Tests detecting cellular immune responses

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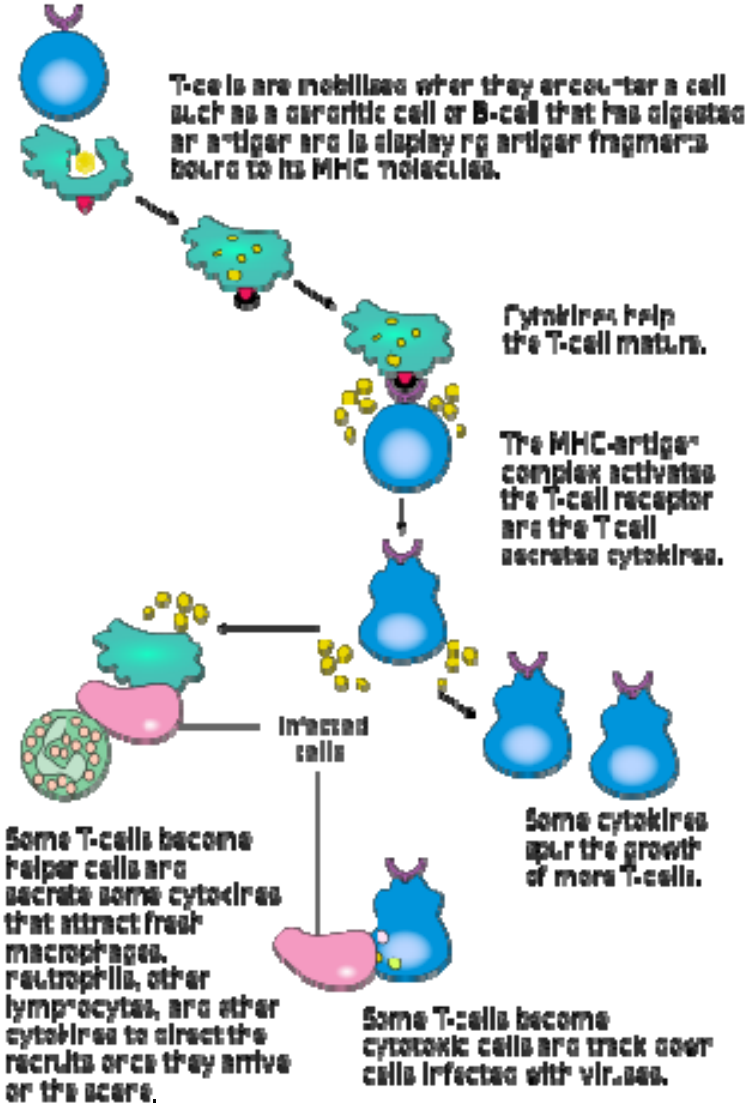
cita
CENTRO DE INVESTIGACIÓN Y TECNOLOGÍA AGROALIMENTARIA DE ARAGÓN

Cell mediated immune response (CMI)

CMI is especially important for destroying intracellular bacteria and eliminating viral infections



Classical approach:

- Antibody mediated immunoresponse (B-cells)
- Cell mediated immunoresponse (T-cells)

Diagnostic tests based on CMI

(macrophages and T-cells activity)

CMI diagnostic techniques

Traditional assays analyzed bulk populations of T cells for proliferation (by ^{3}H-thymidine incorporation) or for cytotoxicity (by 51Cr release assays). These methods tend to be long and labor-intensive, and their results usually cannot be compared quantitatively.

"Hightech assays" of antigen-specific T cells have come into use:

MHC-peptide tetramer staining, enzyme-linked immunospot (ELISPOT) assays, intracellular cytokine assays, gene expression...

CMI diagnostic techniques:

- Blastogenesis
-In vitro cytokines detection (IFN-γ, TNF-α, IL…)
-Differential gene expression
-In vivo Delayed Type Hypersensitivity (DTH) reaction

Lymphocite Blastogénesis Tests

When T cells meet their specific antigen they are stimulated to undergo division. This mitogenic response is usually accompanied by morphological change to a blast cell

The degree of lymphocite stimulation can be assayed either by determining the number of blast cells in the culture or by quantifying the amount of radioactive DNA incorporated into newly sythesized DNA

Sample heparinized blood (peripheral blood mononuclear cells extract)

Method

Lymphocites extraction → Lymphocites are account and cell concentration is adjusted.

Lymphocites are account and cell concentration is adjusted. → Cells are cultured in tubes or microplates with adequate tissue medium

Optimum concentration of antigen is added for lymphocites stimulation.

Negative control: cells cultured without antigen

Mitogenic control: non-especific mitogen

Cells are cultured in tubes or microplates with adequate tissue medium → Incubation → Account of T-cells:

-Scintillator (^{3}H-thymidine)

- Colorimetric reaction

- Flow citometry

...

In vitro cytokines detection after T-cell stimulation

T-cells from infected animals, when exposed and incubated with the specific antigen *, produce cytokines…

* The antigen must be compound by proteins / peptides to elicit especific CMI

Cytokines

IFN-γ. Commonly used for diagnostic puposes

Interleukines (IL2, IL4…)

Samples

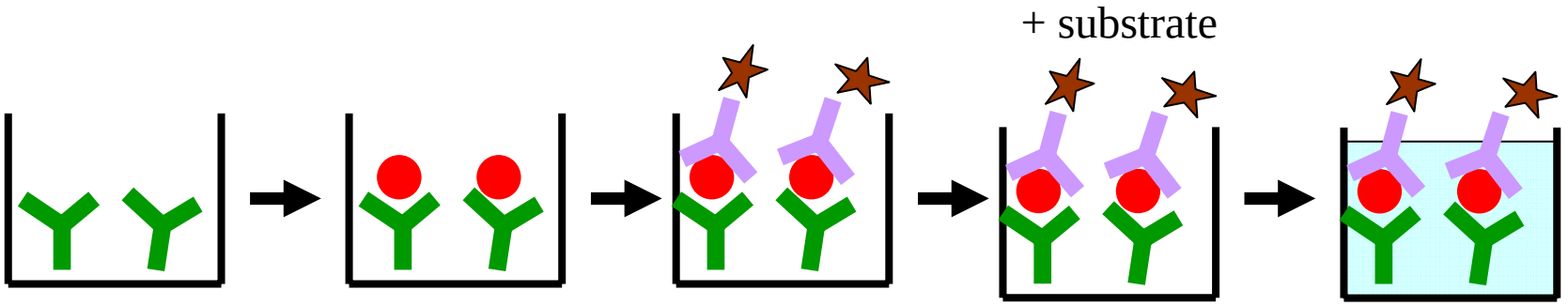
Blood (complet heparinized blood or peripheral blood mononuclear cells)

Lymphoid tissues, Spleen…

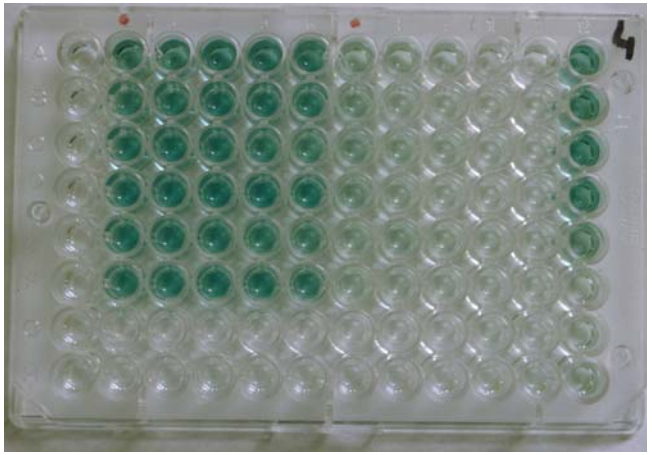
Methods Cytokines production can be quantified by different methods:

- ELISA
- ELISPOT / FLUOROSPOT
- FLOW CITOMETER / LUMINEX
- INTRACELLULAR CYTOKINE STAINING

ELISA (sandwich enzyme-linked immunosorbent assay)



