Development of an equine IFNγ release assay (IGRA) for glanders

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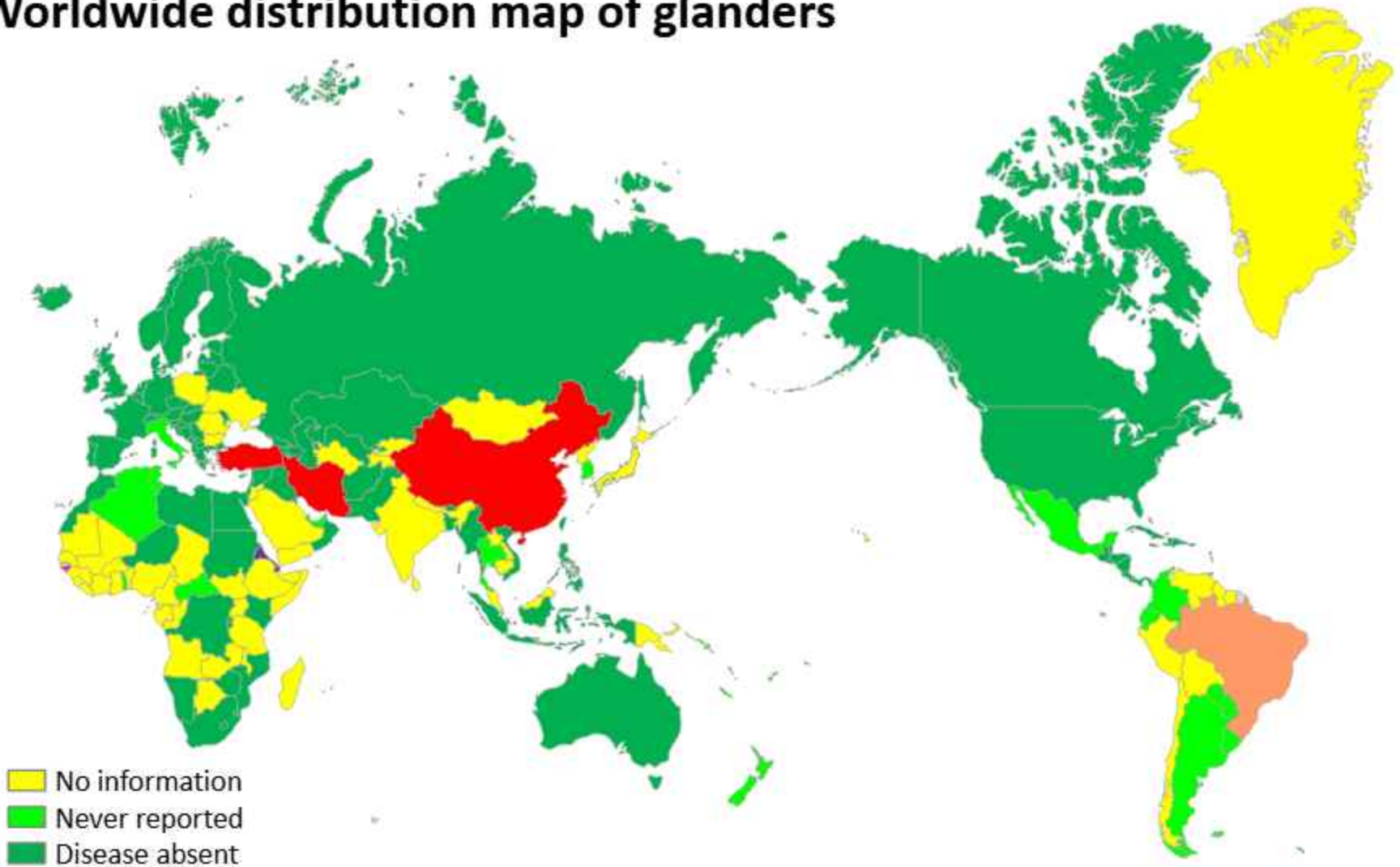
B. mallei and *B. pseudomallei* are closely related intracellular bacteria that often cause fatal infections in animals and humans

- *B. pseudomallei* is a motile Gram-negative rod, commonly found in tropical water and moist soil, infecting most mammals.

- *B. mallei* is a non-motile Gram-negative, non-spore-forming aerobic rod that cannot persist for extended periods outside a host. **It is the causative agent of glanders, infecting not only horses, donkeys, and mules but also humans.**

  ⇒ It causes acute symptoms in donkeys and mules.
  Mucopurulent nasal discharge, pulmonary lesions, nodules in the liver and spleen, along with high fever and respiratory symptoms such as nasal swelling, difficulty breathing, and pneumonia. **Death occurs within a few days.**
  ⇒ It causes chronic symptoms in horses.
  From mucopurulent to hemorrhagic secretions, nasal septal ulcers, and sticky yellow secretions, accompanied by enlargement of hard submandibular lymph nodes. High fever, weakness, etc. The pathology occurring majority in the lungs and airways. **The incubation period is several years.**

Worldwide distribution map of glanders



World Animal Health Information Database (WAHIS Interface), 2020

The usual diagnostic methods for glanders

- **Complement fixation test(CFT)**
  - ⇒ A labor-intensive test
  - ⇒ Generates false positive and negative
  - ⇒ Effectively testing horse serum with antagonistic complementarity can be challenging
- **Mallein test (allergic hypersensitivity test)**
  - ⇒ Low sensitivity
  - ⇒ Takes time (48h)
  - ⇒ Generates false positive
- **Rose Bengal test (RBT)**
  - ⇒ Low sensitivity

⇒ Developing an IGRA that have high sensitivity and specificity. Finally, we developing an immunochromatographic methods that is easy for Mongolian nomadic people to use.

