RESEARCH ARTICLE

Sequencing, Evolutionary Relationship and 3D-Structure Analysis of Interferon-ε Gene of Rabbit (*Oryctolagus cuniculus*)

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Abstract

The present study was carried out with the objective to clone and characterize the full-length gene of IFNε of rabbit. The cDNA of type I interferon, *viz.,* Interferon-ε from the liver of a rabbit was amplified by conventional RT-PCR and subsequently cloned for sequence analysis. The obtained nucleotide sequence and the corresponding amino acid sequences of IFN-ε gene were analyzed by using standard bioinformatic tools. A 3D model structure of rabbit IFNe was constructed using a multi-template model employing MODELLER algorithm. Like other IFNε, the rabbit IFNε cDNA also contained a 573-bp open reading frame encoding a protein of 190 amino acids with an estimated molecular weight of 20.90 kDa. Sequence analysis revealed that rabbit from India shared 100 % both at nucleotide and amino acid levels with that of two other sequences available in the NCBI database. Three Cys residues at positions 53, 163 and 175 were found to be conserved across the primary structure of orthologs. Phylogenetic analysis based on amino acid sequences indicated the close relationship in Interferon-ε gene between rabbit and other lagomorphs. The model structure of rabbit IFNε also had the similar pattern to that of any determined type-1 interferon structures, which contained five alpha helices. The 3D-structure was predicted to be stabilized by a disulphide bond, which was found between the residues 53 and 163 and is conserved across the ortholog IFNε protein sequences. It was observed that the genetic diversity between human and rabbit is lower when compared to that between human and mouse. The study also predicts that the genetic conservation within rabbit species is high across the world; however, the available data for analysis is less to support the statement. The obtained sequence information would be useful for the generation of Type-I interferon based therapeutics for rabbit and other mammalian species.

Key words: 3D structure analysis, Bioinformatic analysis, Liver, Interferon-ε, Rabbit.

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Introduction

Cellular proteins possessing antiviral effects have been called Interferons (IFN), which were discovered by Isaacs and Lindenmann in 1957. There are three families in the Interferon group (Savan, 2014). Type-I IFNs comprises 13 IFN-α variants, a single IFN-β and numerous other IFNs (IFN-ε, -κ, -ω and -δ) (Ivashkiv and Donlin, 2014). Across the phylum Chordates, Type I IFNs are further playing a vital role in host defence, characterized by the possession of conserved multicomponent, species-specific molecules (Stifter *et al.,* 2018). Among the type I IFN family, Interferon epsilon (IFNε) is a recently identified molecule having constitutive expression in the lung, brain, small intestine, and reproductive tissue; it is therefore hypothesized that IFNε would be playing an antiviral role in the reproductive tract or help in the early development of the placenta in mammalian species (Demers *et al.,* 2014).

IFNε possesses both anti-bacterial and anti-viral activities (including HIV-1 infection) within the female reproductive tract (Fung *et al.,* 2013). Being the unique prototype of type I IFN, IFNε acts by binding through the IFNAR1 and IFNAR2 receptors and thereby activating interferon-regulated genes (IRGs) (Fung *et al.,* 2013). When compared with other IFNs, the

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level of expression or availability of IFNε varies between the estrous cycle in animals (Fung *et al.,* 2013; Fischer *et al.,* 2018) and the menstrual cycle in humans (Bourke *et al.,* 2018). In mammalian species, the antiviral activities of recombinant bovine (Guo *et al.,* 2015), cameline (Abdel-Fattah *et al.,* 2019), ovine (Guo *et al.,* 2020) and canine (Yang *et al.,* 2013) IFNε proteins were demonstrated in cell culture systems.

Therefore, it is imperative to explore the immune system of rabbit for the development of diagnostics and therapeutics against human pathogens. Due to the constitutive expression of IFNε in the female reproductive tract of women and other mammalian species, it is hypothesized that IFNε would also be playing a defensive role in the female reproductive tract of rabbit against the potential infectious agents causing abortions. Further, evaluation of the antiviral activities of IFNε is highly inevitable in the uterus of highly prolific mammals such as rabbits. In this direction, while searching for Interferon-ε gene of rabbit in the literature, the baseline information about this important type I interferon is not available. Since rabbits are well known for their large litter size and induced ovulation, as a first step, it is important to investigate its innate immune responses, afforded particularly by IFNε within the reproductive tract of the does. Keeping the aforementioned information in view, the present study was carried out with the objective to clone and characterize the full-length gene of IFNε of rabbit. The deduced amino acid sequences were then used for phylogenetic relationship analyses by comparing them with published sequences from other mammalian animal species available at NCBI.