Colour reaction quantified by spectofotometry

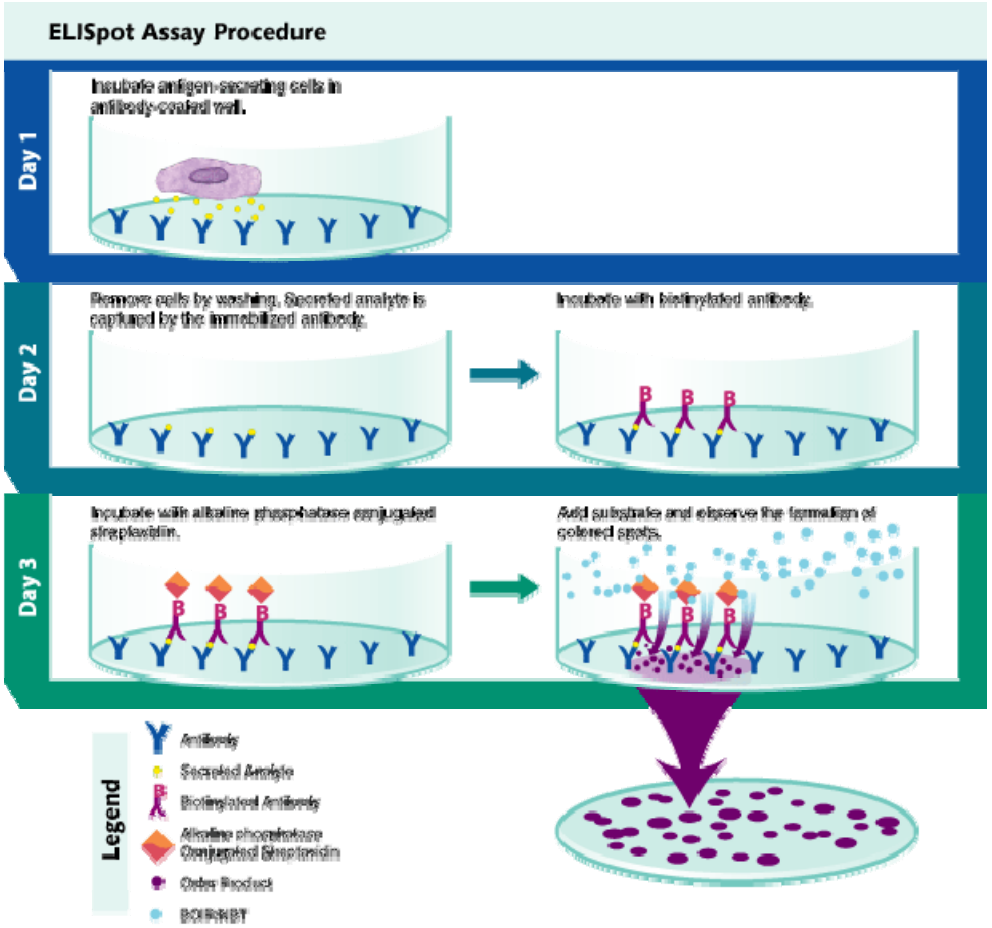


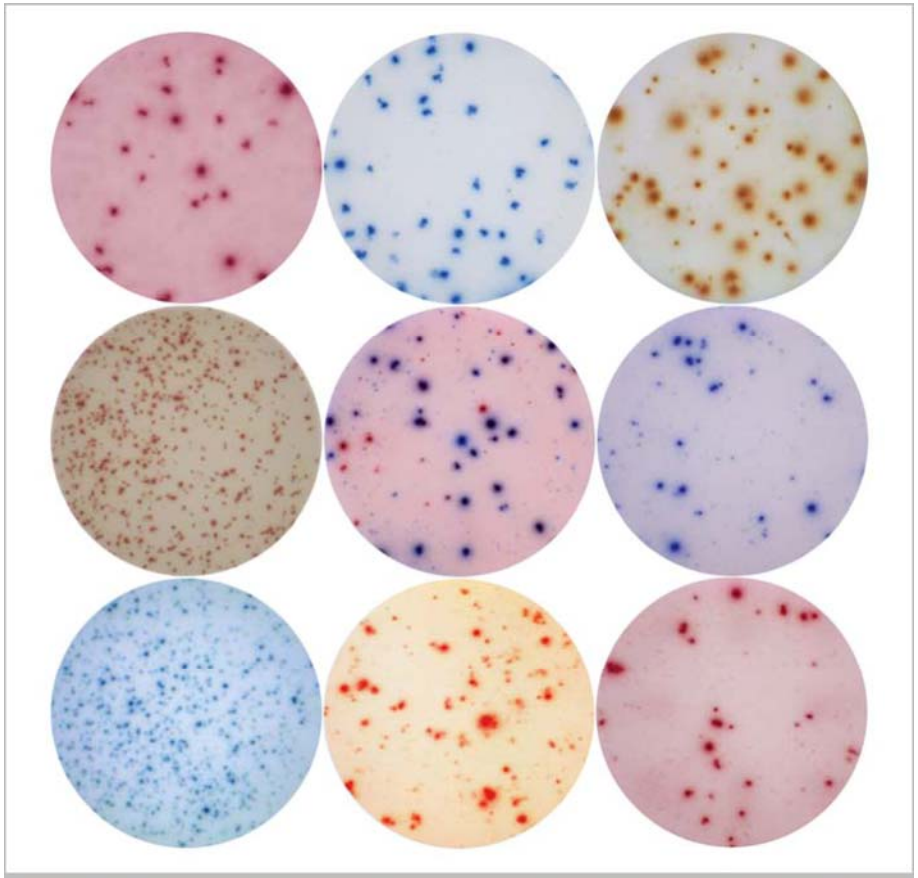
Antibody anti IFN-γ

IFN-γ secreted after stimulation of the sample with the specific antigen

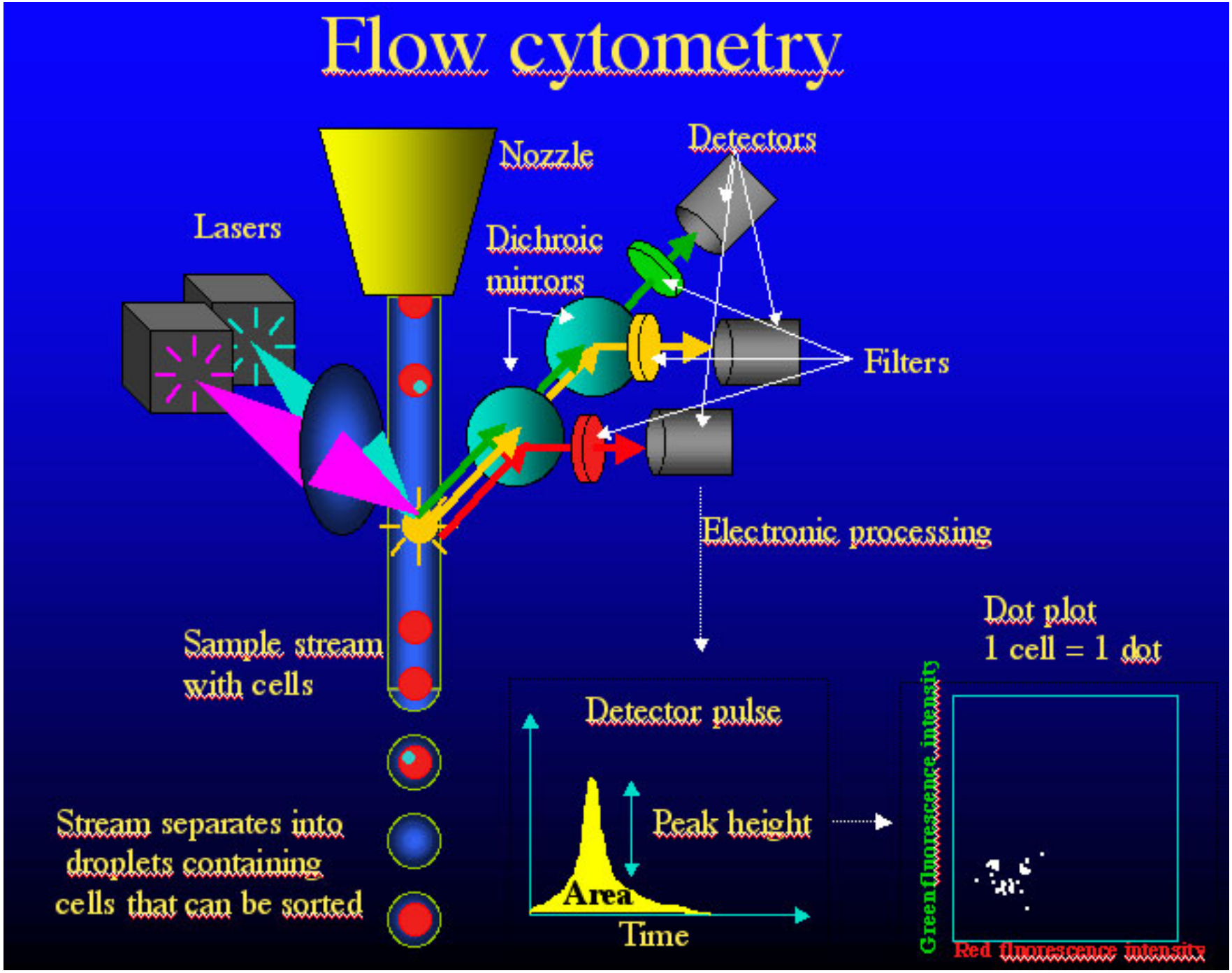
Antibody anti IFN-γ conjugated (HRP, Streptavidin…)

