Indian J Anim Health (2025), 64(2) : 342 - 349
DOI: https://doi.org/10.36062/ijah.2025.16225

Research Article

Immuno-informatics and expression of caprine interferon gamma

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Abstract

Interferon gamma (IFN-γ) is an important cytokine that has antiviral and antitumor activity by stimulating type I immune response. It is also extensively evaluated for its potential as an adjuvant in vaccine formulations and used in assays for the diagnosis of tuberculosis. Considering its significance in practical applications, immuno-informatic analysis of caprine interferon gamma (cIFN-γ) was performed and the designed cIFN-γ was commercially synthesized, cloned, expressed and characterized in a prokaryotic expression system. For immuno-informatic analysis of cIFN-γ, the Alphafold, ABCpred and IEDB servers were used. The majority of residues fell into the "very high confidence" category (pLDDT>90) by Alphafold. Using the ABCpred server, about sixteen non-overlapping linear B-cell epitopes were identified, while the BepiPred analysis predicted seven sequential epitopes. The Kolaskar and Tongaonkar method (IEDB) based analysis revealed four antigenic peptides. Additionally, ElliPro analysis predicted five linear and eight conformational discontinuous epitopes. Since the immuno-dominant epitopes spread across the protein, the complete cIFN-γ gene excluding the signal sequence was cloned into the pET32a prokaryotic expression vector system and expressed. The expression of recombinant cIFN-γ, along with the fusion tag, with the molecular weight of approximately 34 kDa, was confirmed by PAGE followed by western blot analysis. Purification of the expressed protein was achieved using Ni-NTA chromatography under denaturing conditions, and its identity was confirmed by SDS-PAGE analysis. The expressed cIFN-γ could be used for the generation of monoclonal and polyclonal antibodies.

Keywords: B cell epitopes, cIFN-γ, Expression, TB

Highlights

- Immuno-informatics of the caprine interferon gamma sequence retrieved from NCBI
- Cloning and expression of caprine interferon gamma
- Purification and characterization of caprine interferon gamma

INTRODUCTION

The interferon-gamma (IFN-γ) is an essential cytokine involved in adaptive as well as innate immunity, with diverse functions in inflammation along with autoimmune disorders (White et al., 2018). It can increase the ability of macrophages and T cells to express class II MHC molecules, which improves their ability to deliver antigens (Gessani et al., 2014). It has strong immuno-modulatory properties (Qin et al., 2016).

Interferon-γ (IFN-γ) is mainly secreted by immune effector cells, including T lymphocytes and natural killer cells. Upon binding to its receptor, IFN-γ mediates diverse biological functions, primarily through activation of the Janus kinase–signal transducer and activation of transcription (JAK-STAT) signalling pathway. Upon binding to its receptor subunit IFNGR1, conformational changes in the intracellular domain facilitate the recruitment and activation of JAK1 and JAK2 kinases, leading to phosphorylation and activation of STAT1. Activated STAT1 translocates into the nucleus and initiates transcription of IFN-γ-responsive genes, which include transcription factors such as IRF1, which amplify the downstream transcriptional cascade (Schroder et al., 2004).

Tuberculosis in mammals, including humans and animals, is a chronic granulomatous disease caused by infection of the host with pathogenic agents of *Mycobacterium tuberculosis* complex (MTBC). The three primary organisms of the MTBC are *M. bovis*, *M. caprae and M. tuberculosis*. While they share a variety of hosts, they have distinct host preferences (Cousins & Florisson, 2005). Instances of caprine TB are typically carried by *M. caprae* and *M. bovis*, which

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are spread from cattle when they coexist or share grazing ground (Gandhi, 2023).

Goats act as amplifier host in which the pathogen replicates very efficiently producing high pathogen load in the animal and also shedding large quantities of virus into the environment, so the disease spreads quickly among the animals in a herd once it has established itself (Matthews, 2016; Dibessa, 2020; Gandhi, 2023). *M. caprae* is the organism that is accountable for animal tuberculosis (TB), which has surpassed *M. bovis* in several areas (Valcheva et al., 2020). Goats are particularly vulnerable, and their incidence might reach 70% if they are housed near sick herds of cattle.

