

RESEARCH ARTICLE

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Production of Antigenic Bovine Interferon- γ Homologue and Antiserum by Improved Codon Usage Bias in *E. coli*: A Combined *In Silico* and *In Vitro* Approach

A. Cobián-García, Angel Vazquez , R. Hernández-Gutiérrez ,
A. Gutiérrez-Ortega , M. Martínez-Velázquez , TA. Camacho-Villegas ,
H. Espinosa-Andrews , J. Bravo-Madrigal  and AH. Alvarez* 

Center of Research and Assistance in Technology and Design of the State of Jalisco,
A. C. Normalistas No. 800., 44270, Guadalajara, Jal., Mexico.

Abstract

Bovine interferon-gamma (b-IFN- γ) is a key cytokine biomarker, the activity of which increases during chronic animal infection with intracellular pathogens. Disease diagnosis can be performed based on detection of the b-IFN- γ blood levels in infected animals via specific antibodies. The production of specific reagents for b-IFN- γ identification involves the gene cloning of native templates followed by protein expression, and often produces misfolded proteins due to improper translation by heterologous cell platforms. In this study, a computational approach combined with gene engineering expression technology was used for the bacterially production of a recombinant b-IFN- γ yielding 2 μ g/ml soluble protein. The rb-IFN- γ was recognized by Western blot with specific monoclonal antibodies and bovine IFN- γ -capture ELISA. The recombinant cytokine demonstrated good *in vivo* immunogenic properties, raising mouse polyclonal antibodies titers of above 51.2K, and allowing recognition of blood plasma b-IFN- γ . The synthesized bovine cytokine is expected to aid in developing b-IFN- γ specific immunoassays for diagnosing bovine infectious diseases.

Keywords: Gene Optimization, Bioinformatics, *Escherichia coli*, Soluble Bovine IFN- γ , Mouse Antiserum, Immunodetection

*Correspondence: aalvarez@ciatej.mx

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INTRODUCTION

Interferon-gamma (IFN- γ) is one of the most structurally conserved cytokines in the immune system of vertebrates; thus, the interest in producing and measuring IFN- γ relies on its therapeutic and diagnostic properties as a biomarker of inflammatory responses during pathogen exposure or for effective vaccination assessment.¹⁻⁵ The *in vitro* diagnosis of bovine infectious diseases, such as tuberculosis, brucellosis, paratuberculosis, mastitis, and coxiellosis, relies on the specific detection of cellular adaptative immune responses, including IFN- γ production, which follow antigenic stimulus in the blood of peripheral blood mononuclear cells⁶⁻¹¹; thus, cytokine standards and specific antibodies are required for devices, such as ELISA assays, bead-arrays, and biosensors.¹²⁻¹⁶ However, the production of specific antibodies is laborious and may require cross-species sources.¹⁷⁻²⁰ The production of cell mediators via different expression systems from bacteria and yeast to insect and mammal cells with different yields has thus been encouraged in the effort to produce IFN- γ and other chemokines from different species due to the therapeutic and prophylactic properties of these molecules.²¹⁻²⁵ Interferons are typically obtained via biotechnological platforms and sophisticated expression systems, such as gene engineering or exogenous gene modification technology.²⁶⁻³² The b-IFN- γ gene was first cloned from a cDNA library using RNA isolated from concanavalin A-stimulated bovine lymph node cells.³³ Obtaining native gene sequences for bacterial cloning is complicated and includes cellular obtention from the spleen or PBMC, gene expression stimulation, and RNA isolation.³⁴⁻³⁶ Thus, early attempts at manufacturing the cytokine cells for IFN- γ gene cloning relied upon the use of native DNA templates from animal cells, which was often fruitless in terms of yield due to mismatches of rare codons in the heterologous biological mRNA translation system. The heterologous production and purification processes that are used to obtain eukaryotic recombinant proteins are tedious, rendering such products costly on both small-scale and automated production.^{37,38} Although some researchers have expressed recombinant b-IFN- γ in baculovirus, yeast, and even *E. coli*, disadvantages

such as the formation of non-soluble recombinant proteins within the inclusion bodies, and the acquisition of heterologous proteins renders the process biotechnologically challenging.^{39,40} With the advent of gene engineering and cloning techniques, several biologically important proteins with high efficiency and solubility for downstream application have been expressed in *E. coli*.⁴¹⁻⁴³ An efficient and preferred strategy to increase protein production is commercial gene synthesis with computationally designed artificial codon-optimized templates for heterologous expression.⁴⁴⁻⁴⁶ Protein synthesis and folding can be altered by synonymous codons without substituting original amino acids.^{47,48} We therefore aimed to produce a recombinant bovine IFN- γ (rb-IFN- γ) as a biologic with immunogenic potential and an optimized synthetic gene by codon bias adjustment for the high-level production of a soluble rb-IFN- γ molecule in *E. coli*. The accomplished molecule was immunogenic in mice, suggesting the production of polyspecific antibodies with diagnostic properties.

