gene and *IL-2* genes and it has been shown to have 50- 100% homology with human *IL-2* gene (Ge *et al*., 2020). Few years' later, chicken IL-2 was also cloned which showed homology with mammalian IL-2 (Li and Li, 2020; Sundick and Gill-Xion, 1997). The size of IL-2 polypeptide studied till 2000 varied in different organisms ranging from smallest human IL-2 (153 amino acids) to largest *M. musculus* IL-2 (169

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amino acids) (Zelus *et al*., 2000). This study aimed at of genetic characterization of IL-2 in 4 indigenous capra hircus breeds and its intra and inter species comparison.

MATERIALS AND METHODS

Blood samples

Blood samples were collected from 55 individuals of four different indigenous goat breeds (Beetal, Nachi, Dera din Panah and Kamori) from different regions of Punjab province, Pakistan. Five ml blood from jugular vein was collected in BD lavender-top vacutainer which already contained anticoagulant.

Isolation of DNA and PCR amplification of IL-2 *gene*

Genomic DNA was isolated from collected blood samples by using method introduced by Mathew (1985), and stored at -20°C. The quantity and quality of DNA was determined by Nanodrop 2000c Spectrophotometer (Desjardins and Conklin, 2011) and agarose gel electrophoresis.

The PCR primers (forward: 5'-AAGAGTCAT-CAGAAGAGGAAA -3') and (Reverse: 5'-AACCTTG-GGCATGTAGAAGT-3') were designed using reported ovine IL-2 precursor reference sequence (GI:14582608) (Pariset *et al*., 2006). This pair of primer was used to amplify the exon 1 of IL-2 precursor gene. The PCR reaction consisted of 100-150 ng genomic DNA, 255 µM of each dNTP, 1 unit of Taq polymerase, 6 pmol of each primer and 1x Taq reaction buffer were used in 30 µL reaction volume. The reaction was carried out through 30 cycles that consisted of 45 sec denaturation at 95°C, 60 sec annealing at 62°C and 90 sec extension at 72°C. During the first cycle, denaturation was done at 95°C for 5 min while the final extension was done at 72°C for 10 min. Gel electrophoresis of the PCR product was done on 1.5% agarose gel containing gel green for visualization. The amplified products were processed using ethanol precipitation method and bidirectionally sequenced through direct Sanger method (Sanger *et al*., 1977).

Bioinformatics analysis

Mega 6.0 (Tamura *et al*., 2013) and Clustal Omega (Sievers *et al*., 2011) were used for sequence alignment, phylogenetic analysis and comparisons. NCBI database was searched by using BLAST algorithm to find homologous sequences of caprine IL-2 in other organisms (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Single nucleotide polymorphism analysis

Sequence alignment of all samples of four goat breeds Nachi, Kamori, Dera din panah and Beetal was done with the reference sequence of IL-2 precursor gene to find SNPs among the breed. One transversion substitution of single nucleotide (T→A) has been observed in the non-coding region of *IL-2* gene in all four breeds when compared with reference sequence (Fig. 1). The polymorphic site was nucleotide 74 in intron 1 in all breeds showing 69.2%, 92.3%, 100% and 81.8% polymorphism frequency in Dera Din Panah, Nachi, Kamori and Beatal goat breeds, respectively (Table I). The phylogenetic tree (Fig. 2) generated by simple agglomerative (bottom-up) hierarchical clustering method UPGMA showed that avian and mammal were making two distinct groups in phylogenetic tree, indicating that many variations had taken place in *IL-2* gene sequence with the passage of time after speciation/divergence from same ancestor. In current study, it was attempted to find out phylogenetic relationship of the collected goat samples with other mammals to closely analyze the evolutionary/ancestral relationship of our indigenous goat breeds with other mammals. In general, species, having similar physical