M. caprae is predominantly reported in European countries, where it represents a major cause of tuberculosis in goats and other livestock. However, it has also been reported outside of European countries like Tunisia (Lamine-Khemiri et al., 2014). More notably, in India, at the Eastern Regional Station of ICAR IVRI in Kolkata, our group recently isolated several *M. caprae* strains from cattle, representing the first such report for India (Unpublished data). These findings suggest that *M. caprae*, although primarily regarded as a pathogen of goats, may in fact be circulating more widely among goat populations and potentially other livestock in India.

Caprine IFN-γ (cIFN-γ) is important in the diagnosis of the intracellular pathogens like *Mycobacterium*, *Neosporum*, *Toxoplasma*, *Corynebacterium* and viral infections where the cellular immunity plays a major role. The first indication of abnormal IFN-γ expression occurs when the host becomes infected with exogenous pathogens. Consequently, the body's immune system and general health may be evaluated by measuring the amount of IFN-γ, which can be utilised as an early diagnostic sign of illness (Kaket al., 2018).

A powerful regulator of the cellular immune system reaction against *M. bovis* infections is IFN-γ. Whenever the cell-mediated immune response to the mycobacterial antigens is established, the circulating leucocytes with an intrinsic capacity to make IFN-γ increase drastically. The immunological response to TB is mostly mediated by T-cells as opposed to antibodies (Pollock et al., 1996). The generation of IFN-γ by sensitised lymphocytes stimulated *in vitro* is a highly reliable predictor of T-cell response and correlates with illness. This formed the basis for the development of Interferon-gamma release assays (IGRAs).

IGRAs have been developed as blood-based alternatives to the tuberculin skin test (TST) for TB diagnosis. Both TST and IGRA detect T-cell responses indicative of TB infection. IGRAs stimulate peripheral blood T-cells with TB-specific antigens, leading to IFN-γ production (Andersen et al., 2000). Two commercial IGRA systems are available: TSPOT.TB (Oxford Immunotec, 2009), which uses ELISPOT to count IFN-γ producing cells, and Quantiferon-TB Gold In-Tube (QFT-G-IT; Cellestis, 2009), which measures IFN-γ via ELISA. However, there is no dedicated diagnostic kit available for the detection of caprine tuberculosis. The kit developed for the diagnosis of bovine tuberculosis is being used for caprine tuberculosis, which has poor sensitivity and specificity in the detection of caprine tuberculosis.

The monoclonal antibodies against IFN-γ can be developed and used for the diagnosis of various caprine-related diseases (Ma et al., 2019). Similarly, the monoclonal antibodies can be raised against recombinant cIFN-γ and used for the development of caprine-specific IGRA assay. In the present study, as a pre-requisite for the development of caprine IGRA, the expression of recombinant cIFN-γ and characterisation of the recombinant protein were carried out.

MATERIALS AND METHODS

Nucleotide and amino acid sequences: The DNA and protein sequences of the cIFN-γ coding sequence were retrieved using the NCBI nucleotide sequence database (Accession No. EF375708; ABN51232).

3D structure prediction and bioinformatic prediction of potential B-cell binding epitopes: The 3D structure of the protein was predicted from the AlphaFold server (https://alphafold.ebi.ac.uk). The prediction of epitopes for the cIFN-γ was done to know the linear epitopes and discontinuous B-cell epitopes, which are important for eliciting the immune response. The two bioinformatics tools used were ABCPred (http://crdd.osdd.net › raghava › abcpred) for the prediction of continuous B-cell epitopes and IEDB (Immune epitope database and analysis resource), containing BepiPred 2.0 (https://www.iedb.org) tool for the prediction of continuous B-cell epitopes. The antigenicity of the protein was assessed using the Kolaskar and Tongaonkar method available in the IEDB analysis resource, which predicts antigenic regions based on physicochemical properties. Finally, discontinuous B-cell epitopes were predicted by using Ellipro (https://tools.iedb.org/ellipro). The PDB file was taken from AlphaFold server (AF-P79154-F1).