ELISPOT / FLUOROSPOT

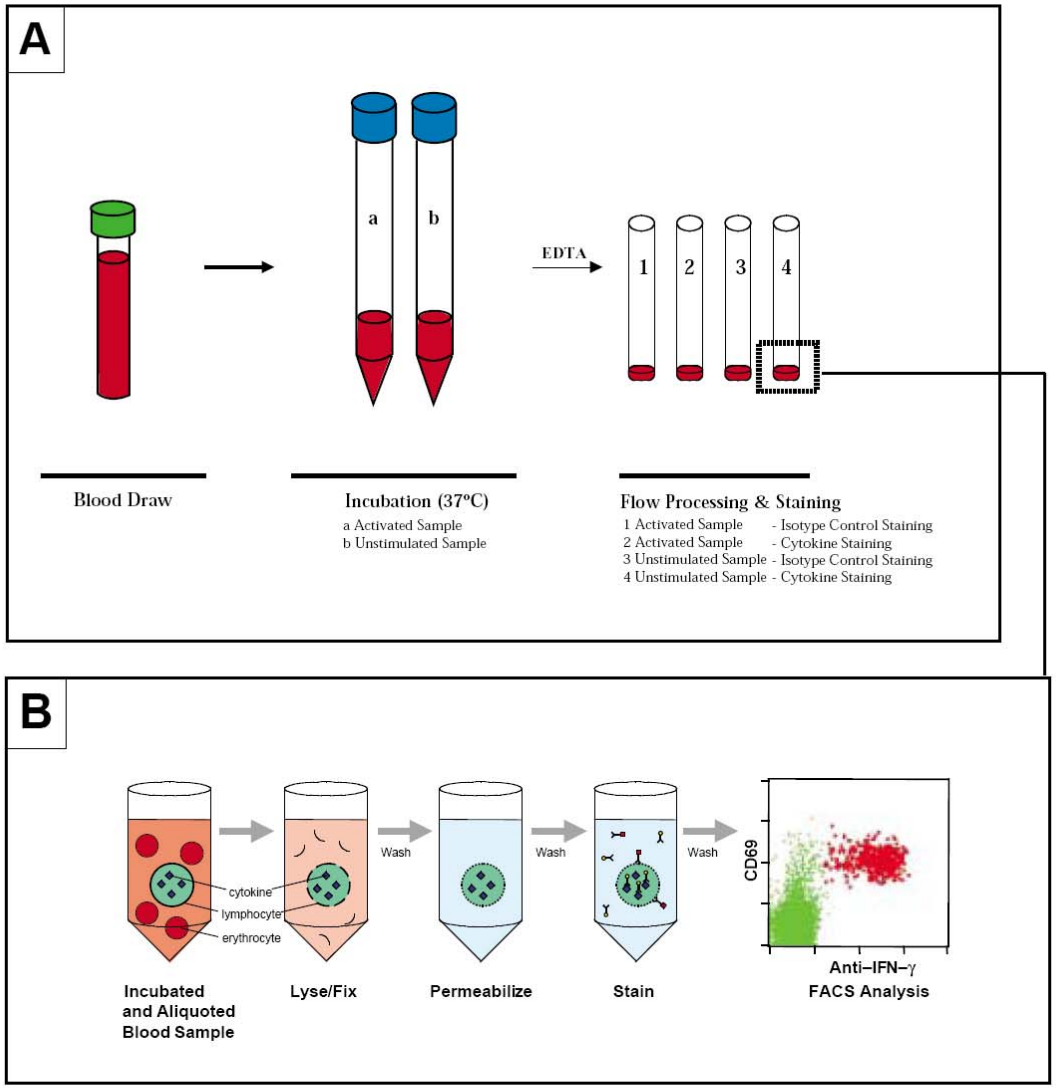




In ELISPOT assays, cellular secretions are captured around the originating cell and manifested as colored secretory footprints. This allows the frequencies of the secreting cells to be measured with an extraordinary level of precision and accuracy. Basically, each cell leaves a "footprint."


Flow cytometry
Nozzle
Detectors
Lasers
Dichroic mirrors
Filters
Electronic processing
Sample stream with cells
Dot plot
1 cell = 1 dot
Detector pulse
Peak height
Area
Time
Stream separates into droplets containing cells that can be sorted
Green fluorescence intensity
Red fluorescence intensity

Intacellular cytokines staining



The samples are stimulated with the especific antigen in the presence of a secretion inhibitor (BFA), allowing for intracellular accumulation of newly synthesized cytokines

Lysis of erythrocytes and fixation of leucocytes

Surface and intracellular staining antibodies are added in a single staining step.

The cells are washed and fixed for flow cytometric analysis

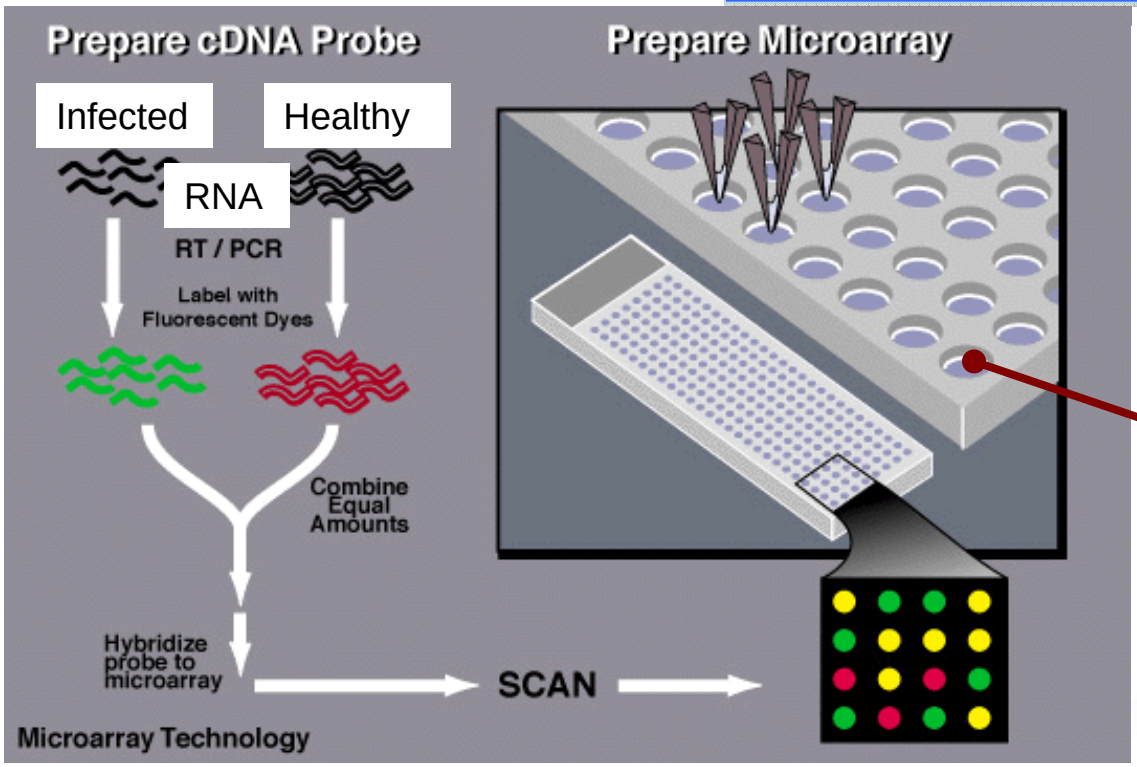
Figure 1 Schematic of whole blood FastImmune antigen-specific assay: Part A From blood draw to sample activation to flow cytometric sample processing; Part B Staining and processing of samples for flow cytometric analysis, applies to tubes 1 to 4 from Part A

