



The Diagnosis of Brucellosis in cattle, sheep, goats & pigs

What is needed?

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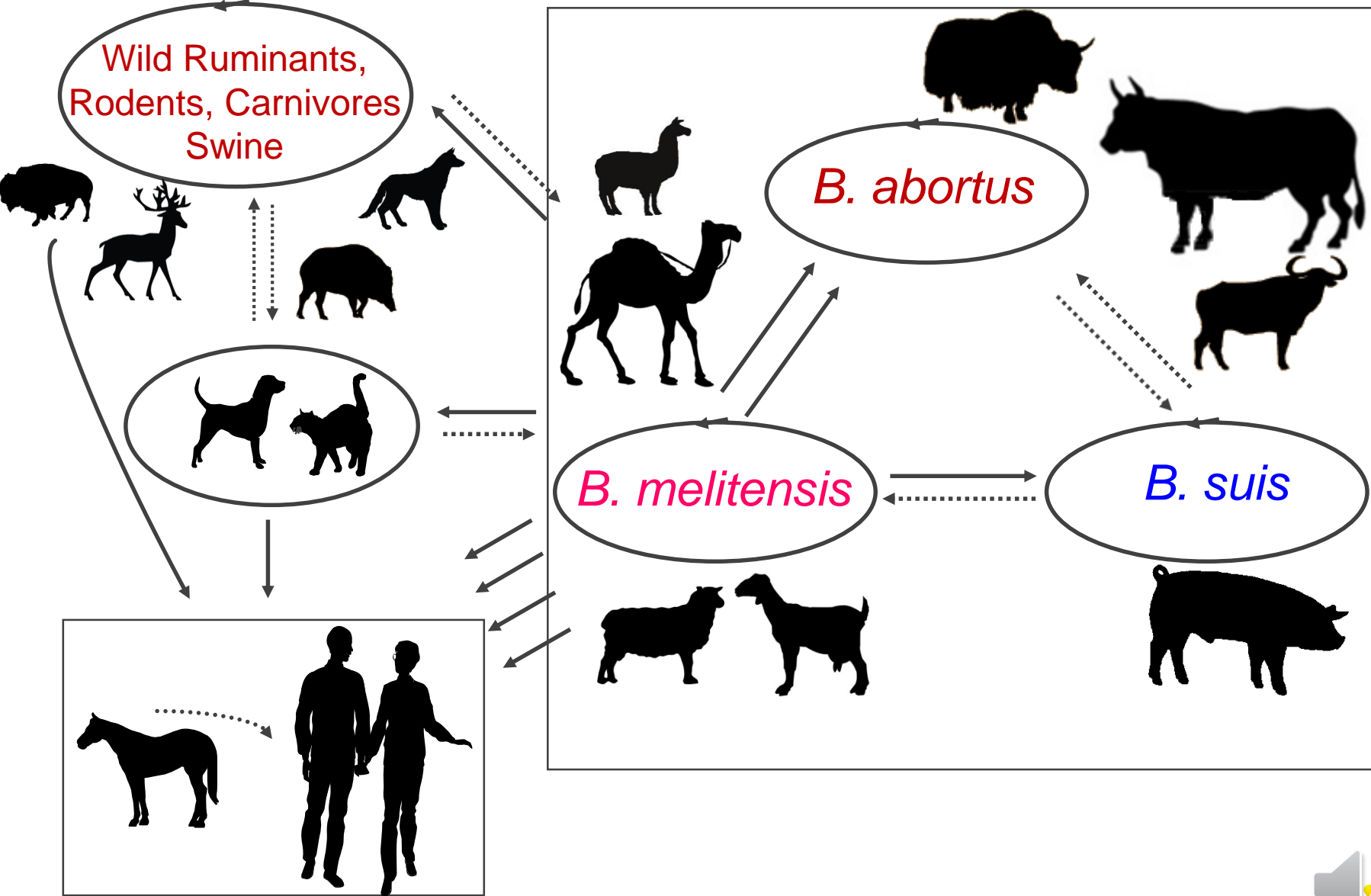
Brucellosis

- Due to *Brucella abortus, melitensis or suis*
 - Gram negative bacteria (α -proteobacteriaceae)
 - Mammals facultative intracellular pathogens
- Geographical distribution
 - Mediterranean countries, near- and middle east
 - Distributed world wide
- Clinical signs (non pathognomonic)
 - abortions, sterility, unthrifty offspring
 - orchitis & epididymitis (+hygromas)
 - *joints may be affected, causing lameness and sometimes paralysis (pigs)*