Amplification of the cIFN-γ gene by PCR and its characterization: The cIFN-γ sequence, excluding the signal peptide sequence, was retrieved from NCBI. The

Table 1. Primers used in the study

cIFN-γ-*NcoI*-For	5'GGAT**CCATGG**GCCATCATCATCATCATCACCAGGGCCCATTTTTTAAAG 3'
cIFN-γ-*BamHI*-Rev	5'**GGATCC**TTACATTGATGCTCTCCGGCCTCGAAAG-3'

sequence was added with *BamH*I restriction sites **GGATCC** to the 5' and 3' ends of the sequence, and a 6×His affinity tag was incorporated at the N-terminal region. A glycine codon was introduced immediately after the start codon and before the His-tag to enhance translational efficiency and minimize potential steric hindrance. The designed sequence was commercially synthesized and cloned into the pUC57 vector. For directional cloning of the cIFN-γ into the expression vector, PCR was performed by using cIFN-γ-*NcoI*-For and cIFN-γ-*BamHI*-Rev primers (Table 1). PCR amplification was carried out with an initial denaturation at 94°C for 30 s, followed by annealing at 55°C for 15 s and extension at 72°C for 30 s. A final extension step was performed at 72°C for 7 min, after which the amplified products were resolved on a 1% agarose gel.

Cloning of cIFN-γ into the prokaryotic expression vector pET32a

The amplified cIFN-γ gene was digested with the *BamHI* and *Nco*I restriction endonucleases, followed by cloning into the pGEMT-Easy vector and then sub-cloning into the pET32a vector, and transformation was done into the *E. coli* top-10 alpha competent cells using standard procedures (Green & Sambrook, 2012).

The recombinant (pET32a-cIFN-γ) plasmid was isolated, and the presence of the IFN-γ gene was confirmed by colony PCR using gene-specific primers as above and also by the restriction digestion with the *Bam*HI and *Nco*I, followed by confirmation of the digested products on 1% agarose gel.

Expression and purification of recombinant cIFN-γ in *E. coli* BL-21 (DE3) pLyS host

The recombinant pET32a-cIFN-γ plasmid was introduced into *E. coli* BL-21 (DE3) pLyS expression host cells after confirming the correct orientation of the IFN-γ gene insert. One colony of *E. coli* BL-21 cells containing the pET32a-cIFN-γ and also the plain pET32a without the insert was kept at 37°C in LB containing 50 µg/mL of ampicillin and 34 µg/mL of chloramphenicol until the OD_{600} reached 0.4-0.6. Then the protein expression was induced with the addition of 1 mM isopropyl-β-D-galactopyranoside (IPTG) and the cells were allowed to grow further for 4-6 h at 30°C. The induced *E. coli* cells were harvested at 1,100xg for 20 min and the lysate was used to confirm the expression through SDS-PAGE using standard procedures (Green & Sambrook, 2012).

The recombinant Histidine-tagged cIFN-γ protein was purified using Ni-NTA affinity chromatography (Invitrogen) under denatured conditions, following the instructions by the manufacturer. Following cell lysis, the pellet was resuspended in buffer containing 6 M urea (pH 8.0) and loaded onto a Ni-NTA agarose column. The bound protein was subjected to three washes with imidazole and then eluted using the elution buffer.

Characterization of recombinant cIFN-γ

The expressed recombinant protein was analyzed in SDS-PAGE along with the induced and non-induced bacterial cell lysates, and finally confirmed by western blot.

SDS-PAGE: A 14% resolving and 5% stacking gel was prepared and allowed to polymerize at room temperature. Harvested cell pellets were treated with SDS loading buffer containing β-mercaptoethanol and boiled at 100°C for 5 minutes. Samples and a pre-stained M.W. marker were loaded onto the gel and run at 110 V until the tracking dye reached the bottom. The gel was visualised by staining with Coomassie Brilliant Blue R-250 for 30 minutes and destained overnight on a slow shaker with periodic replacement of the destaining solution to visualize protein bands.

Western blotting: This technique was performed by transferring proteins from SDS-PAGE gels onto PVDF membranes (4.5 × 5 cm) using chilled transfer buffer with freshly added methanol (10 mL per 40 mL). A transfer sandwich was assembled with filter paper, membrane, gel, and another filter paper, transferred for 1–1.5 h. The membrane was blocked in 5% skim milk powder with 0.05%BSA for 1 hour, washed with PBS, and incubated overnight at 4°C with anti-Histidine primary antibody (1:1000 in PBS). After washing with PBST and PBS, HRP-conjugated anti-mouse secondary antibody (1:30,000 in blocking buffer) was added for 1 hour, followed by final washes and detection was done by using the chemiluminescent substrate. The recombinant protein was expressed at 34 kDa.