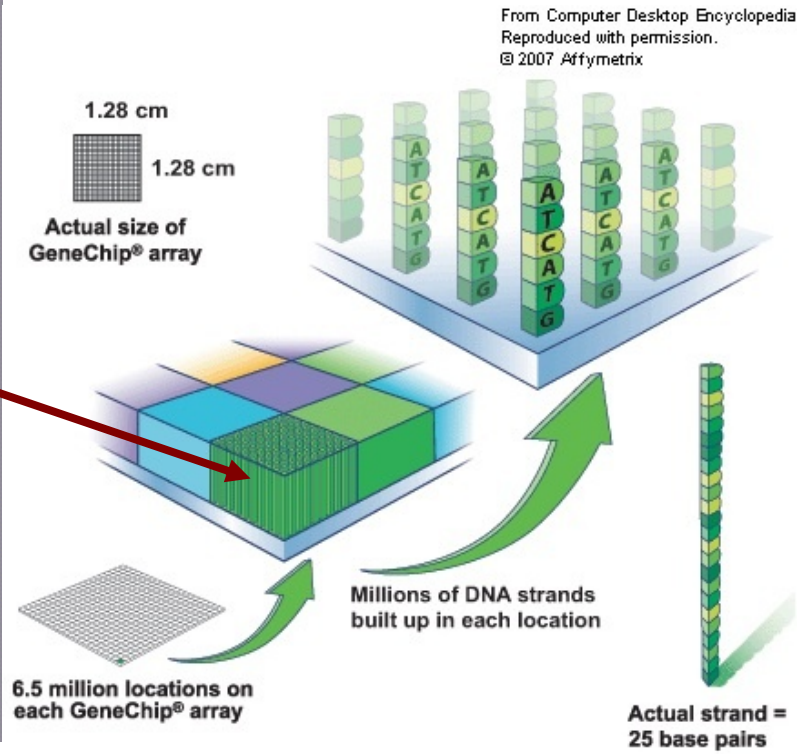
Differential gene expression

- Macrophages or T-cells are activated with the specific antigen Incubation → The IFN-γ or T-cells mRNA expression is determined by microarray hybridization or real-time PCR

- Characterization of the differential expression of inflammatory and immune response genes (look for up-regulated or down-regulated genes in infected animals *vs* uninfected ones)

Microarray hybridization





In the animal health context…

Most of these tests are used exclusively for research purposes:

- Monitoring of immune status during disease

- Vaccine efficacy studies

❑ Expensive equipment and reagents are required

❑ The setting up is quite labourious. Optimal reagents (adequate antigen, reagents, controls, sample dilution, reagent concentrations…) and conditions (temperatures, incubation times..).

❑ Highly experienced staff is required to perform the assay and interpret results correctly

Standarization and validation for the obtention of comparative results is almost imposible (results can be highly variable from one lab to another).

Just the INF-γ assay (assessed by ELISA) is applied for diagnostic purposes in control and eradication programs (e.g, Tuberculosis)

Advantages

- Useful when cellular immune response predominates over the antibody mediated response (Tuberculosis)
- Allowing the analysis of many samples in a relatively short time (2 days)

Drawbacks

- Samples must be analysed within 8h to avoid IFN-γ degradation (Rothel et al. 1992)
- Cross-reaction due to similar protein antigens (e.g, M. bovis and M. avium)

Disagreement about test performance:

- Different methods for intrepetation of results
- Different criteria to determine the cut-off
- Absence of Local Gold Standard

↓

Commercial tests are not always properly validated under local conditions

INF-γ assay

Specific Bovine Brucellosis Diagnosis Based on In Vitro Antigen-Specific Gamma Interferon Production

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In order to improve the specificity of the diagnosis of bovine brucellosis, we developed a test which can be regarded as an in vitro correlate of the delayed-type hypersensitivity test (DTH). A mixture of cytoplasmic proteins from *Brucella melitensis* B115 was used as a specific antigenic stimulus in bovine whole blood culture. Supernatants harvested at 18 to 24 h after the in vitro antigenic stimulus were assayed for their gamma interferon (IFN-γ) content by using a commercial sandwich enzyme-linked immunosorbent assay kit. The IFN-γ assay was evaluated with 10 heifers during the course (80 days) of an experimental infection and with 14 cows from an ongoing brucellosis outbreak. All of these animals were slaughtered, and pertinent organs were subjected to classical bacteriological analyses. In addition, we analyzed 23 field cases in which false-positive serological reactions occurred. The IFN-γ results were compared with those of the standard DTH and a battery of serological assays, and they were correlated with bacteriological data. Both for the experimental infection and for the field brucellosis outbreak, the IFN-γ assay detected infection in more animals than any combination of the serological tests, and it detected infection earlier than these tests. Finally, none of the samples from cows showing false-positive serological reactions was classified as positive by the IFN-γ assay, attesting to its specificity and to its usefulness in interpreting ambiguous serological results. A rapid and convenient alternative to the DTH, the IFN-γ assay appears to be an ideal method that is complementary to the serological diagnosis protocols.

Experimental data with a reduced number of samples

No field studies

INF-γ assay

Field comparison of the interferon-gamma assay and the intradermal tuberculin test for the diagnosis of bovine tuberculosis

PR WOOD*, LA CORNER*, JS ROTHEL*, C BALDOCK†, SL JONES‡, DB COUSINS§, BS McCORMICK¶, BR FRANCIS§, J CREEPER¶ and NE TWEDDLE**

SUMMARY: An extensive field comparison of the gamma Interferon (IFN-γ) assay and the single intradermal tuberculin test for the diagnosis of bovine tuberculosis was conducted in Australia. The specificity of the IFN-γ assay was determined by testing more than 6000 cattle from tuberculosis-free herds and varied from 96.2% to 98.1%, depending on the cut-off point chosen to define a positive reactor. For the sensitivity trial, cattle from herds being de-populated because of bovine tuberculosis were examined with both assays. The sensitivity of the IFN–γ assay was shown to be significantly higher than the single intradermal tuberculin test and varied from 76.8% to 93.6% depending on the method of interpretation. A maximum overall sensitivity of 95.2% was obtained by testing with the IFN-γ and the tuberculin test in parallel. The superior sensitivity of the IFN-γ assay and the ability to adjust the sensitivity of the system depending on the task involved, will provide the Australian Tuberculosis Eradication Campaign with a valuable additional test to enable it to accomplish its goals.

Aust Vet J **68:** 286 – 290

INF-γ assay

Comparison of the sensitivity of the caudal fold skin test and a commercial γ-interferon assay for diagnosis of bovine tuberculosis

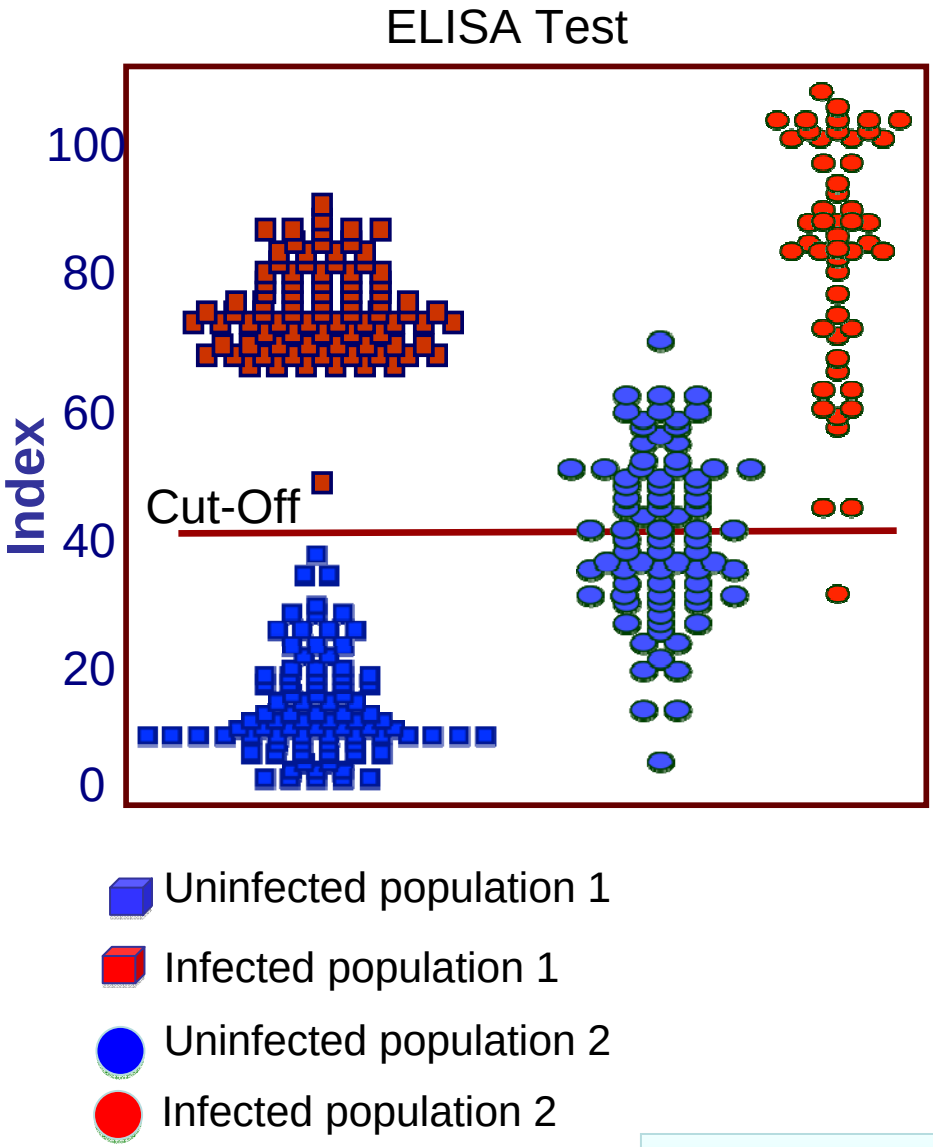
Diana L. Whipple, MS; Carole A. Bolin, DVM, PhD; Arthur J. Davis, DVM; Jerald L. Jarnagin, MS; Dorothy C. Johnson; Richard S. Nabors, BBA; Janet B. Payeur, DVM, PhD; Dennis A. Saari, DVM; Arach J. Wilson, DVM; Mary M. Wolf, BA

"Sensitivity of the γ-INF assay[a] ranged from 55.4 to 97.1%, depending on the standard of comparison and on the method of interpretation".