The cellular immune response to *B. mallei* of infected mice

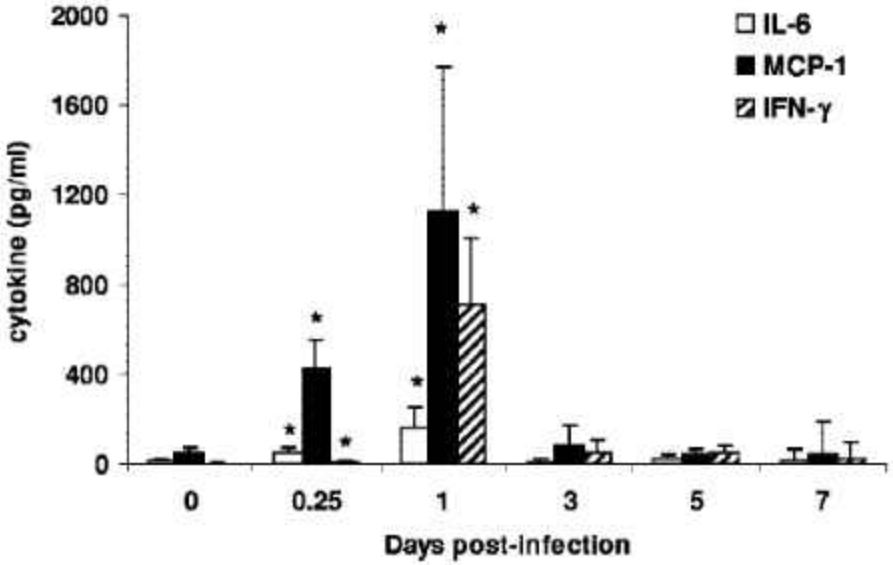


FIG. 3. Median cytokine levels in the sera of *B. mallei*-infected animals. The serum from BALB/c mice (eight mice per group) infected with 1×10^6 CFU *B. mallei* or uninfected controls was removed, and the IFN-γ, MCP-1, and IL-6 levels were determined at several times postinfection. The error bars indicate 99% confidence intervals, and the asterisks indicate statistical significance ($P < 0.01$).