MATERIALS AND METHODS

Reagents, antibodies, plasmids, and bacterial strains

Restriction enzymes *Nco*I and *Xho*I were provided by NEB (New England Biolabs). Anti-His6x tag monoclonal antibody was provided by R&D Systems. Rat anti-b-IFN- γ monoclonal antibody was obtained from R&D Systems Mab, and recombinant bovine IFN- γ (*E. coli* derived) from R&D Systems. Plasmid pET28a(+) was purchased from Novagen/Sigma-Aldrich (USA). One-Shot TOP10 and BL21 (DE3) *E. coli* strains were obtained from Invitrogen (USA). Plasmid pUC57 with the rb-IFN- γ gene optimized for codon usage translation in *E. coli* was manufactured by GenScript (Piscataway, NJ) via the OptimumGene Codon-Optimization platform (<https://www.genscript.com/codon-opt.html?src=cft>), with the complete amino acid sequence retrieved from the NCBI Protein Bank Database Accession No. NP_776511.1 used to obtain the analog gene template for the efficient *E. coli* translation of heterologous protein, including the initial signal peptide (amino acids 1-23), to achieve an extended mouse polyspecific IgG humoral response, as the

initial protein sequence has an antigenic epitope, as a robust immunoreactive biologic for diagnostic purposes. A commercial *in vitro* *Mycobacterium bovis* Gamma IFN- γ -microplate ELISA Test kit (BOVIGAM™, Prionics AG, USA), which is based on bovine tuberculin purified protein derivative (PPDB) and a monoclonal anti-b-IFN- γ as biologics for tuberculous infection diagnosis in cattle, was used for specific cytokine identification. The Bio-Scale Mini Profinity Ni-charged IMAC cartridge for immobilized metal affinity chromatography was from Bio-Rad Laboratories.

***In silico* analysis of amino acid sequence and antigenicity prediction**

Protein sequence in FASTA format was subjected to: (1) secondary and tertiary structure confirmation using the I-TASSER (<https://zhanglab.ccmb.med.umich.edu/I-TASSER>) and parallel comparison of the 3-D model via the protein structure AI machine learning system AlphaFold (<https://alphafold.ebi.ac.uk/>), (2) solubility prediction by Protein-sol (<https://protein-sol.manchester.ac.uk/>), and (3) disulfide bonding and antigenicity predictions by Scratch Protein Predictor (<https://scratch.proteomics.ics.uci.edu/>). Antigenic peptides were identified using the Kolaskar and Tongaonkar's approach (<http://imed.med.ucm.es/Tools/antigenic.pl>). Predictions reflect the occurrence of epitopes that have most probable residues that may elicit an antibody response.

Construction and transformation of recombinant expression plasmid

The purchased plasmid pUC57 (with synthetic rb-IFN- γ gene) was recovered together with the expression vector pET28a(+) from independent *E. coli* transformant strains. Plasmids were digested individually with the *Nco*I and *Xho*I restriction enzymes, and purified from the agarose gel using the MinElute Gel Extraction Kit (QIAGEN) to subclone the gene insert in pET28a(+) with a 5:1 DNA ratio after quantification with a NanoDrop ND-1000 spectrophotometer. Enzyme ligation was performed using T4 DNA ligase at 16 °C for 18 hrs and transformed into *E. coli* TOP10 for plasmid recovery, after which transformants were obtained

on LB agar with selective kanamycin. Plasmid was recovered and confirmed by restriction pattern analysis before final introduction by chemical transformation into the *E. coli* BL21 (DE3) cells in LB-kanamycin 50 μ g/ml agar via clone confirmation.

Culture conditions and protein expression confirmation

Protein induction was performed using a selected BL21 (DE3) clone using standard cell culture conditions described previously.⁶ Briefly, bacteria were cultured by shake flask incubation in 250 ml of LB medium at 37 °C to an OD₆₀₀ of 0.7. Isopropyl- β -D-thiogalactopyranoside (IPTG, 1 mM) was then added to induce expression of the recombinant protein over 4 hrs. Cells were pelleted, and the supernatant medium was discarded. Cells were then lysed by ultrasonication in PBS and 0.1% Triton X-100 with a protease inhibitor cocktail. Protein supernatant was obtained by centrifugation, and rb-IFN- γ was confirmed by SDS-PAGE according to Laemmli using a 12% polyacrylamide gel with 5 μ g of total protein per lane, Coomassie staining, and Western blot on nitrocellulose sheets using monoclonal mouse anti-His6x and rat anti-b-IFN- γ as primary antibodies.

Recombinant protein purification

Recombinant b-IFN- γ was recovered from the supernatant by His-tag metal affinity chromatography using standard methodology as described previously.⁶ Bound proteins were then eluted with elution buffer (300 mM KCl, 50 mM KH₂PO₄, 250 mM imidazole, pH = 8.0) before subjecting fractions to dialysis by membrane tubing (25 mM HEPES, 10% glycerol, 0.15 M NaCl, pH = 7.5) and SDS-PAGE using 12% polyacrylamide gel with Coomassie staining and Western blot as described above.