Materials and Methods

Collection of Rabbit Liver, RNA Isolation and RT-PCR

All animal experiments were performed according to protocols approved by the IAEC at Southern Regional Research Centre, ICAR-Central Sheep and Wool Research Institute, Mannavanur, Kodaikanal, Tamil Nadu, India. A male rabbit around four months of age (White Giant breed) was sold for slaughter at SRRC, Mannavanur on 1st week of March 2022. From this animal, the liver tissue was collected in Trizol^{TM} reagent and brought to the laboratory in ice. Subsequently, liver tissue containing Trizol^{TM} reagent was ground in a mortar with the help of pestle using liquid nitrogen. The resultant ground tissue in the form of powder was used for the isolation of total cellular RNA using RNeasy Plus Universal Kits (RNA isolation from Animal tissues) as per the manufacturer's instructions. The quality and quantity of the RNA isolated from liver tissues were determined by Quawell Nanodrop UV-Vis Spectrophotometer (NanoDrop 2000 C, USA). An aliquot of the total RNA (5 µg) was reverse-transcribed using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara) in a 20 µL volume reaction mixture according to the manufacturer's instructions.

Polymerase Chain Reaction

Reaction volumes for the PCR of 50 µL were used and contained 5 µL of 10X buffer with 15 mM $MgCl_2$, 10 mM of each dNTPs, 100 pmol of each oligonucleotide primer, 100 ng of cDNA sample and 3U Taq DNA polymerase. The primers used for the amplification of Interferon-ε gene of rabbit were the forward primer, RAIFNE-F (28 mer): 5′ ATGACTTACAA GTACTTCTTTGAAATTG 3′ and reverse primer, RAIFNE-R (28 mer): 5′ CTAGCTCTTTTT GTTCAGCATTTCTGTG 3′. The primer sequences were designed based on Interferon-ε gene sequences of rabbit reported in NCBI Accession No. XM_051845009.1.

The reaction mixture was subjected to initial denaturation of the template at 95°C for 3 min in a thermal cycler (BioRad, USA). Cycling conditions for PCR were 35 cycles of 60 s at 94°C, 60 s at 55°C and 60 s at 72°C, followed by a final extension for 10 min at 72°C. The total cellular RNA isolated from the blood of rabbit was included as a negative control in the PCR.

Cloning and Sequencing of Interferon-ε Gene of Rabbit

Resultant PCR products were separated on 1.2% agarose gels containing ethidium bromide (10 mg/mL), and visualised under UV light. The PCR products were purified using QIAquick Gel Extraction Kit and cloned into pTZ57R/T vector (Fermentas) using the protocol according to manufacturer's instructions. The plasmids were transformed into *Escherichia coli* DH5α. Colonies harbouring the recombinant plasmid were inoculated into LB (Luria Bertani) broth containing Ampicillin (50 µg/mL) and incubated at 37°C overnight with horizontal shaking. The plasmid DNA was extracted from culture using QIAprep Spin Miniprep Kit. The recombinant plasmids were confirmed by PCR using the gene specific primers. The sequencing of three positive clones was carried out in both directions using Sanger sequencing method by M/s. Eurofins Genomics India Pvt. Ltd., Bengaluru-560048, Karnataka, India and the firm used both M13 forward and M13 Reverse primers for the sequencing experiments.

Sequence Analysis

Using BLAST (Biological Local Alignment Search Tool) software of NCBI (Altschul *et al.,* 1990), the nucleotide sequences provided by M/s. Eurofins Genomics India Pvt. Ltd., Bengaluru-560048, Karnataka, India, were analysed. Upon BLAST search, the top most sequences displaying 100% alignment with the nucleotide sequences of the present study were Interferon-ε gene of rabbit reported in NCBI Accession No. XM_017348927.1 and eventually, the corresponding amino acid sequences of Interferon-ε gene of rabbit were deduced using the same BLAST software. The determined nucleotide sequences of Interferon-ε gene of rabbit from India were then submitted to GenBank and the accession No. ON007366 was obtained.