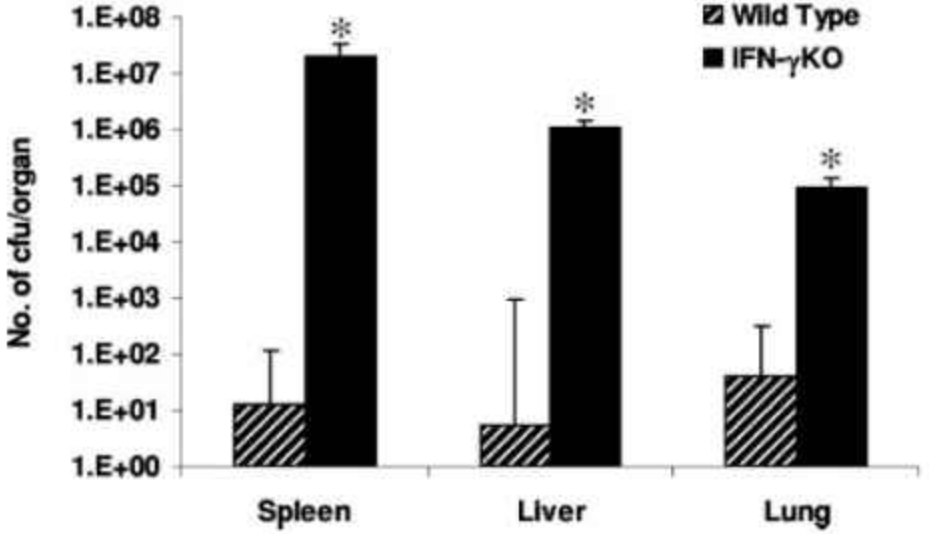
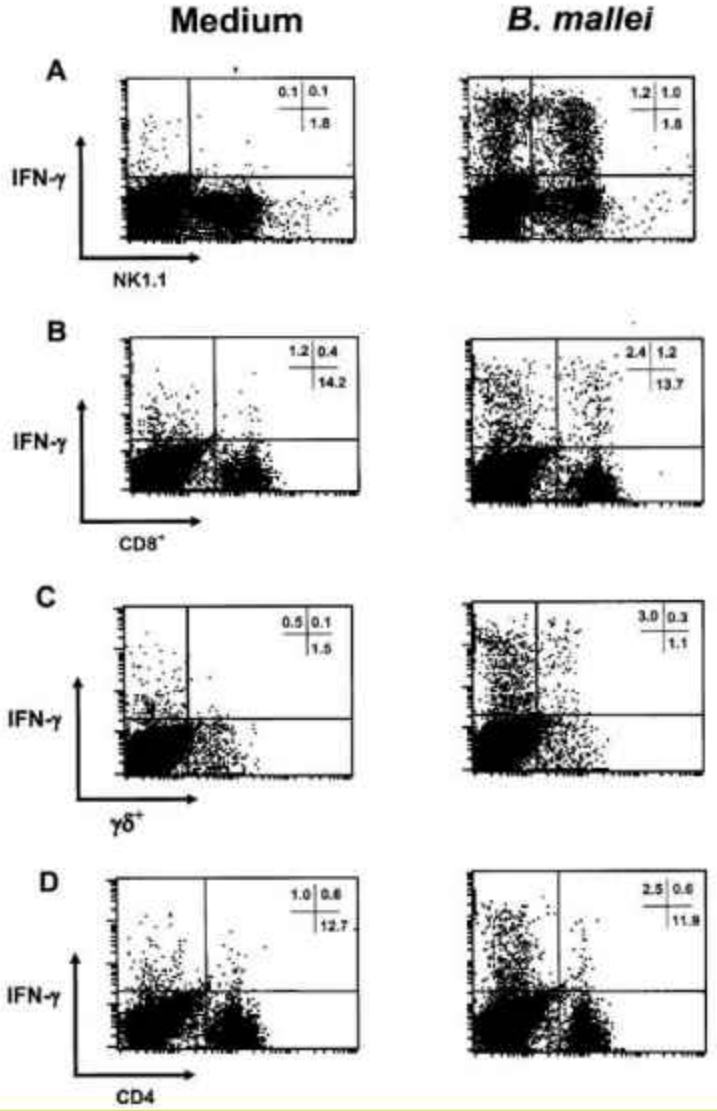
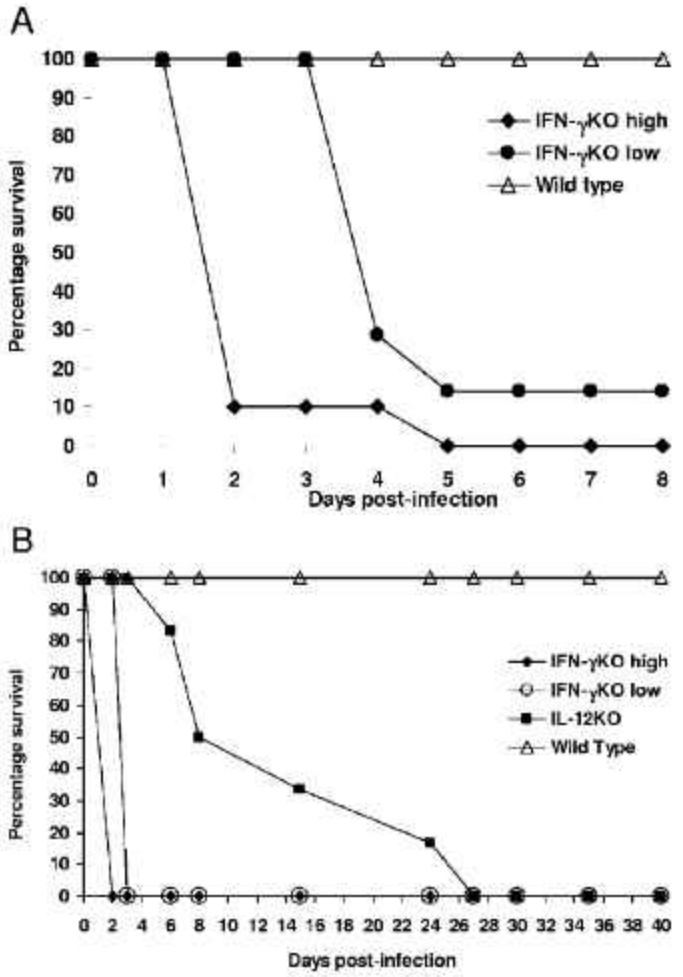


FIG. 5. IFN-γ is essential for controlling bacterial replication: number of bacterial CFU isolated from the spleen, liver, and lungs of IFN-γ KO or wild-type C57BL/6 mice 3 days postinfection for mice challenged with a low dose (41 CFU) of *B. mallei*. The bars indicate the average numbers of CFU per organ, and the error bars indicate standard deviations. Asterisks indicate statistical significance ($P < 0.05$).

Rowland, C. A. et al, 2006. *Infection and immunity*, *74*(9), 5333-5340.