Thermal denaturation studies

Differential scanning calorimetry (DSC) measurements of the structure were assessed using a DSC2000 TA Instrument. Briefly, 5 mg of freeze-dried recombinant protein was placed in a platinum hermetic pan and heated from 20-50 °C at 10 °C/min in a nitrogen atmosphere.

Immunological confirmation, immunogenicity, and polyvalent serum

In vitro immunologic identity of rb-IFN- γ was confirmed in dialyzed recombinant protein dilutions using the specific *in vitro* ELISA Test kit BOVIGAM™ in accordance with its technical protocol. Briefly, the initial recombinant protein solution was subjected to serial dilution at 1:100 to 1:6400 before analysis using ELISA anti-b-IFN- γ . Analysis was performed in triplicate, and OD was contrasted to a standard b-IFN- γ curve with a top concentration of 2 μ g/ml. For *in vivo* testing of immunogenicity and polyclonal antibody production, four 8 week-old female Balb/c mice (ENVIGO, Harlan Laboratories) were housed and treated following the ARRIVE guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>) from the EU Directive 2010/63/EU for animal experiments. The study was approved by the Internal Committee for Care and Use of Laboratory Animals (license number: CICUAL-2018009B). Mice were immunized subcutaneously using 50 μ g of recombinant rb-IFN- γ emulsified with Freund's Incomplete Adjuvant (FIA) on a weekly basis, with three intramuscular boosts of Freund's Complete Adjuvant (FCA) to a final volume of 200 μ l in a 1:1 ratio on days 7, 14, and 21. Mouse tail veins were bled one week after each immunization, and serum aliquots were pooled as a whole polyvalent antiserum in the fifth week. Recognition of mouse antibodies to rb-IFN- γ was then performed by serial dilution of pooled mouse antiserum ranging from 1:200 to 1:51200 on 2 mm thick nitrocellulose foil strips (electroblotted with rb-IFN- γ from a preparative SDS-PAGE gel) by Western blot to form the first antibody. A goat anti-mouse IgG-HRP was used as the secondary antibody. For immuno-testing of mouse antiserum, Dot-Blot analysis was performed on a nitrocellulose sheet with 1 μ g/ml of rb-IFN- γ and commercial b-IFN- γ (*E. coli* generated) at a 1:100 antiserum dilution as primary antibody, while 5 μ l of a pooled bovine plasma from 10 whole-blood samples (pre and post-stimuli with the PPDB-Tuberculin reagent), which have a strong positive BOVIGAM™ test result (with an intense optical density), was analyzed by Western blot in parallel with 1 μ g/ml of commercial rb-IFN- γ .

Protein yield

The concentration of total protein of the solubilized rb-IFN- γ was detected colorimetrically with two standard curves, bovine serum albumin, and specific bovine IFN- γ , with Bradford assay and the anti-b-IFN- γ ELISA kit BOVIGAM™, respectively. GraphPad Prism 10.2.0 software was used for graphical analysis.

RESULTS AND DISCUSSION

In silico screening of protein structure, solubility, and antigenicity predictions

Uniprot ID: P07353 was used to elucidate the reposit protein sequence of b-IFN- γ with the NCBI Protein ID NP_776511.1. Secondary structure analysis using the I-TASSER platform showed a predominant α -helix core and good solvent accessibility (Figure 1A), with the predicted scaled solubility of 0.688 obtained using Protein-sol better than that of other average soluble *E. coli* proteins. A Grand Average of Hydropathicity (GRAVY) index of -0.495 was obtained using the ProtParam in ExPASy tools, indicating the hydrophilic nature of the protein. The solubility of a protein can influence its heterologous overexpression, affecting protein folding, with misfolded proteins degraded as part of the normal protein turnover. However, protein 3-D structures, which are useful parameters to predict protein aggregation, are not always available.⁴⁹ Early attempts to predict the tertiary structure via an α -helix docking algorithm and crystallographic analysis suggested conserved α -helix folding of the interferons.^{50,51} The predicted 3-D structure with predominant helical motifs was obtained using I-TASSER and confirmed via the AlphaFold Protein Structure Database (<https://alphafold.ebi.ac.uk/entry/P07353>, Figures 1B and C), a novel AI system machine learning approach that incorporates physical and biological information about protein structures.⁵²

The structure has less than two cysteines and probably no disulfide bonding; thus, protein folding does not alter the binding interface with the Major Histocompatibility Complex (MHC) in immune cells.⁵³ According to the Scratch Protein Predictor, the average predicted probability of antigenicity of 0.582472 renders it a good antigen

for immunological assessment. Antigenic peptides should be located in solvent accessible regions and contain both hydrophobic and hydrophilic residues. Thus, any modification to the native protein sequence and structure for successful expression of an antigenic recombinant protein in *E. coli* was elucidated, showing five clear epitopes in the primary protein sequence that influence an antibody response (Figure 2A). However, the expression of a foreign protein in *E. coli* is influenced by a range of factors, including the host codon usage preference; therefore, we opted to optimize the DNA template of the native gene sequence to produce a precise template for heterologous translation, avoiding mismatches and punctual mutations of the final recombinant molecule (Table). In other similar research