Using the same method and cut-off for interpretation of results	Sn	Sp
Australia (Wood et al, 1991)	93,6	96,2
E.E.U.U (Whipple et al, 1994)	73,0	Not done!

[a] IDEXX Laboratories Inc, Westbrook, Me.

Field conditions and immunological background can be quite variable among different populations



INF-γ assay

Skin test

In vivo Delayed Type Hypersensitivity (DTH) reaction

When the allergen (proteins or peptides) is intradermicaly inculated in an infected (sensitized) animal, macrophages are inmovilized in the area and realease enzymes causing:

Local edema

Granuomatous inflamation

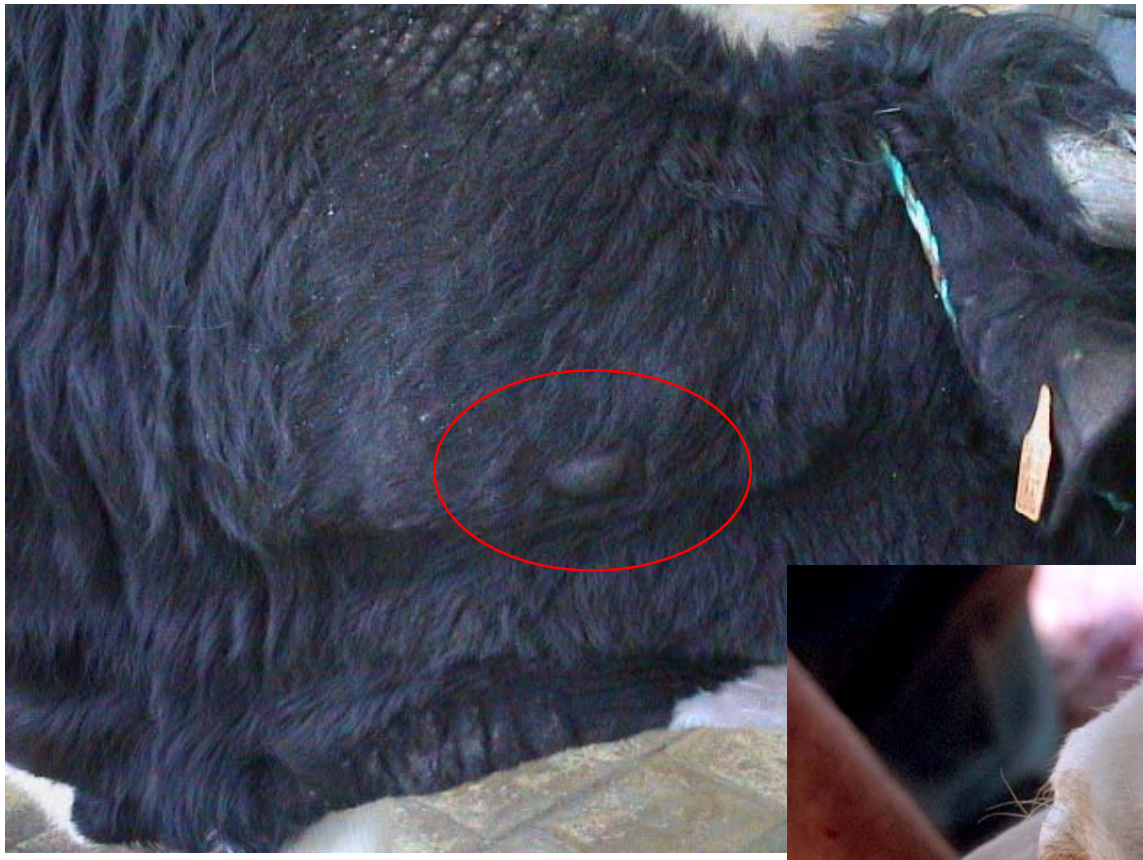
Vascular thrombosis and necrosis.

Method:

Intradermal injection of an allergen (Tuberculin, Brucellin, viral peptides..) in the caudal tail fold, neck or lower eyelid.

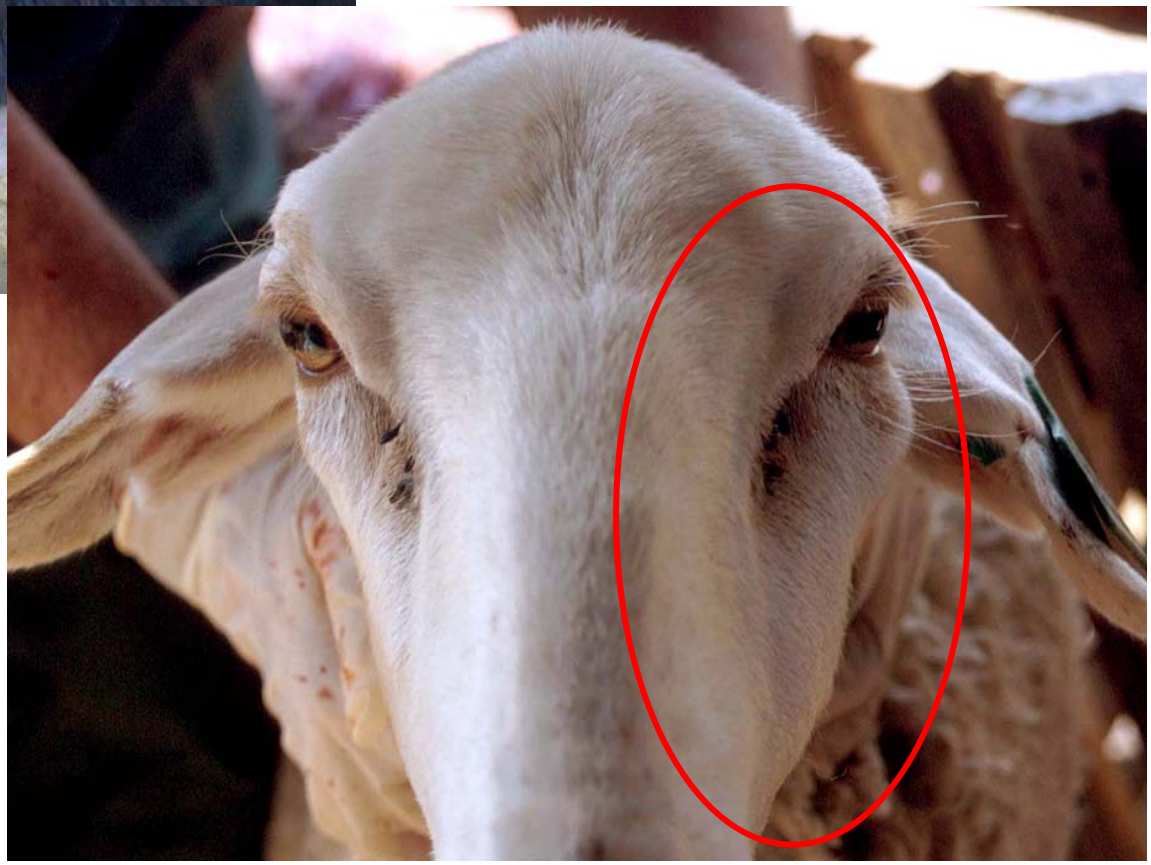
After 48-72 hours the site is examined for signs of swelling.