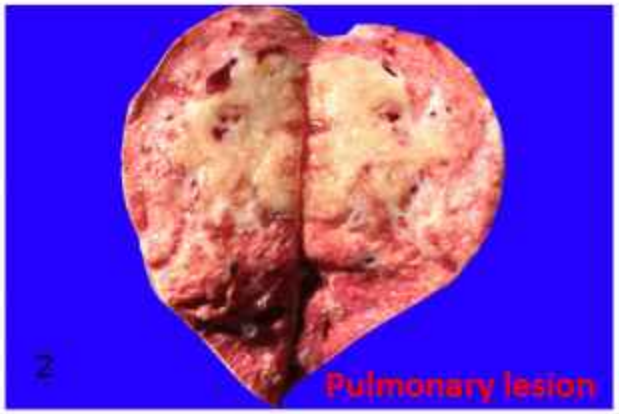
The cellular immune response to *B. mallei* and survival rate of infected mice





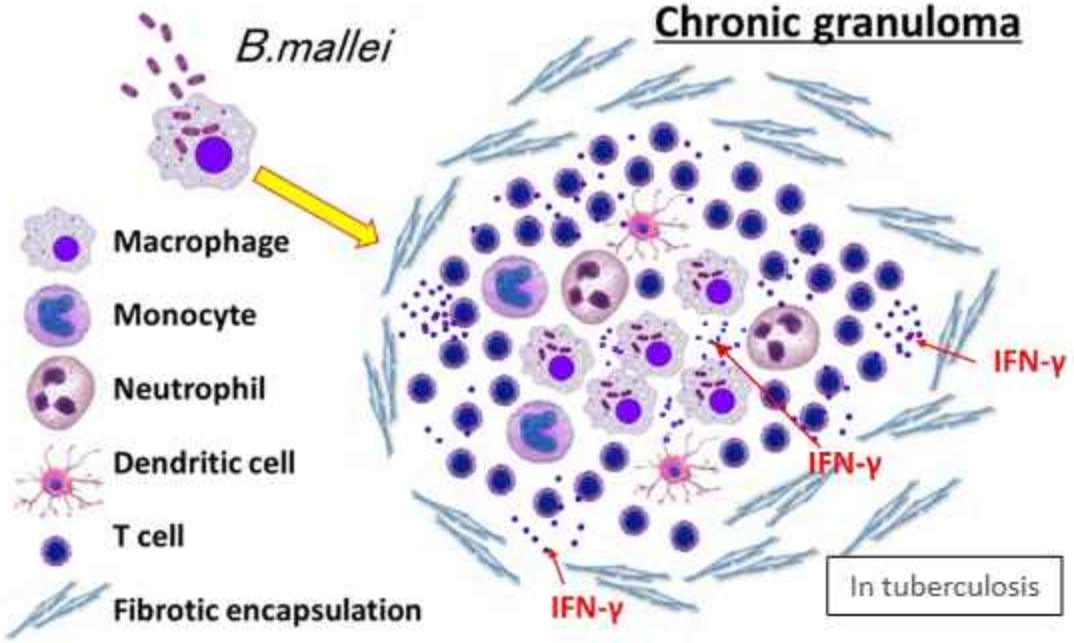
Rowland, C. A. et al, 2006. *Infection and immunity*, *74*(9), 5333-5340.

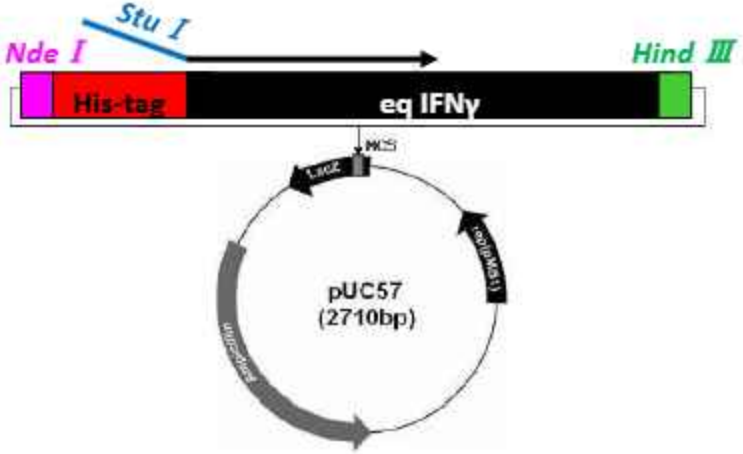
Interferon-gamma (IFNγ) plays a crucial role in cellular immune responses, especially in chronic granuloma



ERDEMSURAKH, Ochbayar, et al. Pathological and Immunohistochemical Analyses of Naturally Occurring Equine Glanders Using an Anti-BpaB Antibody. *Veterinary Pathology*, 2020, 57.6: 807-811.

IFNγ is a cytokine that is primary secreted from macrophage, $CD4^+$ T Th1, NK, and $CD8^+$ T cells and it is critical for innate and adaptive immunity against viral, some bacterial and protozoan infections.