investigating the expression of an analog molecule using *Pichia pastoris*, two codons were removed that code for two cysteines at position 64-69 bp, where they form a disulfide bridge between the structure of IFN- α , possibly leading to inclusion body formation.⁵⁴ Yin et al.⁵⁵ synthesized an optimized bovine IFN- α gene by obtaining a 36 kDa recombinant protein from inclusion bodies in *E. coli*. This protein has six cysteines, with two potential disulfide bonds that could lead to protein aggregation. One hypothesis is that aggregates may affect the immunogenicity of recombinant therapeutic proteins.⁵⁶ Therefore, when overexpressing foreign genes in *E. coli*, it is important to consider structural analysis before template optimization to ensure an efficient protein production bioprocess.

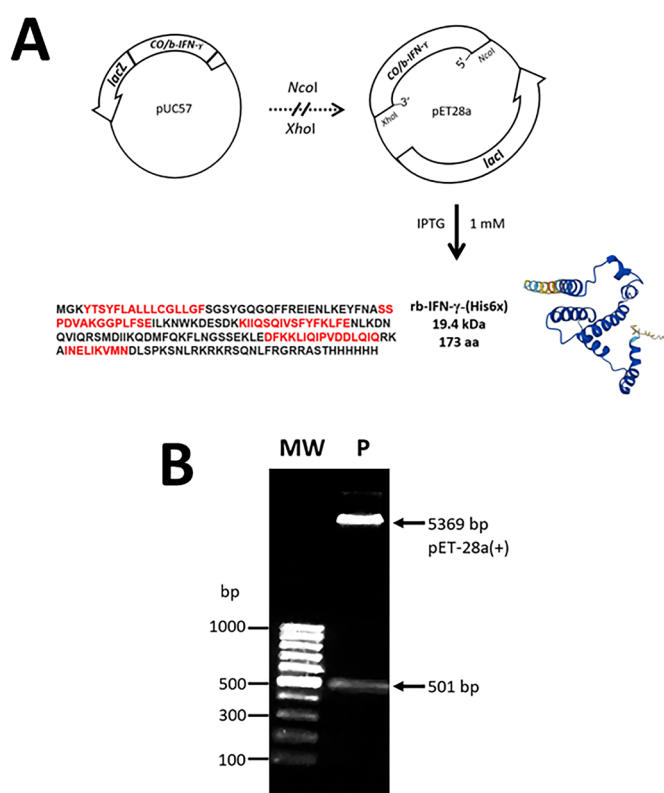


Figure 2. The subcloning strategy for efficient *E. coli* expression of codon-optimized (CO) b-IFN- γ gene for the obtention of recombinant protein after IPTG-induced culture. (A) Artificial CO/b-IFN- γ gene from pUC57 was subcloned into expression plasmid pET28a(+) by enzyme restriction with *NcoI* and *XhoI*. (B) Confirmation of final pET28a(+) with rb-IFN- γ gene from plasmid (P) of a single *E. coli* BL21 (DE3) clone was obtained by enzymic restriction. Protein 3D model and most probable epitopes (red) of linear amino acid sequence are shown. MW, 100 bp DNA ladder

Construction and expression of rb-IFN- γ in the bacterial system

The optimized gene sequence of b-IFN- γ was synthesized via a codon adjustment, obtaining an open reading frame of 501 bp in pUC57. The synthetic gene encodes the native and complete mammal protein (Table). The 5'-terminus included the *NcoI* restriction site in-frame with the initial methionine, and the stop codon was deleted by adding the *XhoI* restriction site at the 3'-ending. The plasmid pUC57 was first transformed into *E. coli* TOP10 strain for plasmid multiplication, with confirmation of the plasmid showing the 501 bp insert, which was subcloned into pET28a(+) using the *NcoI* and *XhoI* restriction sites before introduction into *E. coli* BL21 (DE3), as confirmed by sequencing and enzyme restriction analysis of a single clone (Figure 2B). The results showed that the insert had a correct 5'-3' transcriptional orientation corresponding to the 501 bp insert, giving to pET28a(+)-rb-IFN- γ plasmid.

After cell culture with IPTG addition and cell lysis, Coomassie staining and Western blot were used to search for the recombinant protein in cell fractions, with an expected and overproduced 19 kDa protein in the soluble fraction (Figure 3) after IPTG induction demonstrating successful

heterologous production of the recombinant cytokine molecule in *E. coli* cells identified by a robust protein band at 19 kDa and by using the His6x-tag monoclonal antibody. Although a faint and less abundant protein band was observed in the insoluble fraction (pellet, not shown) following culture lysate, it was considered merely part of unlysed bacteria due to the overexpressed bacterial product that resulted from inefficient lysis.

From the Western blot analysis (Figure 3B, bottom), it is evident that there are truncated fragments of rb-IFN- γ , probably by gene overexpression, however no effects were observed in terms of the protein obtention in the soluble fraction, which was thus used for downstream purposes, with protein purification performed on the soluble fraction via Ni-affinity chromatography.