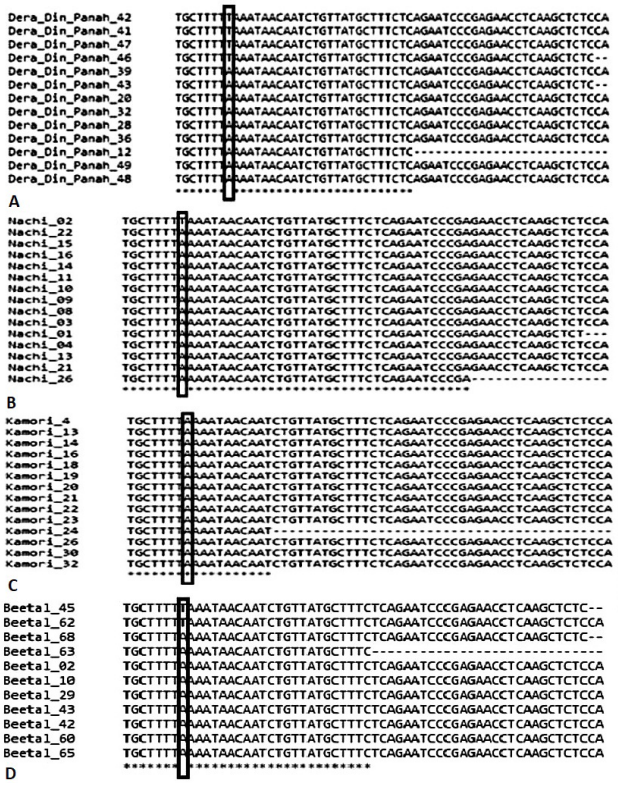
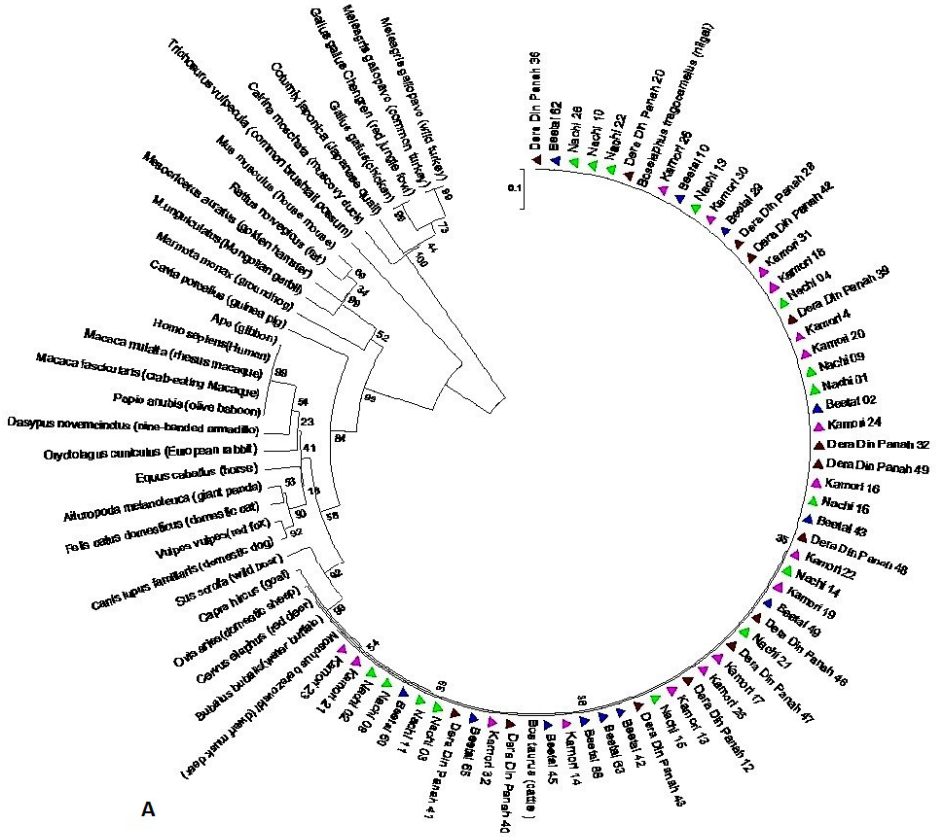


Fig. 1. Transversion substitution of single nucleotide (T→A) observed in the non-coding region of *IL-2* gene in multiple sequence alignment of Dera Din Panah (A), Nachi (B), Kamori (C) and Beetal (D) goat breed. Rectangular box is showing site of polymorphism.



Percent Identity Matrix - created by Clustal2.1

1: Meleagris	100.00	72.03	28.24	23.88	24.44	27.21	27.94	27.21	27.21	27.21
2: Gallus	72.03	100.00	28.24	23.13	22.96	25.00	22.79	23.53	23.53	23.53
3: Trichosurus	28.24	28.24	100.00	35.46	35.21	37.32	35.92	36.62	35.92	36.62
4: Homo	23.88	23.13	35.46	100.00	98.04	65.36	66.01	65.36	65.36	66.01
5: Macaca	24.44	22.96	35.21	98.04	100.00	64.94	65.58	64.94	64.94	65.58
6: Moschus	27.21	25.00	37.32	65.36	64.94	100.00	95.48	95.48	94.84	95.48
7: Bos	27.94	22.79	35.92	66.01	65.58	95.48	100.00	97.42	96.13	96.77
8: Boselaphus	27.21	23.53	36.62	65.36	64.94	95.48	97.42	100.00	96.13	96.77
9: Ovis	27.21	23.53	35.92	65.36	64.94	94.84	96.13	96.13	100.00	99.35
10: Capra	27.21	23.53	36.62	66.01	65.58	95.48	96.77	96.77	99.35	100.00