Brucella : species & biovars

Species	Biovars	Preferred natural host	Main geographical area	Pathogenicity for man
<i>B. melitensis</i>	1, 2, 3	Sheep, Goats, Wild ungulates	Mediterranean countries Middle & Near East	High
<i>B. abortus</i>	1, 2, 3, 4, 5, 6,(7), 9	Bovines, Wild ungulates	Europe, Americas, Africa, Asia	Moderate
<i>B. suis</i>	1	Suids	Americas, Asia, Oceania	High
	2	Suids, Hares	Central & Western Europe	Unknown
	3	Suids	USA, China	High
	4	Reindeer	USA, Canada, Russia	Moderate
	5	Wild rodents	Russia	High
<i>B. neotomae</i>		Desert wood rat <i>Neotoma lepida</i>	USA	Unknown
<i>B. ovis</i>		Sheep (males)	Mediterranean countries	No
<i>B. canis</i>		Dogs	USA, South America Central Europe	Low
<i>B. ceti</i>		Cetaceans	-	High / Unknown
<i>B. pinnipedialis</i>		Pinnipeds	-	High / Unknown
<i>B. microti</i>		Common vole	Central Europe	Unknown

Brucellosis - *the global cycle*



Epidemiology of Brucellosis....

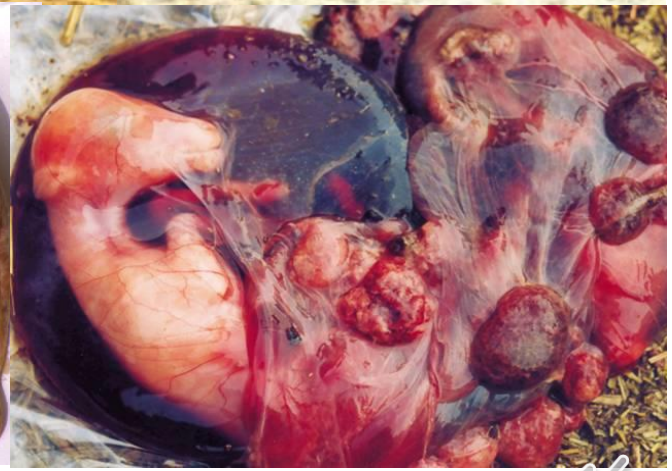
☞ Brucellosis is a "**multi-species**"
infectious and contagious disease...