The expressed rb-IFN- γ protein was efficiently obtained from *E. coli* BL21 (DE3) harboring the pET28a(+)-rb-IFN- γ plasmid with metal affinity His-tag cartridge and recovered with elution buffer containing 250 mM imidazole in almost 95% of pure protein in final elution fractions, and recognition of the produced protein was assessed by a rat anti-b-IFN- γ monoclonal antibody which confirmed its immunological

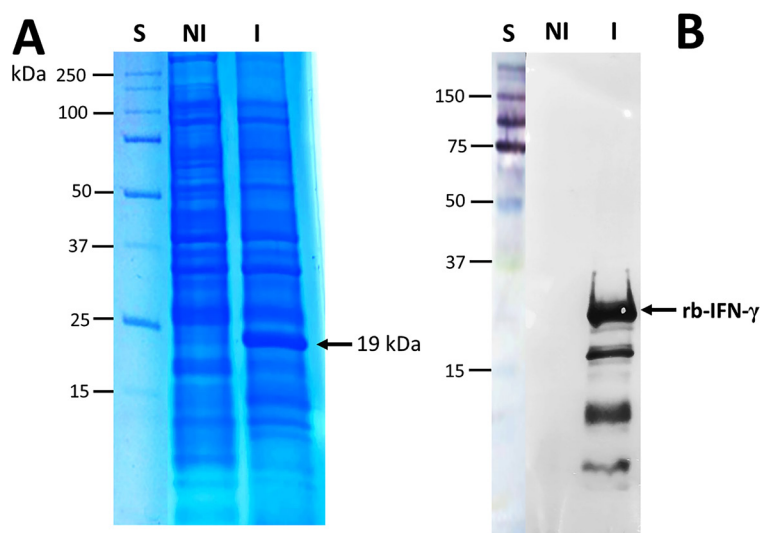


Figure 3. SDS-PAGE and Western blot analysis of soluble fractions following IPTG induction of *E. coli* culture. (A) Aliquots of supernatant of IPTG-induced (I) culture were compared with a cell lysate of non-induced (NI) culture and observed by Coomassie staining, and (B), Western blot with mouse anti-His6x tag monoclonal antibody. S, molecular size standards (kDa)

cytokine molecule identity in all the final aliquots of elution fractions from affinity chromatography evaluated (Figures 4A and B).

Characterization of rb-IFN- γ by DSC and ELISA

DSC has been used to characterize protein thermal stability, overall conformation, and domain folding integrity by the biopharmaceutical biomolecule industry. The thermal denaturation of proteins is an endothermic process that reflects heat absorption, with conformational changes occurring due to the breaking of intramolecular non-covalent and disulfide bonds.

Thus, in this study, DSC was used to search for the heat-denaturation molecular fingerprint of the recombinant protein, with a denaturing temperature (T_m) above 32 °C (Figure 5), which is similar to another recombinant analog IFN- γ molecule from human nature,⁵⁴ with a relatively low T_m , as has been reported somewhere else with other four-helical-bundle cytokines, with data variations depending on pH stability and the instrumental DSC scanning method.⁵⁷

Specific secondary and tertiary structures directly influence antigenicity through linear and conformational epitopes of the amino acid

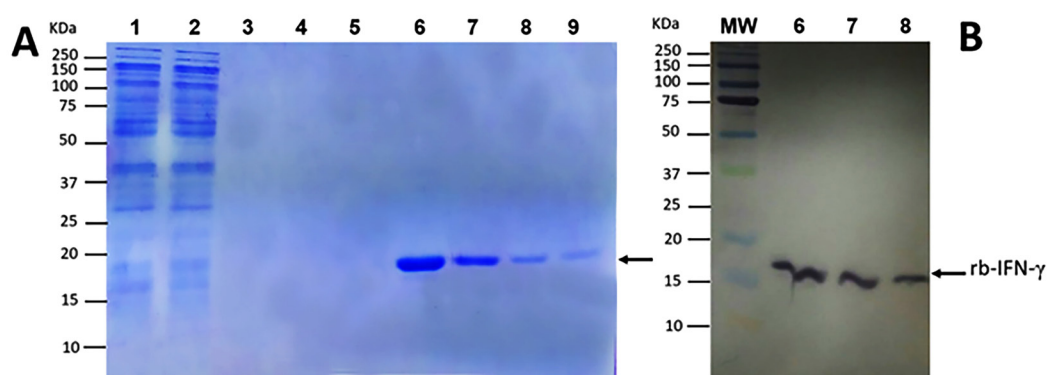


Figure 4. Purification of rb-IFN- γ by Ni-affinity chromatography using the soluble fraction of *E. coli* BL21 (DE3). (A) SDS-PAGE analysis of fractions, lanes: 1, eluate; 2-5, washes (50-70 mM imidazole); 6-9, elutions (250 mM imidazole). (B) Western blot with rat monoclonal anti-b-IFN- γ for three of the 250 imidazole elutions (lanes 6, 7, and 8). MW, molecular size standards (kDa)