Skin test

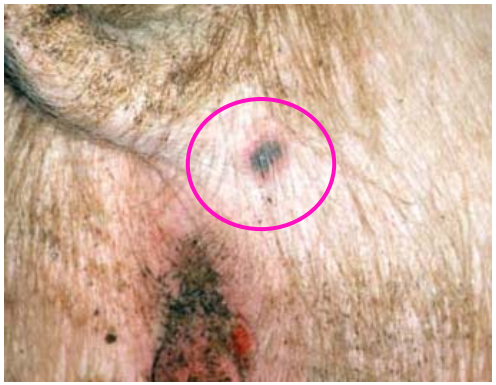


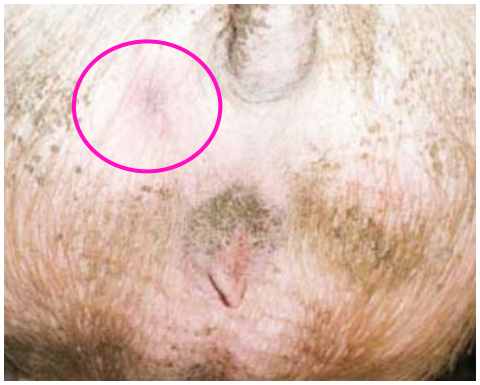
DTH reaction in Brucella infected cow

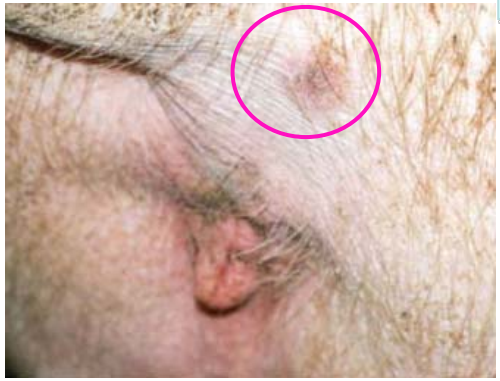
DTH reaction in Brucella infected sheep



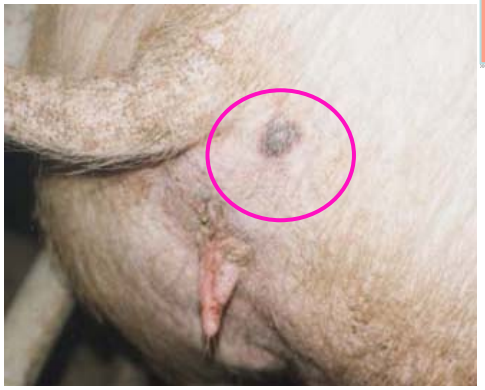
Skin test

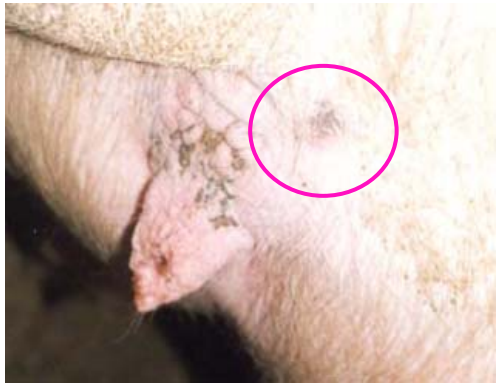


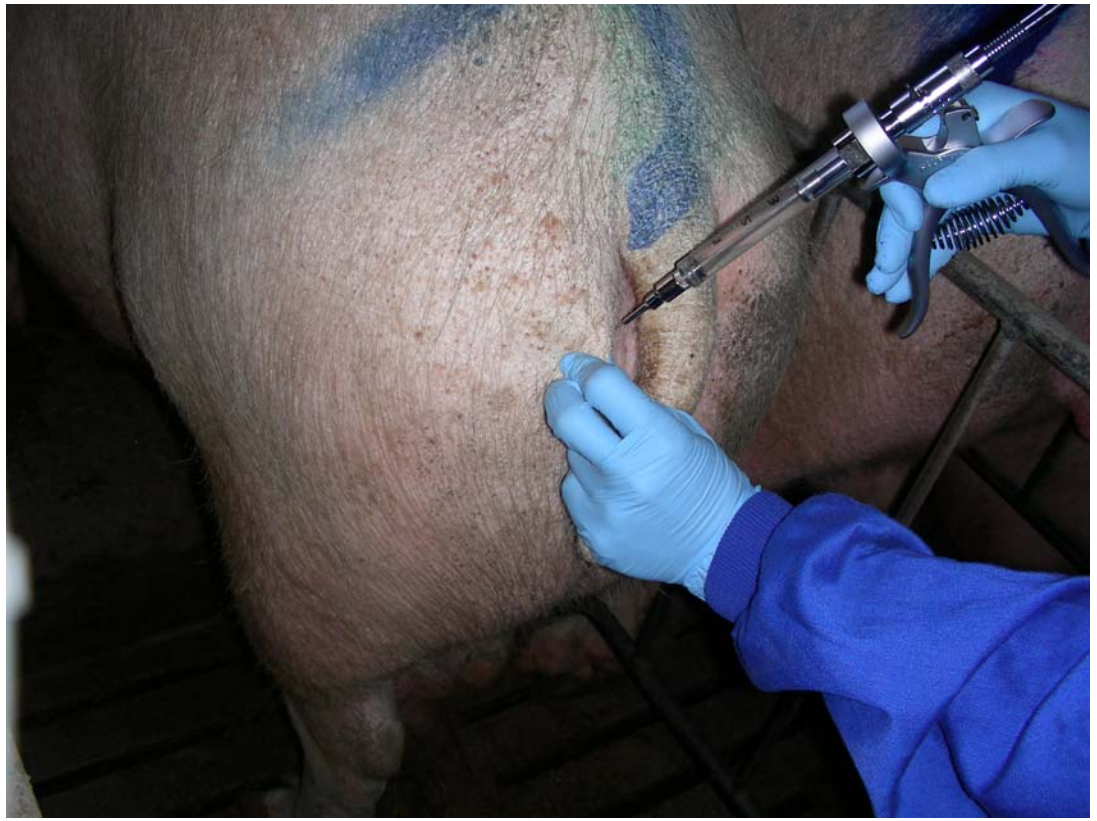




DTH reaction in Brucella infected pigs



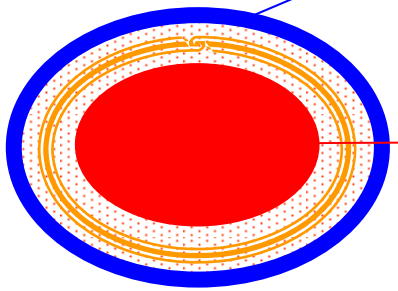




Skin test

Useful when false positive serological reactions (FPSR), due to other common antigens (like lipopolysaccharides), are present.

S-LPS /O-PS share *Y. enterocolitica* O:9 epitopes



Brucella

CYTOSOLIC-PERIPLASMIC PROTEINS (BRUCELLIN)

Are mostly shared by *Brucella* species, and in contrast to S-LPS, they elicit DTH immunoresponses

Are fully (mostly) genus-specific, and not shared with *Y. enterocolitica* O:9

can solve the problem of FPSR

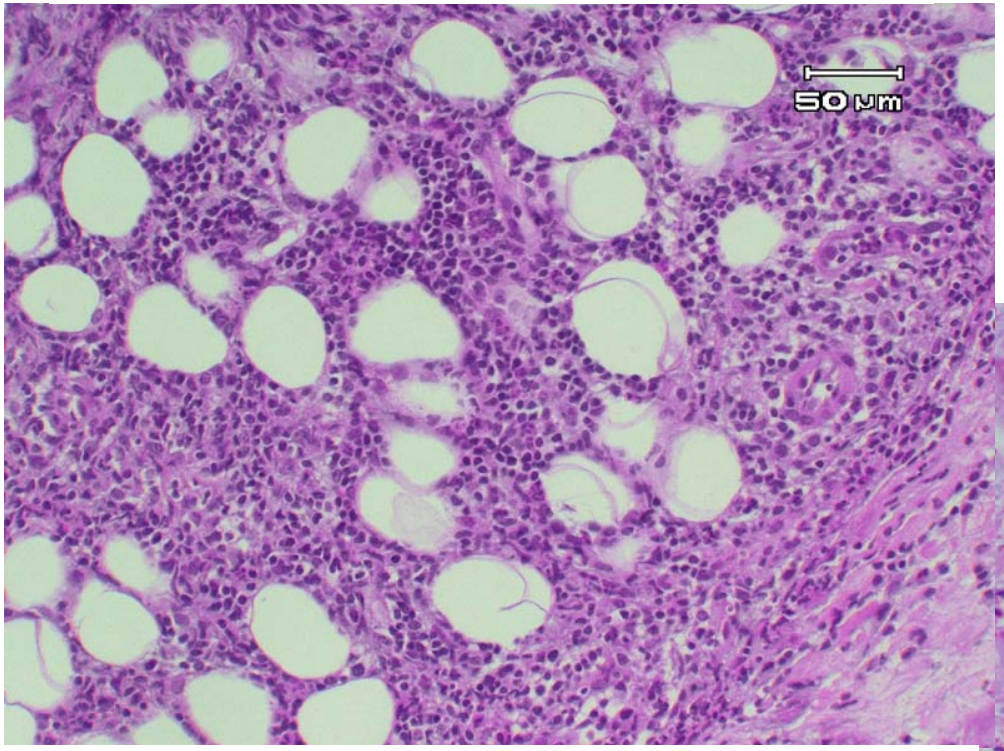
***in vitro* tests**
IL/IFNg

poor results

in vivo
Skin test (DTH)
(herd level)