- *different animal species*
- *different Brucella species*

.....to be considered



Abortions

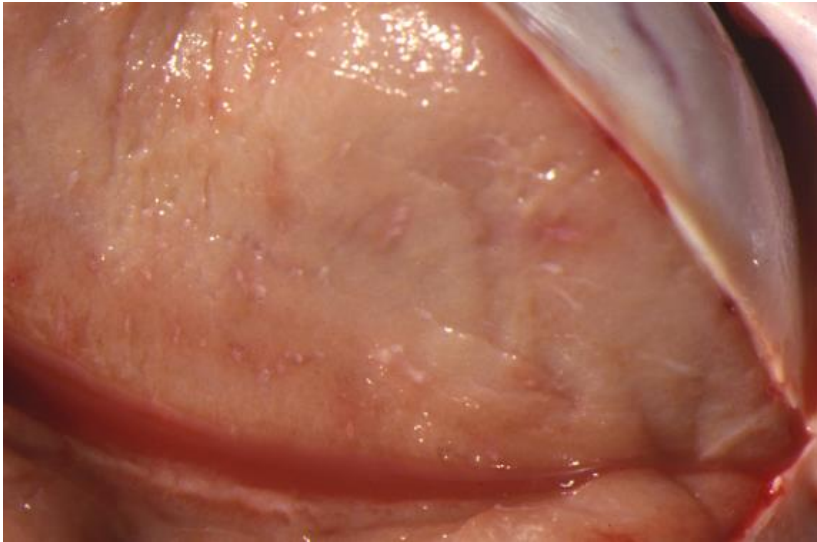


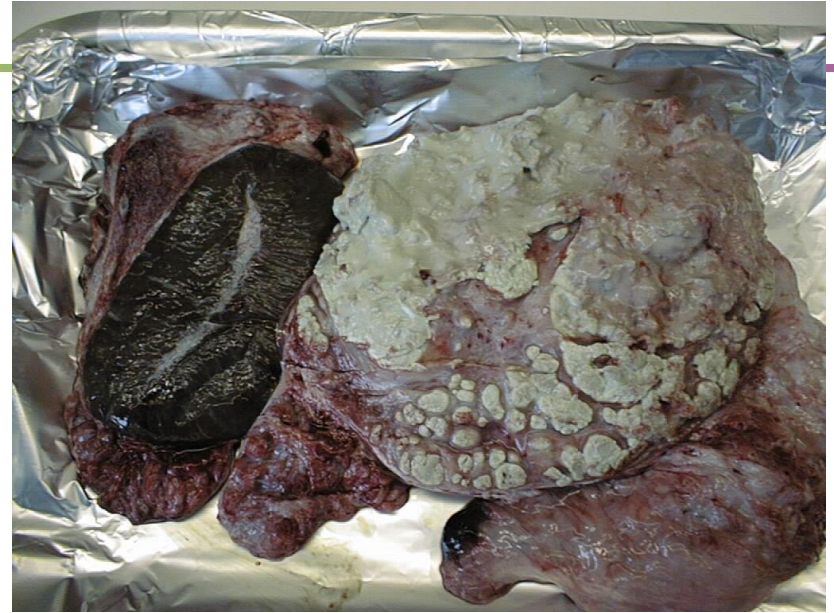


Endometritis



Orchitis in rams





Orchitis in pigs



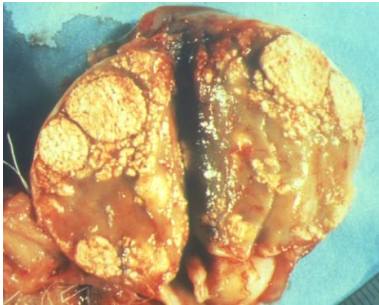
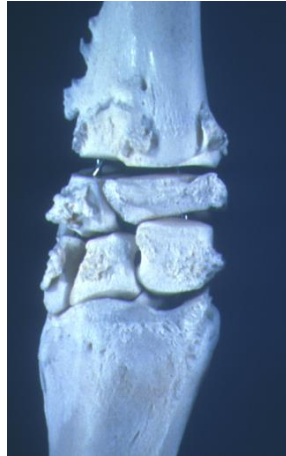
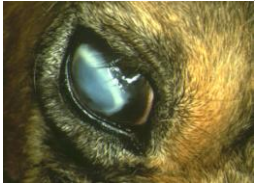
Wild ruminants – e.g. in the EU



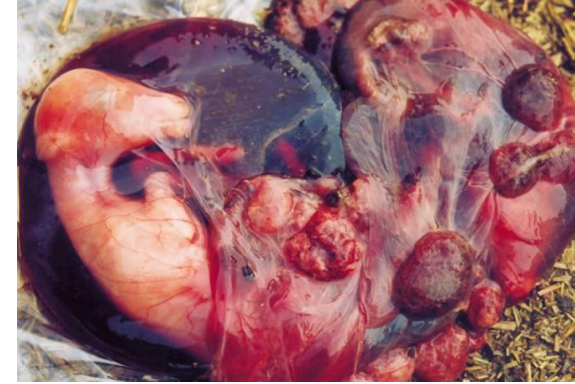
Chamois (*Rupicapra rupicapra*)



Alpine ibex (*Capra ibex*)



Epidemiology of Brucellosis



- ☞ Abortion is the main sign of brucellosis...
- ☞ **But, most infected females give birth normally...**
- *In both cases, huge and durable excretion of Brucella*



Diagnostic tools

- **Direct:**
 - **Detection of the *Brucella* and/or their specific components (Ag, Genes)**
- **Indirect**
 - **Measure of the immune response**

Diagnosis of Brucellosis....