RESULTS

Sequence analysis showed that the full-length cIFN-γ gene encodes a putative polypeptide of 166 amino acids (aa), with the N-terminal 22 aa predicted to form a hydrophobic signal peptide. Upon removal of this signal sequence, the mature form comprised a 144-aa polypeptide.

In order to know the 3D structure of cIFN-γ, the sequence was analyzed with the AlphaFold server. The analysis showed that the model consisted of 166 amino acids and was predominantly α-helical. The predicted three-dimensional structure of cIFN-γ, obtained from the AlphaFold Protein Structure Database, was then assessed based on per-residue confidence scores (pLDDT). The model exhibited a high overall confidence, with a majority of residues falling into the "very high confidence" category (pLDDT>90), indicated by dark blue colouring in the structure visualization (Fig. 1). Several regions displayed "confident" predictions (pLDDT between 70 and 90), shown in light blue, suggesting reliable structural modelling in those areas. A smaller number of residues were assigned "low confidence" (pLDDT between 50 and 70), represented in yellow, typically corresponding to loop or flexible regions. Very few residues were classified as "very low confidence" (pLDDT<50), shown in orange/red, often located at the termini or disordered segments. This distribution of confidence scores supports the structural integrity of the model, particularly within the core functional domains of the protein (Fig. 1A). These confidence scores reflect the reliability of the predicted 3D structure at the individual residue level. In the context of B-cell epitope or antigenic region mapping, structurally high-confidence areas offer more precise spatial resolution, thereby enhancing the accuracy of epitope identification (Jumper et al., 2021).

For expression studies, it is essential to identify and remove the hydrophobic signal sequence present in caprine interferon gamma (IFN-γ). The cIFN-γ protein consists of a signal peptide spanning residues 1 to 23, which is predicted based on sequence similarity and is likely cleaved during maturation (Fig. 1B). The mature protein chain begins at residue 24 and continues through residue 166, corresponding to the active form of IFN-γ. The N-terminal residue (position 24) undergoes a post-translational modification to form pyrrolidone carboxylic acid, a modification inferred by similarity to other species. Additionally, two N-linked glycosylation sites have been observed at asparagine residues 39 and 106 through sequence analysis, suggesting the presence of GlcNAc-type glycan attachments that may influence protein folding, stability, or immunogenicity.

B-cell epitope prediction

To locate the antigenic regions within the cIFN-γ, the full-length amino acid sequence was subjected to sequential B-cell epitope prediction using two independent bioinformatic tools. Using the ABCpred server with a stringent threshold score of 0.89, a total of 16 non-overlapping linear epitopes were presented (Table 2). In parallel, the BepiPred-2.0 server was employed with a default threshold value of 0.5, resulting in the identification of 7 sequential B-cell epitopes (Table 3). Further, the antigenicity of the cIFN-γ protein sequence was assessed using the method of Kolaskar and Tongaonkar available through the IEDB

Table 2. B-cell epitope predicted via ABCpred server is presented along with its position and antigenicity scores

Rank	Sequence	Start position	Score
1	FKEIETLKEYFNASNP	28	0.89
2	SGSYGQGPFFKEIETL	19	0.87
3	SMDIIKQDMFQKFLNG	92	0.86
4	RKAINELIKVMNDLSP	130	0.84
5	EILKNWKEESDKKIIQ	54	0.83
6	PDVAKGGPLFSEILKN	43	0.81
7	KVMNDLSPKSNLRKRK	138	0.80
8	NGSSEKLEDFKKLIQI	106	0.77
9	KKLIQIPVDDLQIQRK	116	0.76
10	RKRKRSQNLFRGRRAS	150	0.75
11	NQVIQRSMDIIKQDMF	86	0.73
12	KEYFNASNPDVAKGGP	35	0.72
13	KKIIQSQIVSFYFKLF	65	0.66
14	QIVSFYFKLFENLKDN	71	0.65
15	YTSSFLALLLSVLLGF	3	0.60
16	SVLLGFSGSYGQGPFF	13	0.59

Table 3. B-cell epitopes predicted via the BepiPred server are presented along with their position

No.	Start	End	Peptide	Length
1	25	29	GPFFK	5
2	32	32	E	1
3	36	50	EYFNASNPDVAKGGP	15
4	58	66	NWKEESDKK	9
5	84	91	KDNQVIQR	8
6	101	128	FQKFLNGSSEKLEDFK KLIQIPVDDLQI	28
7	144	163	SPKSNLRKRKRSQNL FRGRR	20

Analysis Resource. These tools identified four antigenic peptides in the sequence (Table 4).