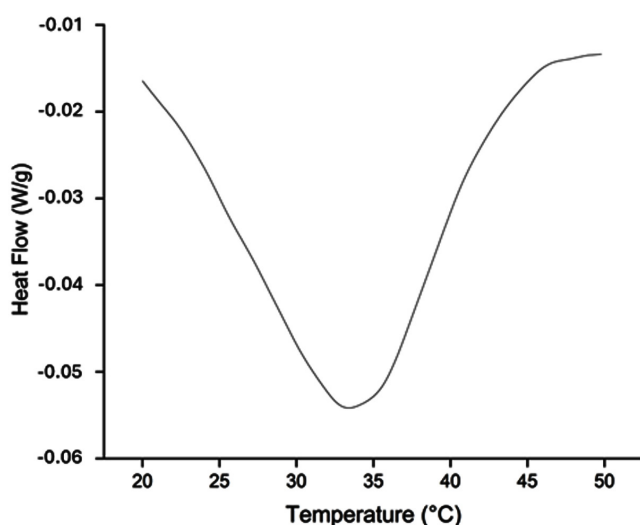


Figure 5. Thermal stability of the rb-IFN- γ . DSC scans were performed from 20-50 °C at a heating rate of 10 °C/min

backbone, with decreasing antigenic levels between 90 and 120 °C. On the other hand, enzyme-linked immunosorbent assay (ELISA) is a common method of measuring epitope binding and antigenicity of a protein source^{58,59}; thus, immunological identity of the purified recombinant protein was confirmed with a specific anti-b-IFN- γ ELISA kit BOVIGAM™. Additionally, immuno-enzymatic analysis of the recombinant protein solution demonstrated a total protein concentration of 2 $\mu\text{g}/\text{ml}$, which was confirmed colorimetrically by increasing dilutions of 1:100 to 1:6400 with the mentioned ELISA kit, as increasing OD reached the top concentration of 2 $\mu\text{g}/\text{ml}$ of the standard control curve in most dilutions (Figure 6A,B).

Another study by Xu et al.³² examined the expression of b-IFN- γ in supernatants obtained from Flp-In™ 293 cells (a stable mammalian expression cell line), with protein yields of 0.33 $\mu\text{g}/\text{ml}$ measured with the BOVIGAM™ specific kit. In another eukaryotic biotechnological platform by *P. pastoris*, expression of human IFN- γ from a gene optimization cassette obtained 200 $\mu\text{g}/\text{L}$ of recombinant product.⁶⁰ Our results confirm that bacterial biosynthesis of heterologous interferon-gamma is also a good choice for stability and reliability for b-IFN- γ obtention, thereby enabling subsequent immunological

investigations. The rb-IFN- γ from *E. coli* lacks the carbohydrate moieties attached to its structure as compared to its natural form. This absence of carbohydrate moieties may reduce the half-life of the cytokine in the bloodstream, affecting its pharmacokinetics, nor the antigenic properties. This considerable difference is important for choosing the heterologous expression system that remains under the downstream application purposes of the biological molecule.

Immunogenic assessment of rb-IFN- γ and mouse polyvalent antiserum

The *in vivo* immunogenicity of the recombinant cytokine was analyzed via the induction of high total IgG antibody levels in the serum of immunized mice following three consecutive shots of recombinant protein in combination with FCA. Although FCA is not recommended for use in laboratory animals because of associated side effects, researchers still prefer FCA to stimulate a higher humoral response; mouse anti-human IFN- γ polyclonal antiserum was achieved with 25-100 μg of recombinant cytokine from *E. coli* using FIA and FCA.⁶¹ In our work, 50 μg doses of the obtained rb-IFN- γ in FCA successfully triggered an active antibody response as shown by Western blot analysis on thick nitrocellulose foil strips, indicating that practically all the

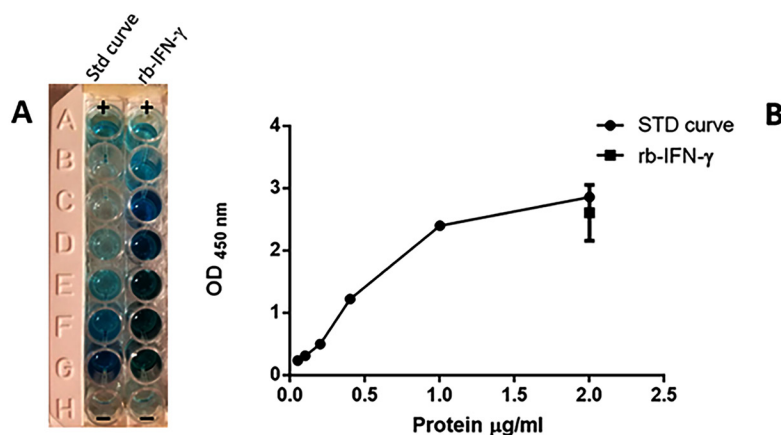


Figure 6. Quantitative immunological evaluation of rb-IFN- γ using a commercial ELISA kit with monoclonal anti-b-IFN- γ . (A) Wells B-G, decreasing dilutions of recombinant molecule (1:6400 to 1:100), and standard commercial b-IFN- γ curve (0.02-2 $\mu\text{g}/\text{ml}$), respectively. (B) Protein standard curve with ODs of protein dilutions. The mean SD of rb-IFN- γ dilutions is shown inside the STD curve. Duplicate positive and negative wells (A and H, respectively) were included as enzyme reaction controls