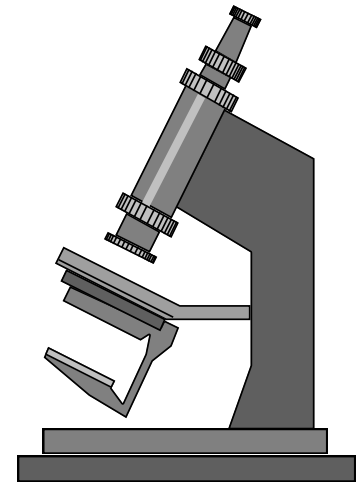
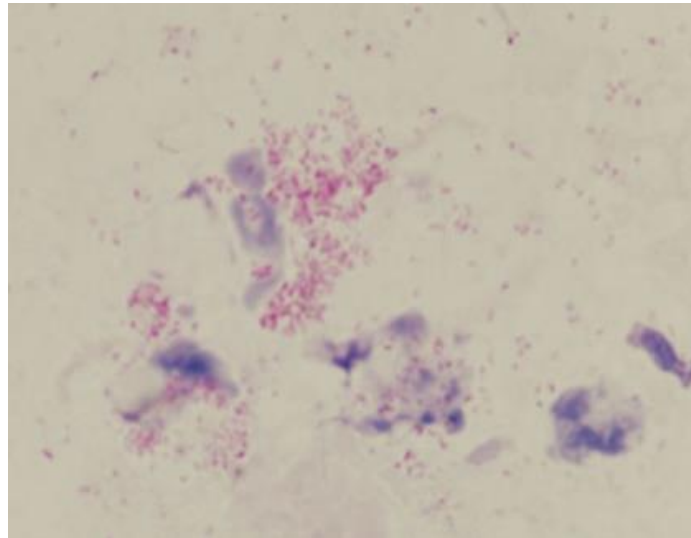
- ➡ No single test able to...
 - identify all infected animals, or
 - certify all free animals
- Tests repetitions needed
- Tests associations (parallel/series) needed
- ➡ BUT a test means... a standardised test which also means a validated test and biologicals regularly checked against standards (see OIE update)

Direct Diagnosis

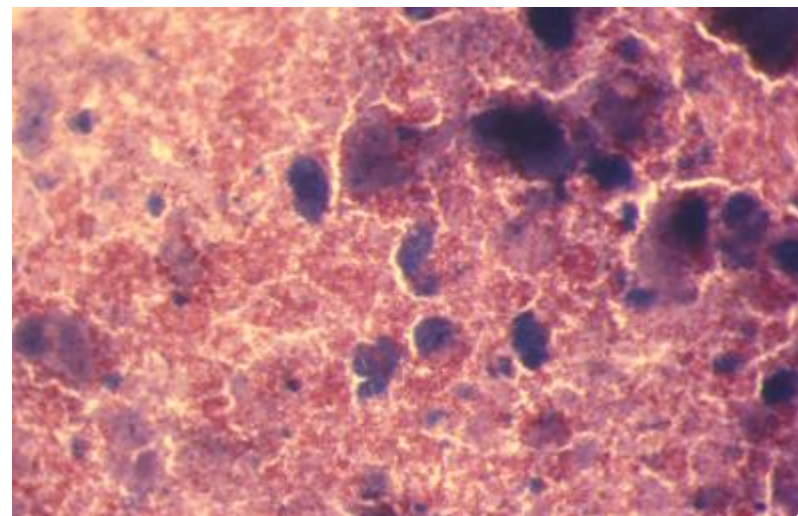
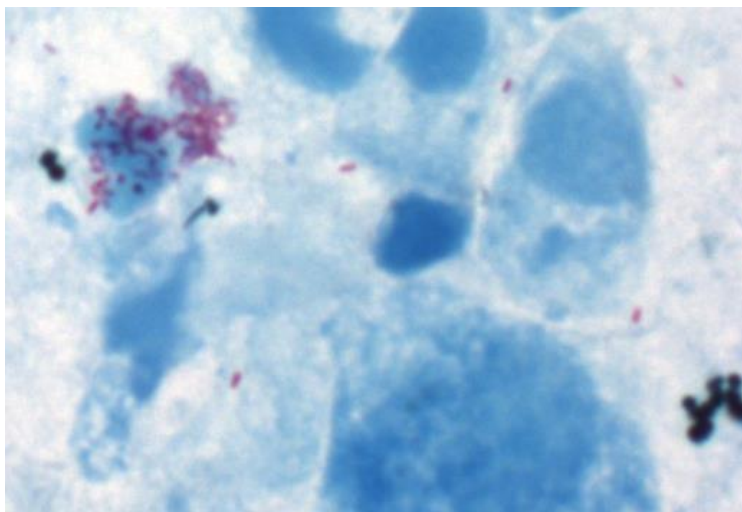
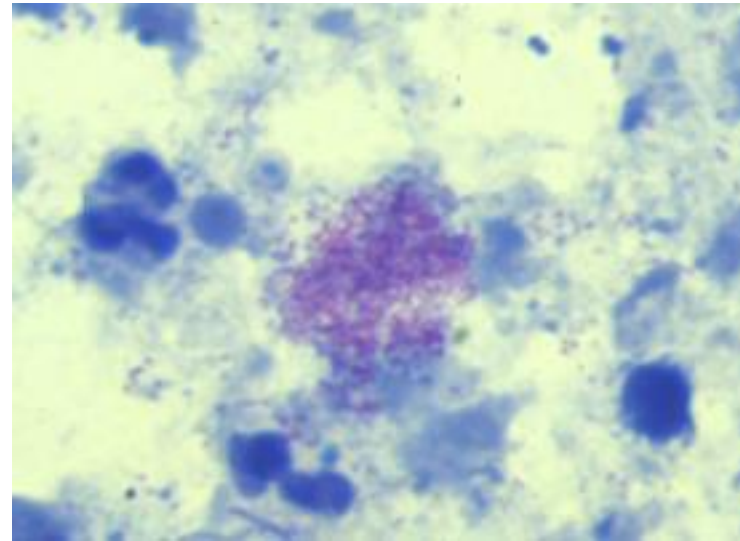
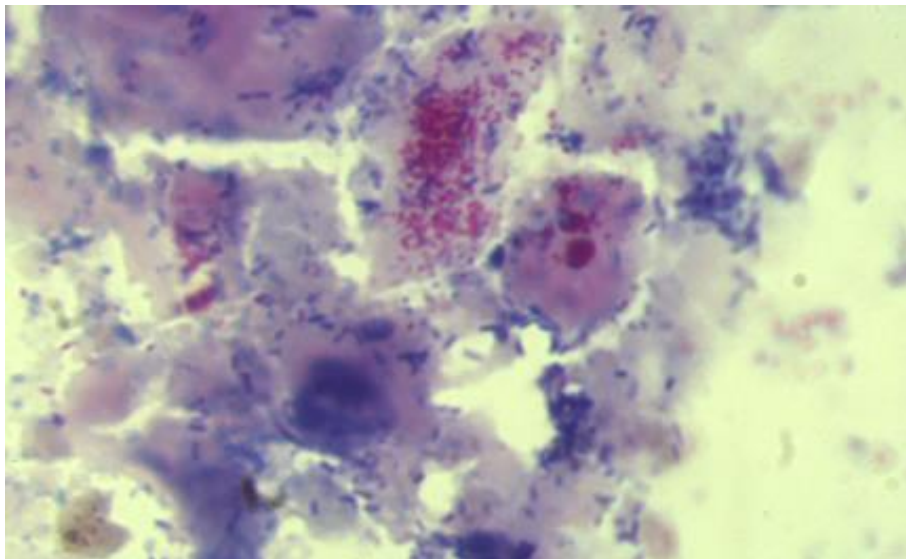
- Bacterioscopy
- Isolation & identification of *Brucella*
- *Antigens: Immuno-enzymology - fluorescence*
 - *Not practicable, no standardisation*
 - *Low specificity, low sensitivity*
- PCR