B

Fig. 2. A, Phylogenetic tree generated by simple agglomerative hierarchical clustering method UPGMA showed that avian and mammal were making two distinct groups in phylogenetic tree. B, Percent Identity Matrix of interleukin-2 protein in 10 genera including Capra.

Table I. showing polymorphic site and frequency of polymorphism in four local Pakistani goat breeds.

S. No.	Breed	Polymorphism frequency (%)	Polymorphic site (Intron 1)
1	Dera Din Panah	69.2	74
2	Nachi	92.3	74
3	Kamori	100	74
4	Beetal	81.8	74

features also have similar genetic makeup to some extent; and this notion has confirmed by evolutionary analysis of many genes like keratin associated proteins families and subfamilies in human and chimpanzee, sheep and goat, cow and buffalo etc (Wu *et al*., 2008; Hua *et al*., 2011). But *IL-2* gene showed surprising results which put sheep and goat at different nodes. Moreover, our phylogenetic tree grouped our sample population close to cattle, dwarf musk deer and nailgai on one taxa due to higher similarity in their nucleotide sequences, but away

from red deer, water buffalo, domestic goat and sheep which have been grouped together on sister taxa due to differences in nucleotide sequences compared to highly similar sequences of previous group. Branch length that is separating these organisms showed that variations have occurred in their *IL-2* gene in very short duration. Interestingly, the domestic cat and giant panda are diverged from same ancestor and have relationship with domestic dog and red fox which are diverged from their previous ancestor (Fig. 2A). The length of branch which is separating red brush tail opossum from rodents and higher animals depicts that with the passage of time large number of nucleotide substitutions has been accumulated in its *IL-2* gene of brush tail opossum. Primate (Gibbon, rhesus macaque, crab eating macaque and olive baboon) *IL-2* gene has same sequence as higher eukaryotes like human, but when compared with our sample population of goat lots of variations has been found, that's why the distance between primates and our sample is population is quite larger on phylogenetic tree.

Protein sequence analysis and comparison with other organisms

By analyzing evolutionary tree, animals were grouped in two groups, first group contains those animals that showed highest evolutionary changes, while second group consisted of those animals possessing lowest evolutionary changes. The protein sequences chosen from NCBI database; [(Less variations: *Bos taurus* (NP_851340.2), *Ovis aries* (NP_001009806.1), *Moschus berezovskii* (AAW27917.1), *Boselaphus tragocamelus* (AAY41281.1) and *Capra hircus* (NP_001274496.1)], [more variations: *Trichosurus vulpecula* (ADV58716.1), *Meleagris gallopavo* (CAB65230.1), *Gallus gallus* (CAE17662.1), *Homo sapiens* (NP_000577.2) and *Macaca mulatta* (NP_001040595.1)], were aligned to get insight into the amino acid protein conserved and variable domain. Percent Identity matrix established by using clustal 2.1 (Fig. 2B) strongly supported our phylogenetic tree data that birds (*Meleagris gallopavo* and *Gallus gallus*) developed many non-synonymous substitutions, thus possessed 23-28 % similarity only with other organisms as compared to 72 % similarity within group. *Capra hircus* had more than 95% similarity with IL2 protein of *Moschus berezovskii, Bos Taurus, Boselaphus tragocamelus* and *Ovis aries*. This showed that IL-2 protein of these organisms have undergone synonymous variations in amino acid sequences in contrast to nucleotide sequence which showed decreased similarity among them (Fig. 1A). *Trichosurus vulpecula* had undergone many nucleotide substitutions that led to non-synonymous changes in IL-2 protein. It has less than 37% similarity with any chosen organism.