dilutions of the obtained pooled mouse antiserum maintained the capacity to recognize the bacterial biosynthetic protein. High antibody titers, ranging from 0.2-51.2 K, demonstrated the high IgG concentrations resulting from the immunogenic biosynthetic protein stimulus in mice (Figure 7A). However, the specificity of the mouse-generated antiserum was demonstrated by Dot-Blot, with positive recognition of b-IFN- γ by the mouse polyspecific anti-b-IFN- γ antibodies observed on the nitrocellulose sheet. Recombinant and commercial b-IFN- γ were also recognized (Figure 7B). Most importantly, the raised antibodies also reacted with a 19 kDa band in the plasma

of a pooled plasma from 10 whole blood bovine samples with previous positive results for *M. bovis* infection, and first diagnosed with the specific BOVIGAM™ anti-b-IFN- γ *in vitro* test. The plasma from blood samples were treated for b-IFN- γ induction using PPDB as whole blood cultures according diagnostic test manual, and did not cross-react with a pooled plasma from 10 blood samples without a previous PPDB stimulus (Figure 7C).

These results demonstrate the specific recognition of b-IFN- γ in the blood plasma of clinical samples, suggesting the potential for diagnostic purposes. It has been reported that

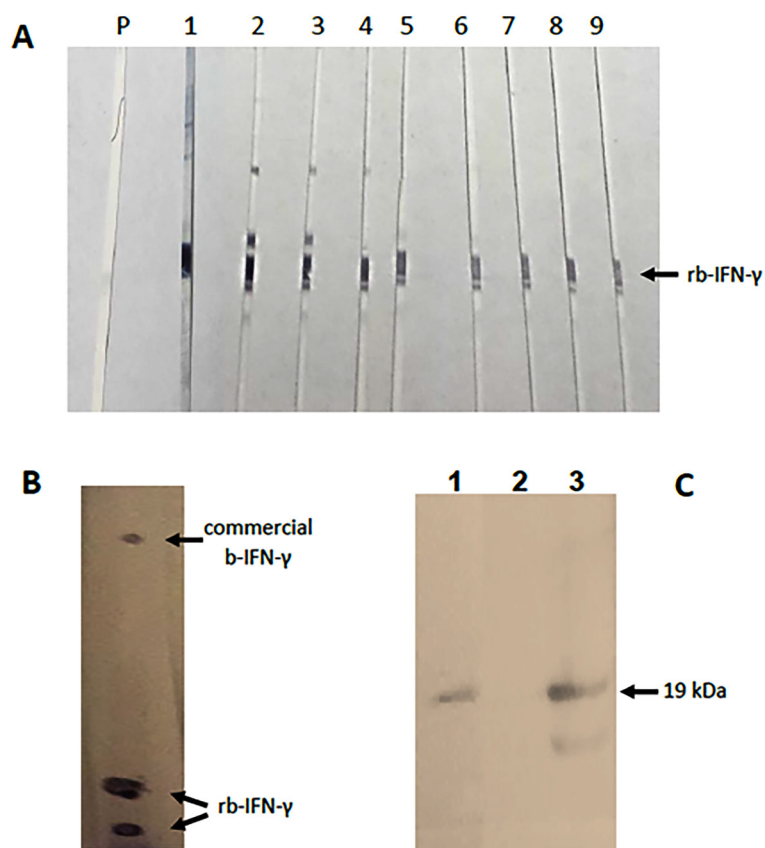


Figure 7. Immuno assessment of mouse anti-rb-IFN- γ antiserum. (A) Western blot of rb-IFN- γ electroblotted in 2 mm thick nitrocellulose foil strips with serial dilution of pooled mouse antiserum:PBS (starting with 1:200 and reaching 1:51,200), lanes: 1-9, respectively; P, pre-immune serum. (B) Dot-Blot analysis of rb-IFN- γ (duplicate) and a commercial b-IFN- γ (*E. coli* generated) with 1:100 mouse antiserum dilution as primary antibody. (C) Western blot with mouse antiserum; lanes: 1, commercial b-IFN- γ (19 kDa); 2, pooled bovine plasma from non-PPDB induced blood; and 3, pooled bovine plasma from PPDB-induced blood

produced monoclonal anti-b-IFN- γ antibodies have recognized recombinant b-IFN- γ overexpressed in the 293 cell line, though not in *E. coli*.³² However, chicken anti-IFN- γ polyclonal antibodies from egg yolk have been developed with a recombinant cytokine molecule and used as secondary antibodies in prototype capture ELISA formats.^{17,19} Strong neutralizing antibody responses have been elicited in mice from the achieved protein with *E. coli* codon optimization bias.⁶² These observations suggest that polyclonal antibodies may be a better choice to cover a wide spectrum of standard biologics for immunological purposes.