1. The eqIFNγ DNA was synthesized by referring to the nucleotide sequence from NCBI

CATATGCATCACCATCACCATCAC**TATTATTGCCAAGCGGCGTTTTTC**AAGGAGATTGAGAACCTGAAGGAGTAC
TTTAACGCGAGCAACCCGGACGTGGGCGACGGCGGCCCGCTGTTCCTGGATATCCTGAAAAACTGGAAGGAA
GACAGCGATAAAAAGATTATCCAGAGCCAAATCGTGAGCTTCTACTTTAAGCTGTTCGAGAACCTGAAGGACAAC
CAAGTGATCCAGAAGAGCATGGACACCATCAAGGAAGACCTGTTCGTTAAGTTTTTCAACAGCAGCACCAGCAA
ACTGGAGGACTTCCAGAAGCTGATTCAGATCCCGGTTAACGATCTGAAGGTGCAGCGTAAAGCGATCAGCGAA
CTGATCAAGGTGATGAACGACCTGAGCCCGAAGGCGAATCTGCGTAAGCGTAAACGTAGCCAAAACCCGTTCC
GTGGT**CGTCGTGCGCTGCAATAA**AAGCTT

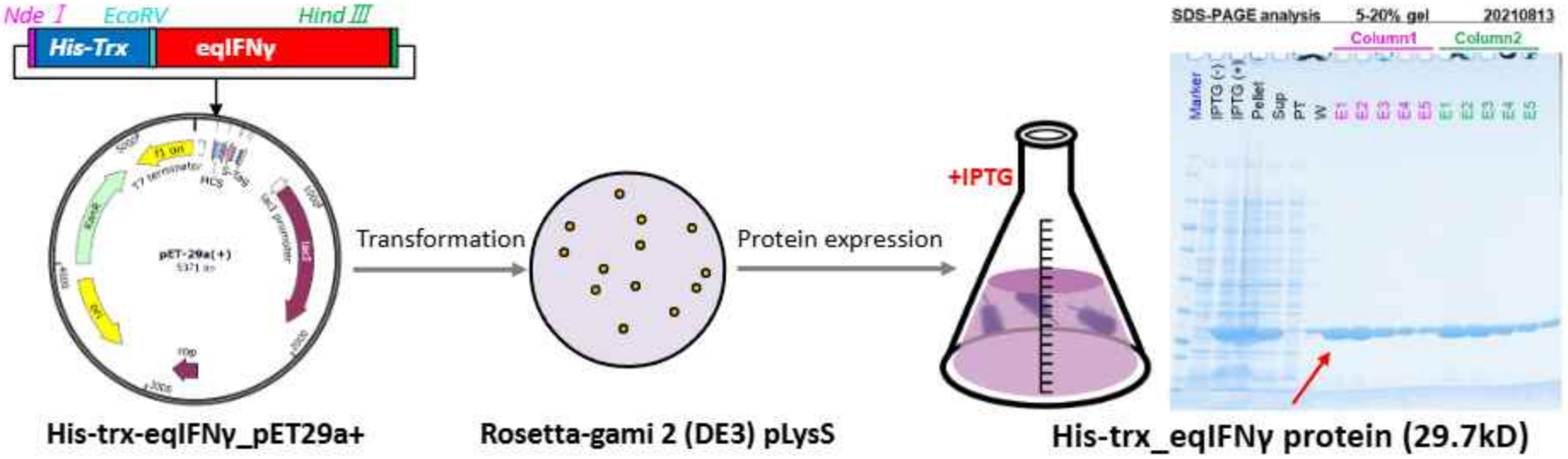
2. The eqIFNγ DNA was amplified through PCR by adding *Stu I* sites

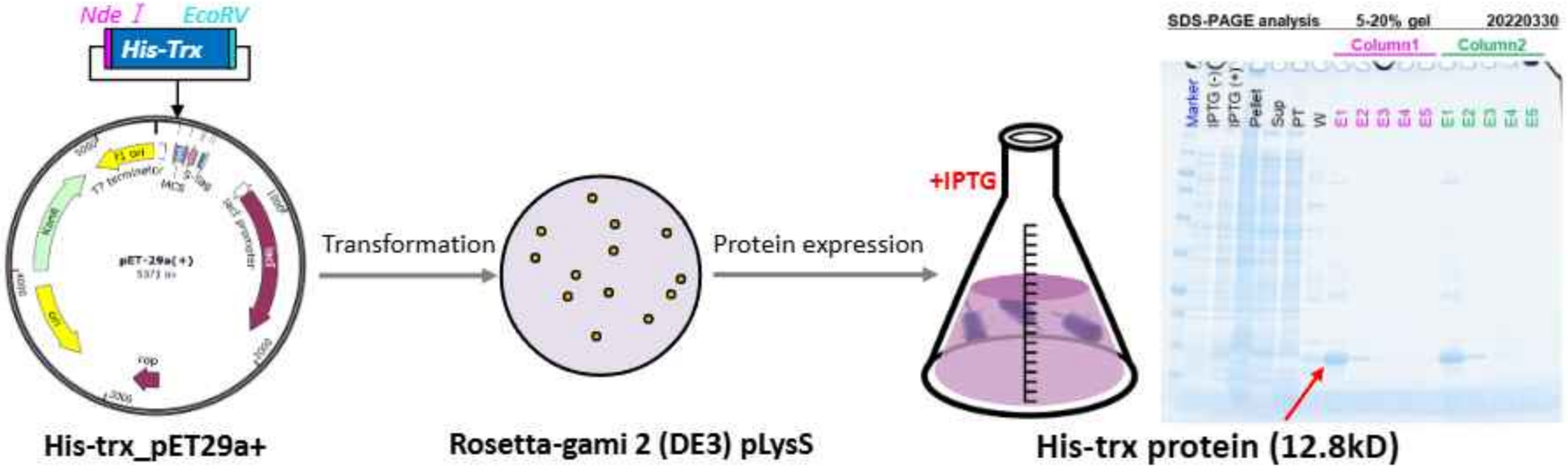
Forward primer: GAAGGCCT**TATTATTGCCAAGCGGCGTTTTTC**