DISCUSSION

Inteleukin-2 is one of the most extensively studied members of cytokine family. It was in 1982 when IL-2 was isolated and purified, responsible for the selective proliferation of T-lymphocytes (Welte *et al*., 1982). It was then cloned (Taniguchi *et al*., 1983) and its orthologue was found in chicken that was cloned in 1997 (Sundick *et al*., 1977). The gene has now been identified in more than 30 species like ruminants, avian, mammals, rodents (Zelus *et al*., 2000). *IL-2* has been described to possess many substitution mutations in its sequence (Zelus *et al*., 2000). These nucleotide substitutions are non-synonymous due to which protein sequence of all species also varies greatly. The present study attempted to find out phylogenetic relationship of indigenous 4 goat breeds with other mammals by characterizing mutational and evolutionary characteristics of *IL-2* gene. Phylogenetic analyses have shown that human IL-2 has least homology with avian IL-2 but has highest homology with other primates (Zelus *et al*., 2000). Birds have undergone rapid evolutionary process, resulting in accumulation of many variations in their sequence. This varying rate of substitution among mammals can be associated with body size, rate of pregnancy, population size and generation time (Nerves *et al*., 2014; Bromham, 2011). Rate of evolution in rodents, because of their smaller in size, shorter life span and generation time and large populations, is higher as compared to other mammals like bovine which have large body size, have few young ones and longer generation time (Elsik *et al*., 2009). Primate's *IL-2* gene has same sequence, but when compared with our sample population of goat, lots of variations were found, that's why the distance between primates and our goat population was quite larger on phylogenetic tree. The four indigenous species of goat, because of higher similarity of their nucleotide sequence were close to cattle and musk deer but were found away from red deer, sheep and water buffalo because of difference of *IL-2* nucleotide sequence from these mammals. Only a single substitution (T→ A) was observed in non-coding region of *IL-2* gene in all four breeds, showing their very close similarity in their *IL-2* nucleotide sequence. Regarding the phylogenetic and ancestral relationship, it was found during the present study that goat breeds were close to cattle, dwarf musk deer and nailgai on one taxa due to higher similarity in their nucleotide sequences, but were away from red deer, water buffalo and sheep due to differences in nucleotide sequences. Branch length that was separating these organisms showed that variations have occurred in their *IL-2* gene in very short duration. Capra hircus have > 95% similarity with IL2 protein of *Moschus berezovskii*, *Bos*

Taurus, Boselaphus tragocamelus and *Ovis aries*. It shows that IL-2 protein of these organisms have undergone synonymous variations in amino acid sequences in contrast to nucleotide sequence which showed decreased similarity among them

CONCLUSION

Phylogenetic analysis of protein and nucleotide sequence has shown that IL-2 has undergone many evolutionary changes, but rate of substitutions is different in mammalian species, probably because of varying rate of non-synonymous substitution, smaller generation time, smaller body size and higher number of young ones.

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IBR approval

This study was approved by Ethical Review Board, Virtual University of Pakistan, Lahore.

Ethical statement

This study was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20220414180446

Statement of conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary Material

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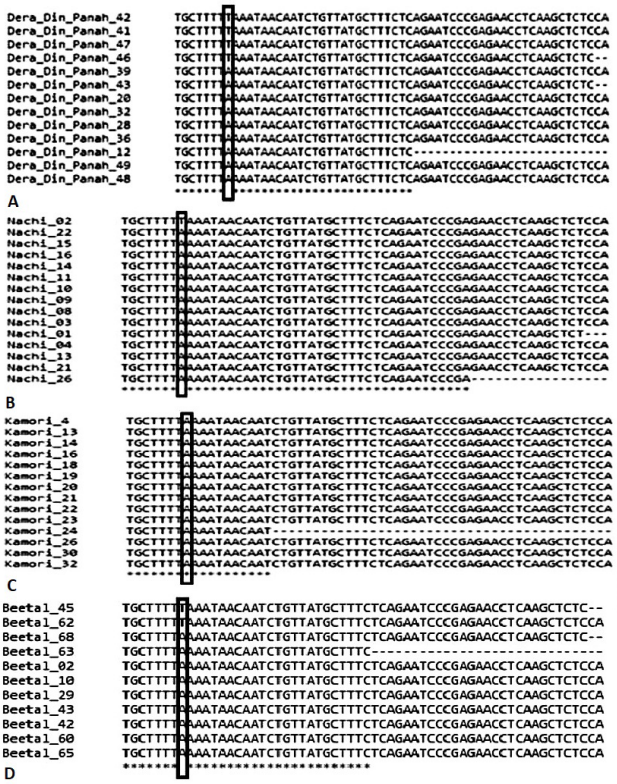

Supplementary Fig. 1. Transversion substitution of single nucleotide (T→A) observed in the non-coding region of IL-2 gene in multiple sequence alignment of Dera Din Panah (A), Nachi (B), Kamori (C) and Beetal (D) goat breed. Rectangular box is showing site of polymorphism.

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