Further, the nucleotide and amino acid sequences of Interferon-ε gene of rabbit from India were aligned with that of 34 mammalian orthologs published earlier in the GenBank (Table 1, Fig. 1) using sequence alignment software Clustal X 2.1 (Larkin *et al.,* 2007). Pairwise nucleotide and amino acid sequence identities among the relevant orthologs were also computed using Clustal X 2.1. The signature motifs in Interferon-ε protein of rabbit were identified as per the reports published earlier (De Castro *et al.,* 2006; Sigrist *et al.,* 2013). The signal peptide of rabbit IFNε protein was predicted by using PREDISI server (Hiller *et al.,* 2004). This signal peptide was removed before constructing three-dimensional structural model of rabbit IFNε.

Phylogenetic and 3D Structure Analysis

The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model (Jones *et al.,* 1992). Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018). The close homologs of rabbit IFNε were identified through BLAST search (Altschul *et al.,* 1990) against protein-structure-sequence database in NCBI. The sequences of two protein structures, human IFNω (PDB id: 3SE4) and human IFNα (PDB id: 3ux9) were found to be close to the query sequence. 3D model structure of IFNε was constructed using multi-template model employing MODELLER algorithm (Webb and Sali, 2021). The protein

Table. 1: Percent nucleotide (nt) and amino acid (aa) identity of IFNε gene of rabbit with that of other mammals closely related to rabbit

Sl. No.	Animal species	Mammalian order	NCBI nucleotide Accession No.	NCBI protein Accession No.	Nucleotide seq. identity (%)	Amino acid seq. identity (%)
1.	*Oryctolagus cuniculus*	Lagomorpha	ON007366	UUB68735.1	This report	This report
2.	*Oryctolagus cuniculus* - Australia	Lagomorpha	LR761101.1	CAB0000288.1	100	100
3.	*Oryctolagus cuniculus* - Autopred	Lagomorpha	XM_051845009.1	XP_051700969.1	100	100
4.	*Lepus europaeus* -Finland	Lagomorpha	XM_062208133.1	XP_062064117.1	98	97
5.	*Ochotona curzoniae*	Lagomorpha	XM_040963600.1	XP_040819534.1	82	68
6.	*Homo sapiens*	Primates	NM_176891.5	XP_007176883.1	80	67
7.	*Colobus angolensis palliatus*	Primates	XM_011930331.1	NP_001098780.1	80	67
8.	*Cercocebus atys*	Primates	XM_012071937.1	XP_005209958.1	80	66
9.	*Macaca fascicularis*	Primates	XM_045373323.1	XP_019821215.1	80	66
10.	*Piliocolobus tephrosceles*	Primates	XM_023203866.2	XP_010851614.1	80	66
11.	*Mus musculus*	Rodentia	NM_177348.2	NP_796322.1	70	54
12.	*Rattus norvegicus*	Rodentia	NM_001402768.1	NP_001389697.1	69	52