CATATGCATCACCATCACCATCAC**TATTATTGCCAAGCGGCGTTTTTC**AAGGAGATTGAGAACCTGAAGGAGTAC
TTTAACGCGAGCAACCCGGACGTGGGCGACGGCGGCCCGCTGTTCCTGGATATCCTGAAAAACTGGAAGGAA
GACAGCGATAAAAAGATTATCCAGAGCCAAATCGTGAGCTTCTACTTTAAGCTGTTCGAGAACCTGAAGGACAAC
CAAGTGATCCAGAAGAGCATGGACACCATCAAGGAAGACCTGTTCGTTAAGTTTTTCAACAGCAGCACCAGCAA
ACTGGAGGACTTCCAGAAGCTGATTCAGATCCCGGTTAACGATCTGAAGGTGCAGCGTAAAGCGATCAGCGAA
CTGATCAAGGTGATGAACGACCTGAGCCCGAAGGCGAATCTGCGTAAGCGTAAACGTAGCCAAAACCCGTTCC
GTGGT**CGTCGTGCGCTGCAATAA**AAGCTT

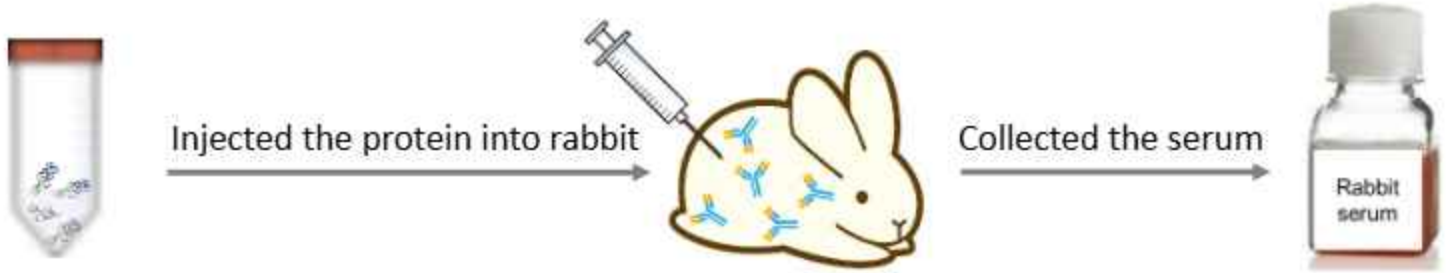
Reverse primer: GCAGCACGCGACGTTATTTTCGAACTCA