Histopathological reaction should be further studied…

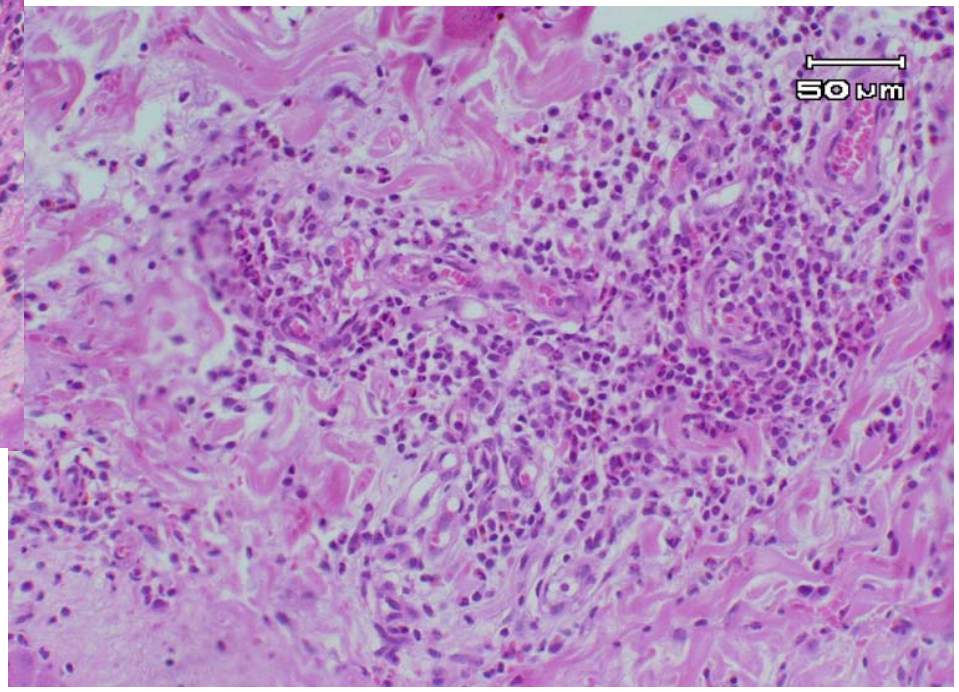
HIPERSENSITIVITY TYPE IV – DTH
(cell mediated response)



Large infiltrates of mononuclear cells

Mixed hypersensitivity reaction (Arthus +DTH) has been observed in pigs

HIPERSENSITIVITY TYPE III – ARTHUS
(antibody mediated response)



Hyperemia and perivascular infiltrates

Possible drawbacks to be taken into account…

- Animals have to be manipulated twice
- Interference with the immune status of the animal after injection :

  Anergy period

  Sensitization

Anergy state produced by the allergen

Local macrophages and T-cell remain inactive during a certain period after the DTH reaction

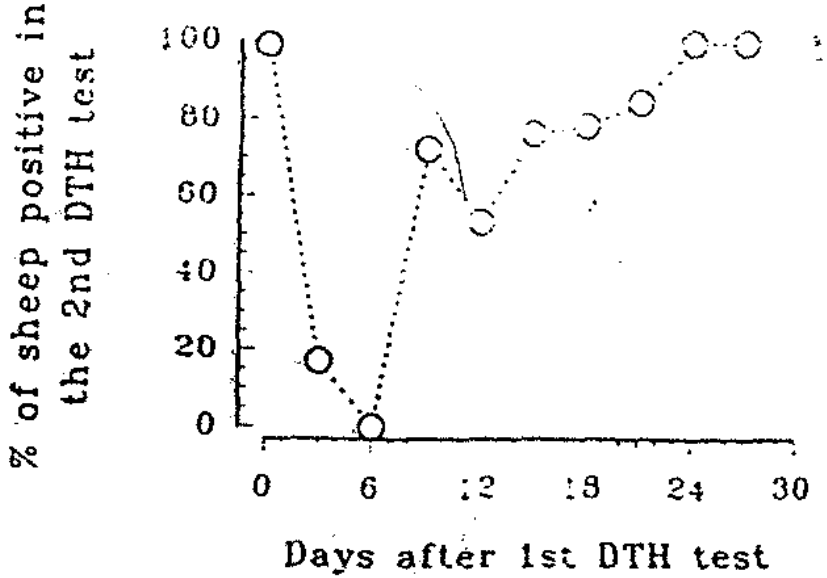
Anergy periods upon inoculation with Tuberculin or Brucellin allergens:

Tuberculin in cows: 60 days

Brucellin in cows: unknown

Brucellin in sheep: 24 days

Experimental data (Blasco *et al*, 1994)



Brucellin in pigs: NO anergy phenomenom observed.

Brucella infected pigs showing positive reaction in DTH test were grouped (n 12) and submitted to a second DTH test (same allergen and dose) at regular intervals (1, 2, 3 ,4 and 6 weeks) after the first inoculation. All pigs gave positive reaction after the second inoculation regardless of the elapsed time from the first one.

Sensitization produced by the allergen

Some allergens elicit the activation of macrophages and T-cells by themselve, leading to undesirables DTH positive reaction in non infected animals. Allergens must be free of sensitizer molecules.

Undesirable DTH reactions reported when using *Brucella* allergens containing hydrolitic polysaccharides (Alton G.C. Animal Brucellosis. CRC Press). No sensitization problems reported using *Brucella* citosolic proteins (Brucellin free of polysaccharide)

Purified protein derivative (PPD) products replaced heat-concentrated synthetic medium tuberculins.

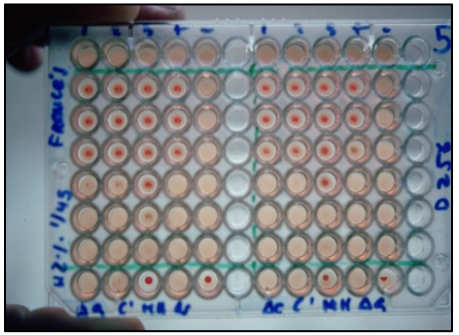
Possible drawbacks to be taken into account…

- Animals have to be manipulated twice
- Interference with the immune status of the animal after injection :
  - Anergy period
  - Sensitization
- Interferences in serological tests
  - Not necessarily when using different antigens
- Cross-reaction due to similar protein antigens