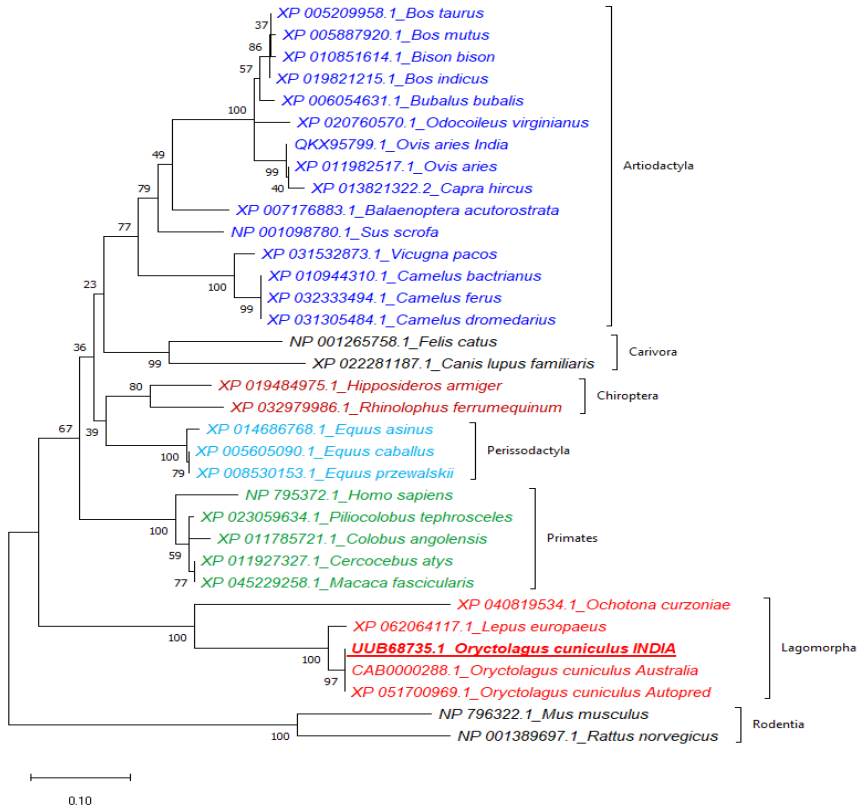

Fig. 1: Phylogenetic analysis of IFNε protein sequences of various mammalian species. The accession numbers shown in the tree represent the NCBI IFNε proteins.

sequences of the templates were extracted employing JOY algorithm (Mizuguchi *et al.,* 1998) and multiple sequence alignment was constructed using Clustal X software tool (Larkin *et al.,* 2007).

Results and Discussion

Nucleotide and Amino Acid Sequences of Rabbit IFNε

The rabbit IFNε cDNA contained a 573 bp open reading frame encoding a protein of 190 amino acids with an estimated molecular weight of 20.90 kDa. A signature motif, "YFLRIRDYLEAQDYSICAW" (description- PS00252, Interferon alpha, beta and delta family signature) was found from the position No. 147 to 165 of IFNε protein of rabbit. The first 21 amino acid residues of rabbit IFNε protein, MTYKYFFEIVLVLLASSTIFS were predicted to be the signal peptide. On multiple alignments of nucleotide and amino acid sequences of IFNε of Indian rabbit with 33 mammalian species, it was revealed that the IFNε of rabbit from India shared 100% sequence identity both at nucleotide and amino acid levels with other two IFNε sequences of rabbit available at NCBI database (LR761101.1 and XM_051845009.1).

With the other Lagomorph, *i.e., Lepus europaeus* (GenBank Accession No. XM_ 062208133.1) and Pika (GenBank Accession No.XM_040963600.1), the range of identity of Indian rabbit was 98% & 97%, and 82% & 68%, at nucleotide and amino acid levels, respectively (Table 1). The multiple sequence analysis (MSA) of IFNε proteins of 12 orthologs revealed that three Cys residues at positions 53, 163 and 175 were found to be conserved across their primary structure.

Phylogenetic and 3D Structure

Phylogenetic relationship was constructed employing MEGA-X utilizing the multiple sequence alignment of IFNε protein sequences of 34 mammalian species (Fig. 1). A three-dimensional model structure (Fig. 2b) has been predicted by employing Modeller algorithm (Šali and Blundell, 1993) through multi-template model approach. The rabbit IFNε protein 3D model was examined and found to have bigger loop disturbing the helix region. Hence, loop refinement was performed for the bigger loop and energy minimized. The final model structure was validated by plotting phi and psi angles on Ramachandran's map employing PROCHECK algorithm (Laskowski *et al.,* 2012), which shows >90% of