3. His-trx_eqIFNγ and His-trx were ligated into the pET29a+ plasmid and the proteins were expressed, respectively



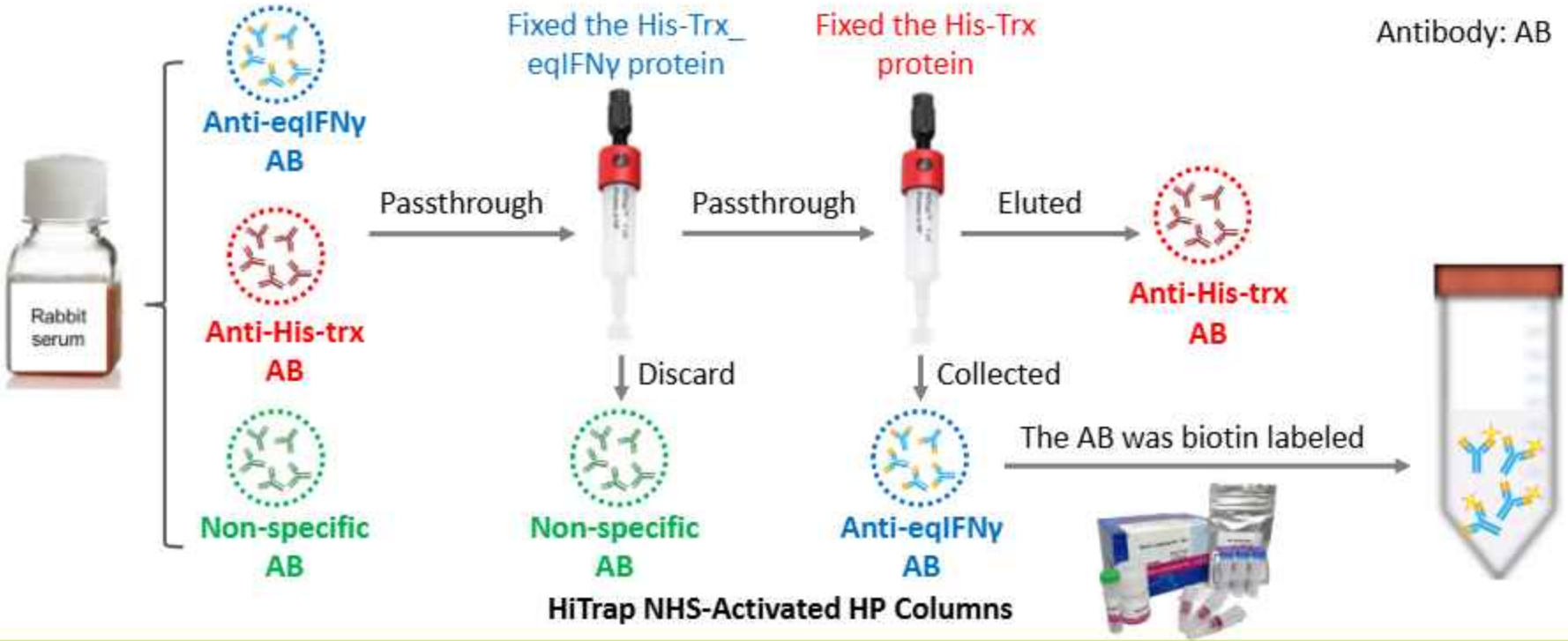


4. Injected the protein into rabbit and collected the serum

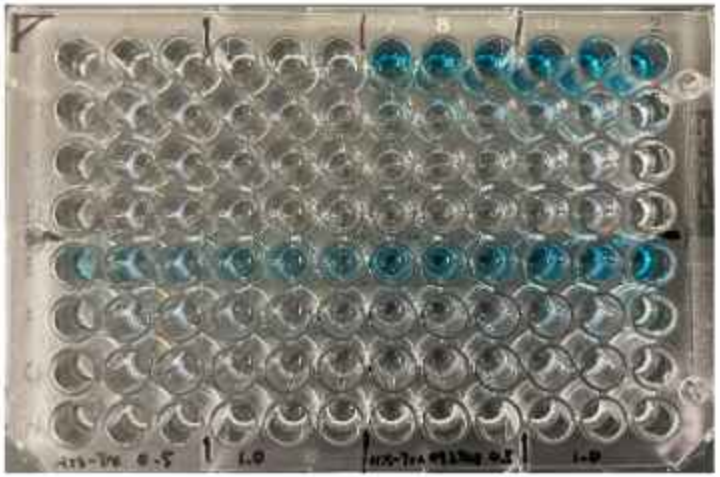


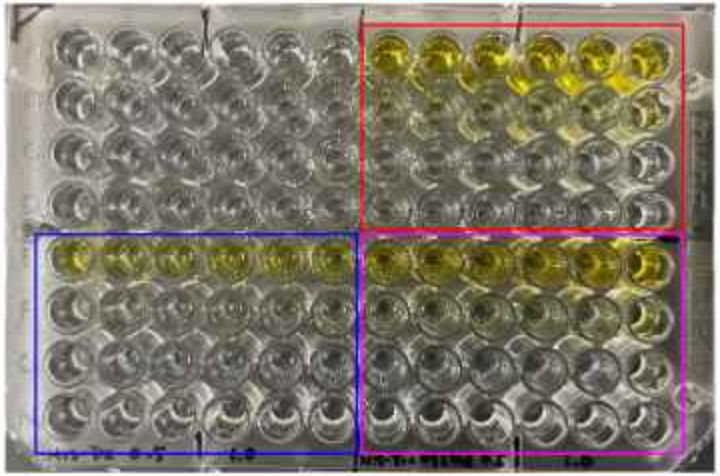
His-trx eqIFNγ protein

5. Specific AB were purified from serum, and the anti-eqIFNγ AB was biotin labeled

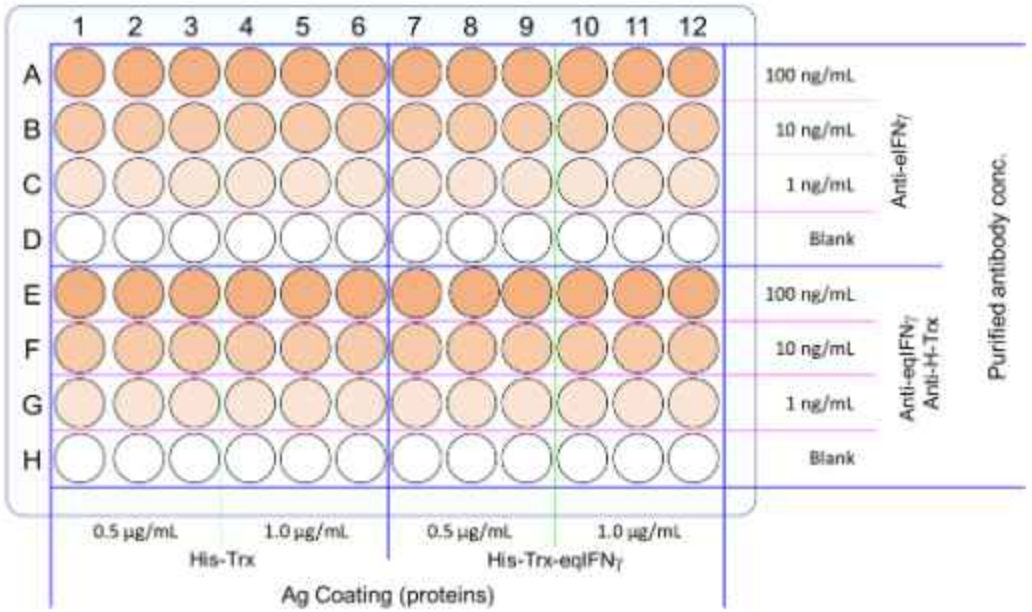


5. The eqIFNγ protein specific antibody was confirmed by ELISA method





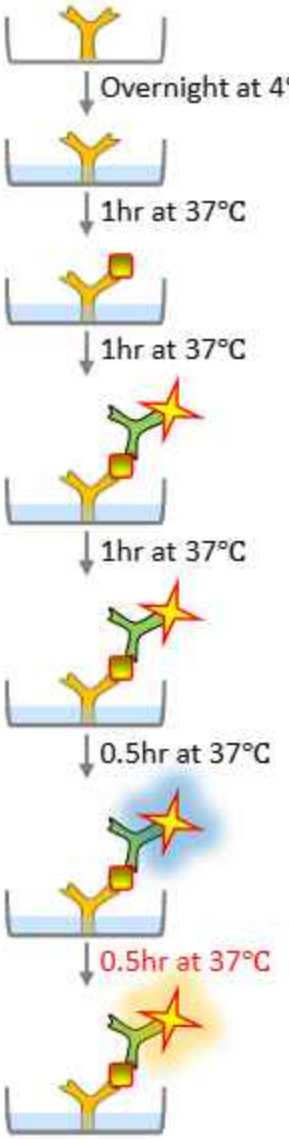
The binding reaction between His-Trx protein and His-Trx specific antibody



The binding reaction between His-Trx-eqIFNγ protein and eqIFNγ specific antibody

The binding reaction between His-Trx-eqIFNγ protein and His-Trx specific antibody

6. ELISA Development of IGRA for glanders



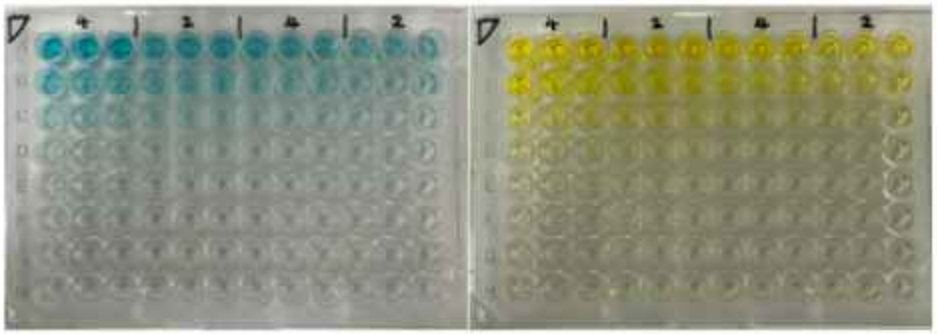
- Capture Ab: Anti-eqIFNγ Ab
- Blocking: Block Ace
- Wash, add standard
  His-trx eqIFNγ protein
- Wash, add detection Ab
  Biotin labeled anti-eqIFNγ Ab
- Wash, add Ultra-Sensitive
  ABC Peroxidase
- Wash, add TMB
  Substrate Solution
- Stopped the reaction