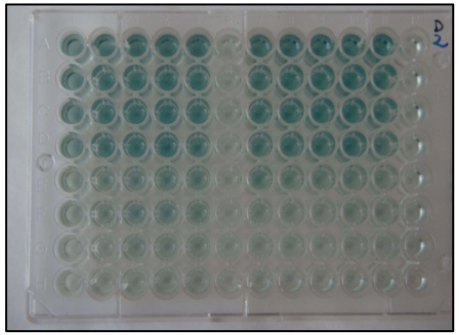
Skin test

Interferences in serological tests

RBT

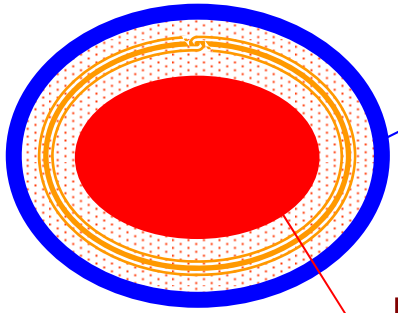


CFT



ELISA

Brucella



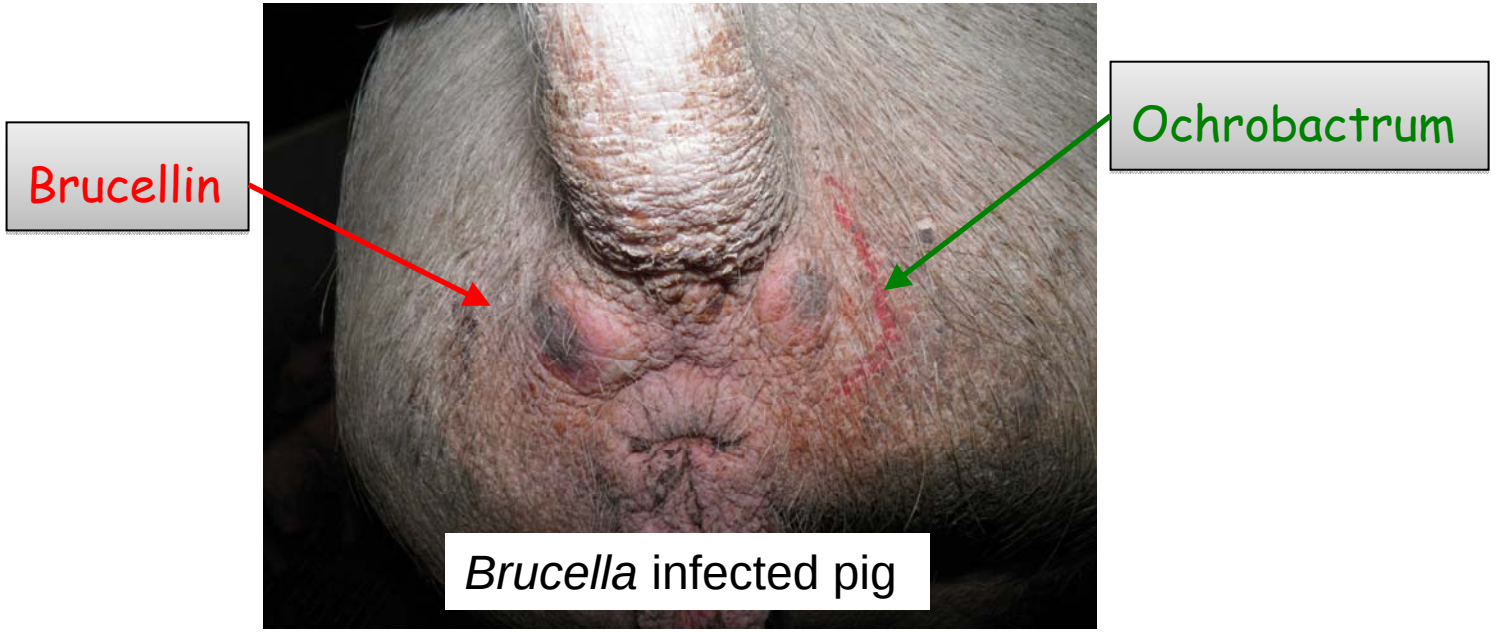
Serological tests applied for Brucellosis routinary diagnostic are based in surface smooth antigens (S-LPS / O-PS)

The use of cytosolic extracts from rough strains (free from O-PS precursors) prevent serological interference

Cross-reaction due to similar protein antigens

The comparative intradermal tuberculin test with bovine and avian PPD is used to differentiate between animals infected with M. bovis and those sensitised due to exposure to other mycobacteria or related genera.

Cross-reaction betwen *Brucella* and phylogenetic neighbors *(Ochrobactrum)* citosolic proteins has been experimentally demonstrated, but cause no significant trouble in field diagnostic



Possible drawbacks to be taken into account…

- Animals have to be manipulated twice

- Interference with the immune status of the animal after injection:

  Anergy period

  Sensitization

- Interferences in serological tests

  Not necessarily when using different antigens

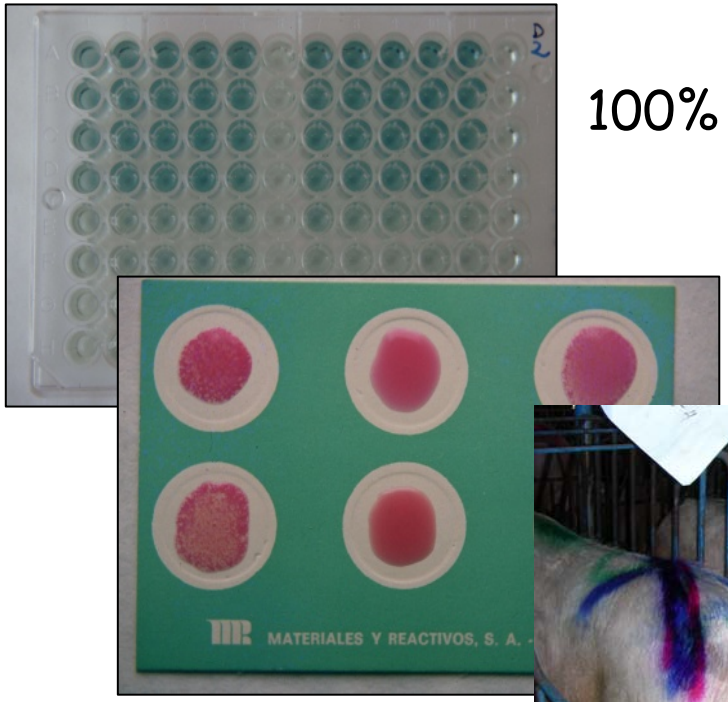
- Cross-reaction due to similar protein antigens

- Lacks specificity after vaccination

  Allergic reactions detected in vaccinated cows several years after vaccination with all Brucella vaccines

Message to take home…

Routinary diagnostic techniques have to be…



100% specific (ideally)

Diagnostic sensitivity as high as possible

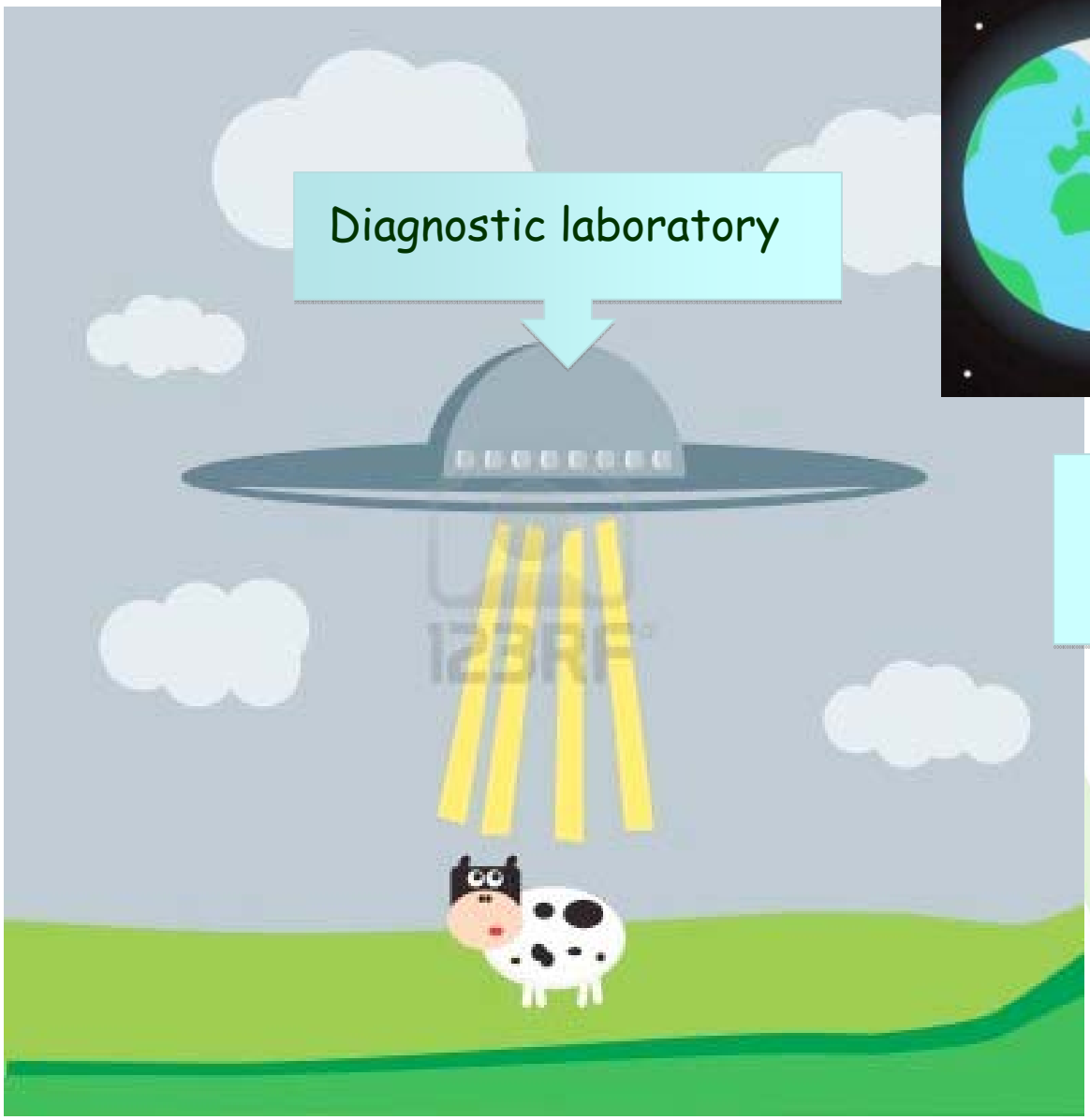
As EASY as possible

As CHEAP as possible

And above all…Well validated!!!

Diagnostic performance and cut-offs should be always determined using LOCAL Gold Standard populations

Message to take home…



Director of Control and Eradication strategies against animal infectious diseases

Thanks for your atention!!

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