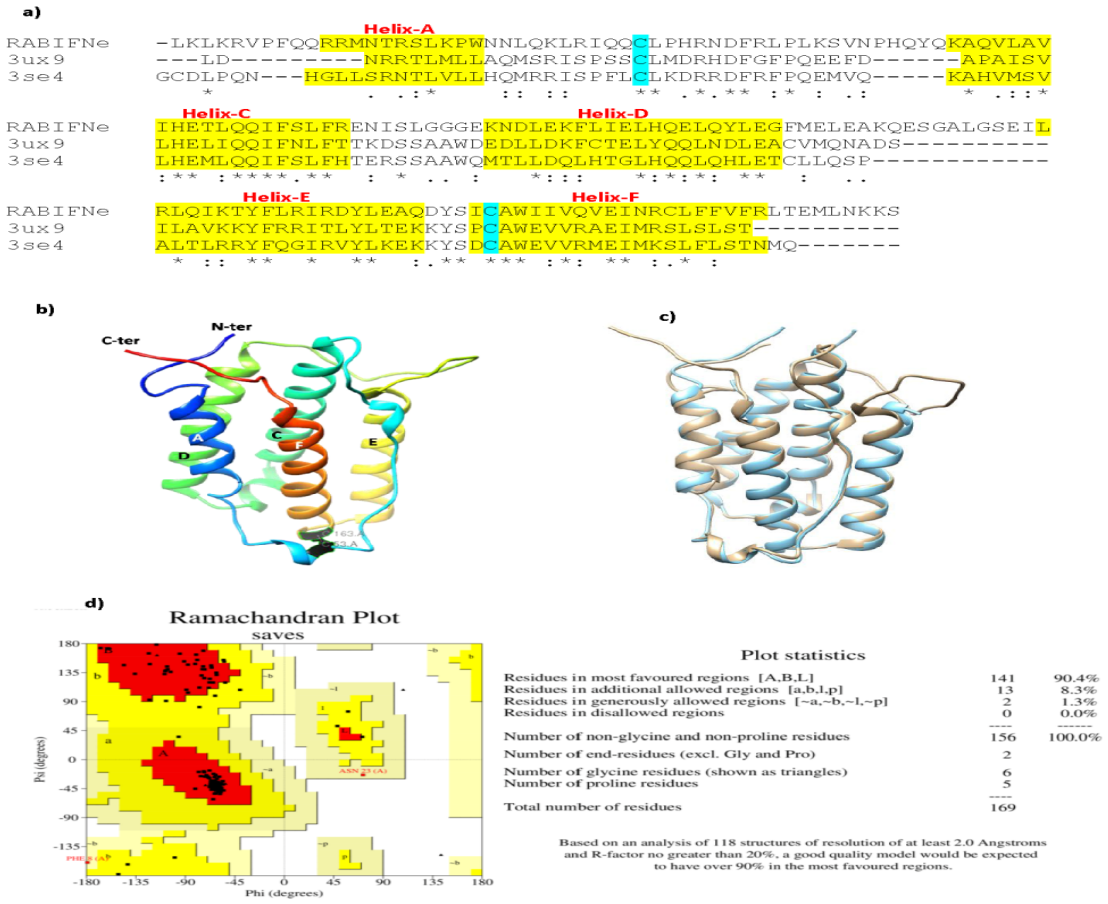

Fig. 2: Three-dimensional model of rabbit IFNε and its validation. a) Target and templates (3ux9 and 3se4) sequence alignment and highlight of helix region range; b) model with disulphide bond interaction between Cys53 and Cys163. c) Superimposition of target structure on templates; d) Ramachandran's plot showing phi and psi values of built 3D model structure

values in the most favoured region (Fig. 2d). Hence, the built 3D model was good and may be used for further analysis. The rabbit IFNε model was compared with the template 3SE4 by superimposing it in Chimera (Pettersen *et al.,* 2021) and found to have the Root Mean Square Deviation (RMSD) value of 0.485 A°. Three conserved cysteine residues, Cys 53, Cys 163 and Cys 175 in the type 1 Interferon were mapped on this model structure (Fig. 2b), in which a disulphide bond found between Cys 53 and Cys 163 is highlighted. The similar disulphide bond was found between Cys 52 and Cys 145 in case of human IFNω, and Cys 29 and Cys 139 in case of human IFNα. There have been five α-helices, labelled from A to E. The residues falling in five helix regions have been highlighted on the sequences of IFNε of *Oryctolagus cuniculus* in the pairwise alignment (Fig. 2a).