  Read the OD value (450nm)

Table1. Coating the Biotin non-labeled anti-eqIFNγ AB into the 96 well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Coating anti-eqIFNγ AB 4ug/ml			Coating anti-eqIFNγ AB 2ug/ml			Coating anti-eqIFNγ AB 4ug/ml			Coating anti-eqIFNγ AB 2ug/ml		
B												
C												
D												
E												
F												
G												
H												

Table2. Dilution of standard (His-trx eqIFNγ protein)

Standard	St1	St2	St3	St4	St5	St6	St7	Blank
Conc. (ng/ml)	30	10	3	1	0.3	0.1	0.03	0



Reaction was slower than in general ELISA

7. The future plan for the development of the IGRA

eqIFNγ1

QAAFFKE IENLKEYFNA SNPDVGDGGP LFLDILKNWK EDSDKKIIQS

eqIFNγ2

QIVSFYFKLF ENLKDNQVIQ KSMDTIKEDL FVKFFNSSTS KLEDFQKLIQ

eqIFNγ3

IPVNDLKVQR KAISELIKVM NDLSPKANLR KRKRSQNPFR GRRALQ

⇒ Fragmenting the eqIFNγ cDNA into three fragments, eqIFNγ1, 2 and 3.

⇒ Inserting them into pGEX-6p-1 vectors to express proteins, respectively.

⇒ Separately purifying specific antibodies against epitopes present on each fragment protein.

⇒ Developing an ELISA assay to confirm sensitivity and specificity.

Thank you for your attention