In addition to the analyses above, a total of 5 B-cell epitopes, which were linear, were predicted through the Ellipro2.0 server, which showed a high threshold score of 0.839 (Fig. 2B and Table 5). Moreover, eight conformational (discontinuous) B-cell epitopes were also identified using the ElliPro tool (Table 6), showing a high threshold score of 0.988 and 0.948 (Fig. 2A). Analysis revealed that antigenic regions are located on the entire cIFN-γ. Hence, it was decided that the complete cIFN-γ gene to be expressed encompassing all the antigenic regions.

Table 4. Antigenicity Results by Kolaskar & Tongaonkar using the IEDB server

Predicted peptides				
No.	**Start**	**End**	**Peptide**	**Length**
1	5	19	SSFLALLLSVLLGFS	15
2	68	80	IQSQIVSFYFKLF	13
3	117	126	KLIQIPVDDL	10
4	134	140	NELIKVM	7

Table 5. Linear B-cell epitopes predicted through the Ellipro2.0 server

No.	chain	start	end	peptide	No. of residues	score
1	A	148	166	NLRKRKRSQNLFRGRRASM	19	0.839
2	A	1	23	MKYTSSFLALLLSVLLGFSGSYG	23	0.776
3	A	121	137	IPVDDLQIQRKAINELI	17	0.699
4	A	35	51	KEYFNASNPDVAKGGPL	17	0.663
5	A	83	89	LKDNQVI	7	0.539

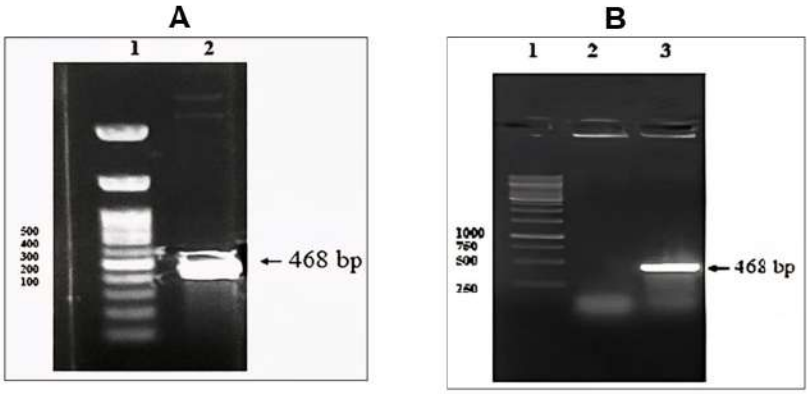

Fig. 3. PCR amplification and colony screening of recombinant caprine IFN-γ (cIFN-γ)

(A) PCR amplified cIFN-ã excluding signal sequence: Lane 1, 100 bp DNA marker; Lane 2, amplified cIFN-ã product at 468 bp. (B) Screening of BL21 colonies: Lane 1, DNA ladder; Lane 2, plain pET32a vector; Lane 3, recombinant pET32a+cIFN-γ showing 468 bp insert.

Expression of caprine interferon gamma

The synthetic pUC57-cIFN-γ clone was transformed into the top-10 alpha competent cells. The mature peptide sequence was amplified from the original sequence by removing the signal peptide, i.e., the first 22 amino acids, by using the primers at 468bp (Fig. 3A). A 468 bp amplicon containing the desired insert with restriction sites and a histidine tag was successfully amplified. The insert was cloned into the pGEMT-Easy vector and confirmed through blue/white screening and restriction digestion. Subsequent subcloning into the pET-32a vector using *BamHI* and *Nco*I sites was achieved, with the correct orientation confirmed by colony PCR for the