In the present study, IFNR1 and IFNR2 3D model of rabbit was built based on the template 3SE4 using chain A and C, respectively. All the built models, IFNR1, IFNε, IFNR2 proteins were superimposed on 3SE4 as chain A, B and C, respectively and the transformed coordinates were represented. The signal peptide of rabbit IFNε protein was equivalent to the length of IFNε protein of human (Marks *et al.,* 2019) and sheep (Guo *et al.,* 2020). Despite that, the last residue of the signal peptide is Ser in rabbit, human and sheep, the mature IFNε protein of rabbit possessed 169 amino acids. Leporine IFNε also retained the three conserved cysteine residues at the positions of 53, 163 and 175 similar to ovine IFNε (Guo *et al.,* 2020), cameline IFNε (Abdel-Fattah *et al.,* 2019), rabbit interferon-γ (Samudzi *et al.,* 1991) and Avishaan sheep IFNε (Nagarajan *et al*., 2024). Additionally, the three amino acid residues such as Ser 38, Glu 112, and Ile 167 were also conserved in rabbit IFNε, as observed in type I INFε of other species (Abdel-Fatah *et al.,* 2019), except in *Ochotona curzoniae* (GenBank Accession No.XM_ 040963600.1) that contained Ile at position 38 in place of Ser.

The number of potential glycation sites present in rabbit IFNε protein also has seven sites as that of camel (Abdel-Fatah *et al.,* 2019) and Avishaan sheep. When compared to the said two artiodactyl species, the positions and the amino acid residues of the said seven glycation sites in rabbit IFNε are entirely different, which include 43NNLQ, 58NDFR, 68NPHQ, 95NISL, 104NDLE, 173NRCL and 187NKSS. Out of seven potential glycation sites, Leporine IFNε protein has retained the similar amino acid residues in only one putative glycation sites, *viz.,* 173NRCL compared to glycation sites predicted in camel IFNε protein (Abdel-Fatah *et al.,* 2019) and Avishaan sheep. Such glycation sites are known to play an important role in the protection of IFNε protein against proteases-mediated hydrolysis and in the process of folding, oligomerization, and stability of the protein. Another finding, Zinc (metal) ion binding residue Gln 143 in Camel and Sheep IFNε protein was also conserved in rabbit IFNε protein similar to other species.

Human IFNε is having 80 % and 67 % identity with rabbit both at nucleotide & amino acid levels, respectively. As far as IFNε is concerned, the percent identity between human & mouse at nucleotide and amino acid levels are 72 % and 58 %, respectively. It clearly indicates that the genetic diversity between human and rabbit is lower compared to human and mouse, which is supported by the reports published elsewhere (Perkins *et al.,* 2000; Soares *et al.,* 2022). It is proposed that the European rabbit would be the better animal model to study the genes of innate immune system of human beings while comparing mouse as reported earlier (Soares *et al.,* 2022).

While looking deeply into the amino acid residues of IFNε protein of all the 34 mammalian species covered under the present study, an unique amino acid substitution at 118 position, *i.e.,* replacement of Glutamic acid (E) by Glutamine (Q) was observed invariably among Lagomorphs (GenBank Accession Nos.ON007366, LR761101.1, XM_051845009.1, & XM_040963600.1), except in *Lepus europaeus* (XM_062208133.1), that contained Leucine (L) in place of Glutamic acid (E) at position 118. It is highly warranted to study the effect of Lagomorph - specific amino acid replacement in IFNε on mucosal immunity.

Further, the Lagomorph specific amino acid substitutions such as E37R and W101G were found in IFNε protein (GenBank Accession Nos. ON007366, LR761101.1, XM_051845009.1, XM_062208133.1 and XM_040963600.1). More precisely, among Lagomorph, one Leporid specific amino acid substitutions was found at the position 187 (S187N) of *Oryctolagus cuniculus* IFNε protein (GenBank Accession Nos. ON007366, LR761101.1, XM_051845009.1 and XM_062208133.1). The predicted amino acid substitutions in IFNε protein of Lagomorphs would provide perception about how Lagomorphs handle pathogens, including viruses.

The presence of conserved Cys amino acids observed at positions 53,163 and 175 across the orthologs was in agreement with the published results on analysis of various interferon sequences (Premzl, 2020). The MSA also reveal that the amino acids present in the helices C and E were found to be highly conserved compared to B and D.

In phylogenetic analysis, a close relationship was observed between rabbit and other lagomorphs. Indeed, *Ochotona curzoniae* (Order Lagomorpha; Family Ochotonidae) IFNε was branched out separately, though it was closely related to *Oryctolagus cuniculus* (Order Lagomorpha; Family Leporidae). This was supported by the recent report on the phylogenetic analysis, which was carried out based on retroposon presence/absence patterns, where in the lagomorphs were widely divided into ochotonids and leporids with *Pronolagus* as the first divergence in the leporid tree (Sparwel *et al.,* 2019). An extensive novel sampling is highly needed in order to understand the genetic relationship of Lagomorpha (Kraatz *et al.,* 2021).