Polyspecific antibodies recognize multiple sites on an antigen and are considered optimal ligands for immunodiagnostics, as almost all protein antigens usually bind to IgG antibodies, with large-scale production lowering costs.⁶³ Besides its technical and economic virtues, the inbred mouse model used for immunogenic development has negligible overall genetic variability, implying high representativeness for the resulting immunity.⁶⁴ However, the synthesis of bioengineered molecules in *E. coli* as a heterologous expression host has been performed using various soluble immunological molecules, including fragments and full-length IgGs, without altering their functional conformation.⁶⁵⁻⁶⁷ Due to the lack of post-translational glycosylation and the corresponding influence on the circulatory half-life of the molecule, the bacterial cell factory has a clear disadvantage, as proposed by Gentilomi et al. and Jalali et al.,^{29,30} who improved biosynthesis of the molecule using eukaryotic cells. It is assumed that the glycosylation of native IFN- γ protects the protein from proteolytic degradation, suggesting a potential role for glycans in therapeutic application.²⁴ Although some contradictory statements assume that glycosylation is not essential for the biological activity of recombinant IFNs,⁶⁸⁻⁷⁰ IFN- γ acts as an adjuvant and enhances the immune response. It would be relevant to analyze whether the synthesized bovine cytokine displays its immunomodulatory activity by promoting gene transcription and lymphocyte recruitment, as indicated in downstream studies of a recombinant b-IFN- γ .⁷¹ Even so, optimization of the *E. coli* gene expression system cassettes is generating a wide range of new recombinant biologics with antigenic and pharmaceutical potential with improved

protein yields.⁷²⁻⁷⁵ Initial strategies for cytokine synthesis and related antibodies consisted of laborious cell and total mRNA isolation steps that considered the native coding sequences for cDNA template generation and expression.^{20,36,76,77} Still, the overproduced recombinant proteins were often present in inclusion bodies as non-soluble products or usually could not sustain continuous high-level expression of the protein, needing improved subcloning strategies, adding fusion protein tags or downstream denaturing treatment conditions to solubilize the target molecule, but often making a more complex structure.⁷⁸⁻⁸⁰ Innovations have been continuously implemented to improve the production of these immunological molecules, overcoming their unfavorable yields and aggregative properties after heterologous overexpression.^{81,82} We used gene retro-engineering for favorable rb-IFN- γ biosynthesis as a biomarker for immunodiagnostic downstream applications with satisfactory protein yields, strategically starting from a rational computational analysis of the protein-retrieved amino acid sequence, overcoming the production challenges inherent in other methods. An IFN- λ molecule was successfully obtained after direct gene sequence retrieval from the NCBI database for gene synthesis with synonymous codon usage bias, with overexpression in *E. coli*.⁸³ Thus, evidence for preferring gene optimization strategies over standard procedures with native sequences for biotechnological and diagnostic purposes in *E. coli* is accumulating.⁸⁴⁻⁸⁹ Attempts to express a fully glycoprotein from buffalo in HEK 293F cell line were not successful, opting for the expression of a 124 aa segment of the target protein in *E. coli* with no glycosylated sites after codon optimization technology.⁹⁰ Production of recombinant chicken TNF- α from a cDNA cloning protocol and expression in transfected COS-7 cells was unsuccessful, not by a codon optimization strategy for the expression of the extracellular domain of the target protein in *E. coli*.⁹¹ In our work, the complete target bovine cytokine was successfully overproduced with the described gene retro-engineering strategy for bacterial expression. Finally, the achieved rb-IFN- γ may have good immunogenic characteristics of a standard biologic for specific immunoassaying, representing a feasible biotechnological product

for accomplishing the immune reagent production for the livestock pharmaceutical industry.

CONCLUSION

This study indicated the feasible biotechnological synthesis of a soluble immunocompetent b-IFN- γ in an *E. coli* expression system by codon usage optimization technology, with good protein yields and the generation of high levels of polyclonal antibodies as biologics specific for b-IFN- γ detection. Polyspecific antibodies recognize multiple sites on an antigen and are considered optimal ligands for immunodiagnosics, with the potential for large-scale production lowering costs. This study adds to others favoring the production of such mediators as specific reagents with immunological control potential for infectious diseases in livestock, with secondary application in the production of biologic standards for the biopharmaceutical industry. To our knowledge, this approach could be more suitable for commercial purposes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

AHA conceptualized the study. RH, AHA, AG, MM, HE, TAC, and JB collected resources and supervised the study. RH, AHA, AG, MM, HE, TAC, and JB applied methodology. AC and AOV performed the investigation. AHA performed visualization, project administration, funding acquisition and wrote the original manuscript. RH, AHA, AG, MM, HE, TAC, and JB reviewed and edited the manuscript. All authors read and approved the final manuscript for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

The study was approved by the Internal Committee for Care and Use of Laboratory Animals (license number: CICUAL-2018009B).

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