Table 6. Discontinuous B-cell epitopes predicted through the Ellipro2.0 server

No.	Residues	Number of residues	Score
1	A:A164, A:S165, A:M166	3	0.988
2	A:R160, A:G161, A:R162	3	0.948
3	A:R154, A:S155, A:Q156, A:N157, A:L158, A:F159	6	0.884
4	A:T4, A:S5, A:S6, A:F7, A:L8, A:A9, A:L10, A:L11, A:L12, A:S13, A:V14, A:L15, A:L16, A:G17, A:F18, A:S19, A:G20, A:S21, A:Y22	19	0.772
5	A:I121, A:P122, A:V123, A:D124, A:D125, A:L126, A:Q127, A:I128, A:Q129, A:R130, A:K131	11	0.732
6	A:K35, A:N39, A:A40, A:S41, A:N42, A:P43, A:D44, A:V45, A:A46, A:K47, A:G48, A:G49, A:P50, A:L51, A:E54, A:K57, A:N58, A:W59	18	0.648
7	A:A132, A:I133, A:N134, A:E135, A:L136, A:I137	6	0.64
8	A:L83, A:N86, A:Q87, A:V88, A:I89	5	0.52

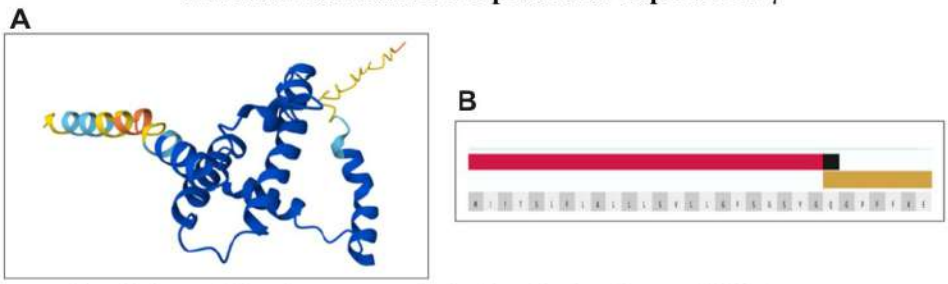

Fig. 1. Structural prediction and signal sequence analysis of caprine interferon-γ (cIFN-γ)
(A) Predicted 3D structure of cIFN-γ generated by AlphaFold. The model is colored based on per-residue confidence scores (pLDDT): very high confidence (pLDDT>90, dark blue), confident (70<pLDDT=90, light blue), low confidence (50<pLDDT=70, yellow), and very low confidence (pLDDT=50, orange/red). Regions with lower confidence are typically unstructured. (B) Predicted signal peptide and N-terminal modification of cIFN-γ. The signal sequence (residues 123, red) is cleaved during secretion, and the 24th residue (glutamine) is predicted to undergo N-terminal modification to pyrrolidone carboxylic acid.

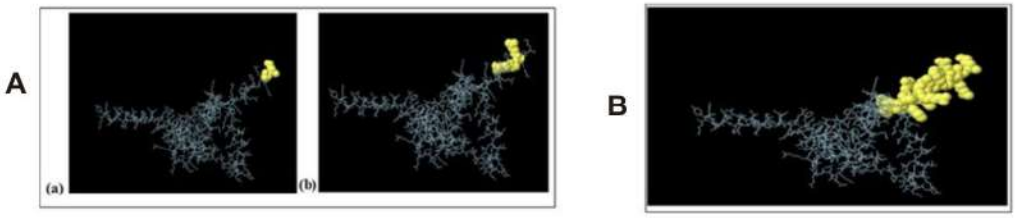

Fig. 2. Predicted 3D structures of B-cell epitopes on caprine IFN-γ generated by ElliPro
(A) Discontinuous epitopes with high threshold scores: (a) A:A164, A:S165, A:M166 (score 0.988) and (b) A:R160, A:G161, A:R162 (score 0.948). (B) Linear epitope with high threshold score (0.839).