Based on earlier reports, the putative IFNAR-1 and IFNAR-2 binding domain residues of camel IFNε (Abdel-Fatah *et al.,* 2019) have been mapped on the amino acid sequence of camel, sheep, rabbit and human as well as the built 3D

model of IFNε of rabbit by the presence of number of 8 and 13 amino acid residues, respectively. The IFNAR1 binding domain residues such as F29, Q30, R33, R36, E37, K40, N43 & K44, reported in camel falls in the alpha A helix region in IFN epsilon of rabbit, where R36, E37 and K44 residues were replaced by T36, R37 and N44. The IFNAR2 binding domain residues such as L54, P55, H56, R57, N58, D59, F60, R61, P63, L64, K65 Q71 and Y72 reported in camel falls in between alpha-A helix and alpha-B helix, which is majorly occupied by the coil or loop region and a small helix region in IFN epsilon of rabbit (Fig. 2b). A few of IFNR2 binding domain residues of camel such as K58, N59, L61 and Q64 were replaced by N58, D59, R61 and L64 in IFNε of rabbit. As per the earlier published reports, this small helix region is marked as alpha B in case of IFN epsilon of camel (Abdel-Fatah *et al.,* 2019); however, which is not being marked for helix region in case of sheep (Guo *et al.,* ,Nagarajan et al., 2024. In the present study, the said helix region of IFN epsilon protein model of rabbit is also not labelled, although a small helix region is found in the characterized IFNAR2 binding domain.

In the built model structure, the long and highly flexible loop region present after the helix-A was observed to be stabilized by the disulphide bridge formed between Cys 53 at this loop region and Cys 163 present at the N-terminal region of Helix-F. Hence, the disulphide bond was predicted to be involved in stabilizing the overall 3D structure of the protein in rabbit IFNε. Three-dimensional structure was found to be stabilized by the hydrophobic interaction between four helix regions that are proximate to each other, which are labelled A, C, D and F. As reported earlier in this study, another Cys residue at position 175 of helix-E was observed, but does not observed to form a di-sulphide bond; however, it forms a main chain hydrogen bond with Glu 171.

As far as the type of amino acids are concerned, the helix-A and loop region found between helix-A and C were found to have more number of positively charged amino acids, polar uncharged amino acids, special amino acids (Cys and Pro) and less number of hydrophobic amino acids. Especially, two prolines were found before and after the helix-A and three prolines were found in the loop region found between helix-A and C. Since proline is a helix breaker (Li *et al*., 1996), which could be one of the major reason for the protein unable to form a proper helix region structurally. It is interesting to note that the residues in helix-A and loop region between helix-A and C were reported to be involved in binding with the receptors IFNAR1 and IFNAR2, respectively. As per the amino acid conservation pattern observed, 36 amino acids were found to be identical, 38 amino acids were found to be similar and 10 amino acids were found to be species specific. Mapping of these amino acids on to the built model structure revealed that most of the conserved (identical and similar) amino acids found in the helix regions, which indicates that these residues are conserved for maintaining the structural properties. However, the species-specific residues warranted further studies.

The present study was carried out only with available five IFNε gene sequences of the order Lagomorpha (including the present study) in the public domain. Hence, a large number of sequence resources are required to predict/ identify lagomorph-specific and leporid-specific functional motifs to understand their mechanism of action and function. Further, it is also recommended that the biological activities of recombinant IFNε protein of rabbit needs to be validated using both *in vitro* and *in vivo* systems for the purpose of developing diagnostics and therapeutics from Lagomorphs.

Conclusion

The complete analysis of nucleotide and amino acid sequences of IFNε of a rabbit from India was carried out for the first time. Similar to other IFN homologs, three cysteine residues at the positions of 53, 163 and 175 are also conserved in IFNε of rabbit. As far as the innate immunity genes are concerned, the level of genetic relatedness between human & rabbit is comparatively higher when compared to that of between human & mouse. The antiviral properties of the recombinant protein of IFNε of rabbit need to be extensively studied under *in vitro* as well *as in vivo* conditions of the mammalian species.

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