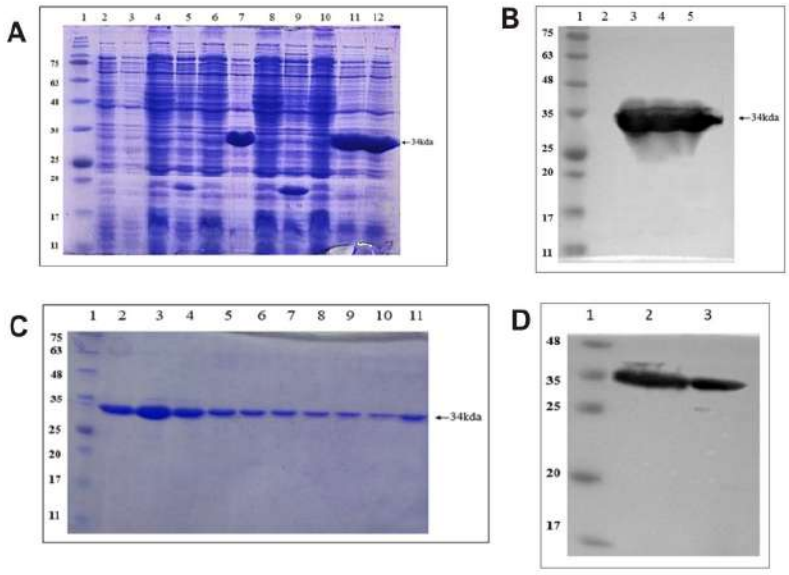

Fig. 4. Expression and purification of recombinant caprine IFN-γ (cIFN-γ)
(A) SDS-PAGE profile of BL21 cells at different induction time points: Lane 1, protein marker; Lane 2, 0 h control (plain plasmid); Lane 3, 0 h test (plasmid+cIFN-γ); Lane 4, 3 h control uninduced; Lane 5, 3 h control induced; Lane 6, 3 h test uninduced; Lane 7, 3 h induced showing ~34 kDa band; Lane 8, 6 h control uninduced; Lane 9, 6 h control induced; Lane 10, 6 h test uninduced; Lane 11, 6 h test induced showing ~34 kDa band; Lane 12, 9 h test induced showing ~34 kDa band. (B) Western blot detection of expressed cIFN-γ (~34 kDa): Lane 3, 3 h test induced; Lane 4, 6 h test induced; Lane 5, 9 h test induced. (C) SDS-PAGE of purified recombinant protein (~34 kDa): Lane 1, protein marker; Lanes 211, eluted fractions of purified protein. (D) Western blot of purified protein: Lane 1, marker; Lanes 23, purified cIFN-γ (~34 kDa).

plasmid with insert and the plain plasmid that which was used as a control (Fig. 3B). Optimal expression was observed upon induction with 1 mM IPTG at 30°C for 3–6 h. SDS-PAGE analysis showed a clear band at ~34 kDa in induced samples, indicating successful expression of the protein (Fig. 4A) and confirmed in Western blot (Fig. 4B). The recombinant protein was purified under denaturing conditions and appeared as a distinct band on SDS-PAGE and Western blot, with no detectable contamination. Purification was performed using 6 M urea with 40 mM imidazole in the wash buffer, and elution was achieved with 300 mM imidazole, confirming the recovery of cIFN-γ along with the fusion tag (Fig. 4C & 4D).

DISCUSSION

The diagnostic accuracy of the Bovigam IGRA kit in diagnosing tuberculosis of small ruminants is significantly lower than its reported performance in large ruminants. This reduced sensitivity indicates that diagnostic assays originally developed for cattle are not showing reliable results when applied to sheep and goats. These findings show the need for developing and validating tuberculosis diagnostic methods specifically for small ruminant species (Roy et al., 2020). In the present quest to develop a caprine IGRA kit, immuno-informatic analysis of caprine IFN-γ was performed to identify the immunodominant epitopes, followed by expression of the protein.

Initially, the bioinformatic analysis of cIFN-γ was performed. The 3D structure of cIFN-ã was predicted using the AlphaFold program. AlphaFold predicts highly accurate protein structures based on amino acid sequences, providing insights into how proteins fold, interact with other molecules, and carry out their functions (Jumper et al., 2021). Analysis of cIFN-γ indicated that over 90% of residues showed pLDDT scores>90, indicating high confidence. Core immune-functional regions were especially well resolved, supporting accurate epitope mapping. Similar high-confidence predictions have been reported for SARS-CoV-2 spike RBD and influenza HA stem, aiding vaccine and diagnostic design (Zeng et al., 2022; Kilim et al., 2023).

Various bioinformatic tools, such as ABCpred, BepiPred-2.0, Kolaskar-Tongaonkar method and Elliprotools were used to locate the B-cell epitopes in cIFN-γ gene. Analysis using ABCpred and Bepi Pred-2.0 tools could detect 16 and 7 linear epitopes, respectively, while the Kolaskar–Tongaonkar method found 4 more based on amino acid properties. ElliPro predicted 5 linear and 8 discontinuous conformational epitopes using the 3D structure from AlphaFold. These tools together helped to identify important surface regions that antibodies can recognize. Similar strategies have been employed in SARS-CoV-2 studies to map spike and nucleocapsid epitopes for diagnostic purposes (Ponomarenko et al., 2008; Chen et al., 2020; Lon et al., 2020; Dashti et al., 2024). Since antigenic regions were distributed across the entire caprine IFN-γ protein, expressing only partial segments could miss key epitopes. Therefore, full-length cIFN-γ was selected for expression.

The cIFN-γ encodes a putative 166 amino acids (aa) polypeptide with a predicted molecular weight (MW) of 19.12 kDa. The first 22 aa of the N-terminal compose a hydrophobic signal sequence, which, when cleaved, results in a mature 144-aa polypeptide with a predicted MW of 17.197 kDa. The mature polypeptide was amplified by using the specific primers designed using the published caprine sequence. This approach of excluding the signal sequence was followed for expression of bovine, caprine and ovine IFN-γ (Beyer et al., 1998; Gupta et al., 2006; Rasool et al., 2006).

In this study, due to the small size of the target protein, the expression was carried out in a vector that expresses the target protein along with the fusion tag to increase the yield of expression of the protein. To express the caprine IFN-γ, the gammapET-32a vector was used. IFN-γ expression in BL-21 DE3 *E. coli* cells was achieved by optimising the induction parameters. Expressed cIFN-γ was verified by SDS-PAGE and western blot using the anti-histidine antibody. The recombinant protein was purified using Ni-NTA column affinity chromatography under denaturing conditions. Together with the fusion tag, the purified protein was found to have a band at the expected size of 34 kDa. Similarly, in previous studies, caprine Interferon gamma was produced in the *E. coli* BL21 cells containing recombinant plasmid pET32a, yielding around 34 kDa (Ma et al., 2019). Similarly, the bovine, ovine and caprine IFN γ genes were expressed using different vectors such as pRSET, pET32b and pET 19b (Beyer et al., 1998; Gupta et al., 2006; Rasoo et al., 2006).

In previous works, IFN-γ was expressed in plasmid vectors in combination with the fusion tags in order to increase the expression and protein yield. The bovine IFN-γ was expressed in the pRSET vector, yielding a 23 kDa protein, though the expected size of the monomer protein was around 16 kDa. This increase in the M.W. of the protein was due to the N-terminal fusion of the target protein with the polyhistidine tag

(Ceretti et al., 1986; Gupta et al., 2006). Similarly, ovine IFN-γ was also expressed in pET32b, yielding a protein of around the M.W. of 34 kDa as a fusion protein with the polyhistidine and thiredoxin fusion tags (Rasool et al., 2006).

In conclusion, in the present study, immuno-informatic analysis of cIFN-γ was performed using various bioinformatic tools followed by expression of the cIFN-γ in a prokaryotic expression system. This recombinant cIFN-γ can be used for developing a monoclonal antibody as a preliminary step in the development of the IGRA assay for detection of caprine TB. In earlier studies, to detect the Orf virus from histopathological samples, an immunofluorescence assay was developed using cIFN-γ mAbs (Ma et al., 2019). Further, this recombinant caprine IFN-γ can also be used for various diagnostic purposes, therapeutics and as a vaccine adjuvant.

Conflict of interest: The present research work has no conflict of interest.

Author's contribution: KM, SG: Performed the experiments, wrote the manuscript; VN, PD: Designed the experiments, wrote the manuscript; BV, PC: Improved the manuscript.

Data availability statement: All data generated or analyzed during this study are included in this article. Additional data will be available from the corresponding author on reasonable request.

Use of artificial intelligence tools: During the preparation of this work, the author(s) used ChatGPT to paraphrase the sentences in correct grammatical format. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

ACKNOWLEDGEMENTS

The Authors are thankful to the Director and the Joint Director, ICAR-Indian Veterinary Research Institute (IVRI), Indian Council of Agricultural Research (ICAR), New Delhi, for providing the facilities to conduct the research. The funding support from the Department of Animal Husbandry and Dairying, Govt. of India, in the form of the National Livestock Mission (NLM) project is highly acknowledged.

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Received - 17.09.2025, Accepted - 19.11.2025, Published - 26.11.2025 (Online), 01.12.2025 (Print)
Section Editor: Dr. Neeraj Shrivastava, Editorial Board Member