Bacterioscopy (Stamp)

- Samples to be ground
- Several smears needed
 - **Advantages: quick and simple**
 - **Disadvantage: presumptive value**
 - False negative
 - False positive (*B. ovis*, *Chlamydia*, *Coxiella*,..)



Stamp staining (modified Ziehl-Neelsen)



Direct Diagnosis

- Bacterioscopy
- Isolation & identification of *Brucella*
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Isolation & identification of *Brucella*

- **The only unequivocal method**
- **Identification = definitive diagnosis**



- High epidemiological value: biotyping
- Relatively expensive, long lasting
- Bio-hazard: needs expertise, procedures and equipment
- Lack of sensitivity
- Sample sometimes unavailable
(milk, foeto-maternal materials, genital secretions, lymph nodes,...)

Specimens for *Brucella* isolation

	Live animal	Slaughtered animal
Female	Vaginal discharges Milk*	Lymph nodes** Spleen** Udder** Uterus**
Male	Semen	Lymph nodes ** Spleen ** Epididymes** Sexual accessory glands **

* Cream + pellet

** Ground (stomacher)

Distribution of *Brucella* infection (cattle)

- **Australia** (Hornitsky, 1986)

➤ Mam.	79.6%
➤ Mam. + Sc.	89.8%
➤ Mam. + Sc. + RP	93.9%
➤ Mam. + Sc. + RP + Mand.	98.0%
➤ Mam. + Sc. + RP + Mand. + Ili	100.0%
<i>(# culture + = 86% CFT+ animals)</i>	

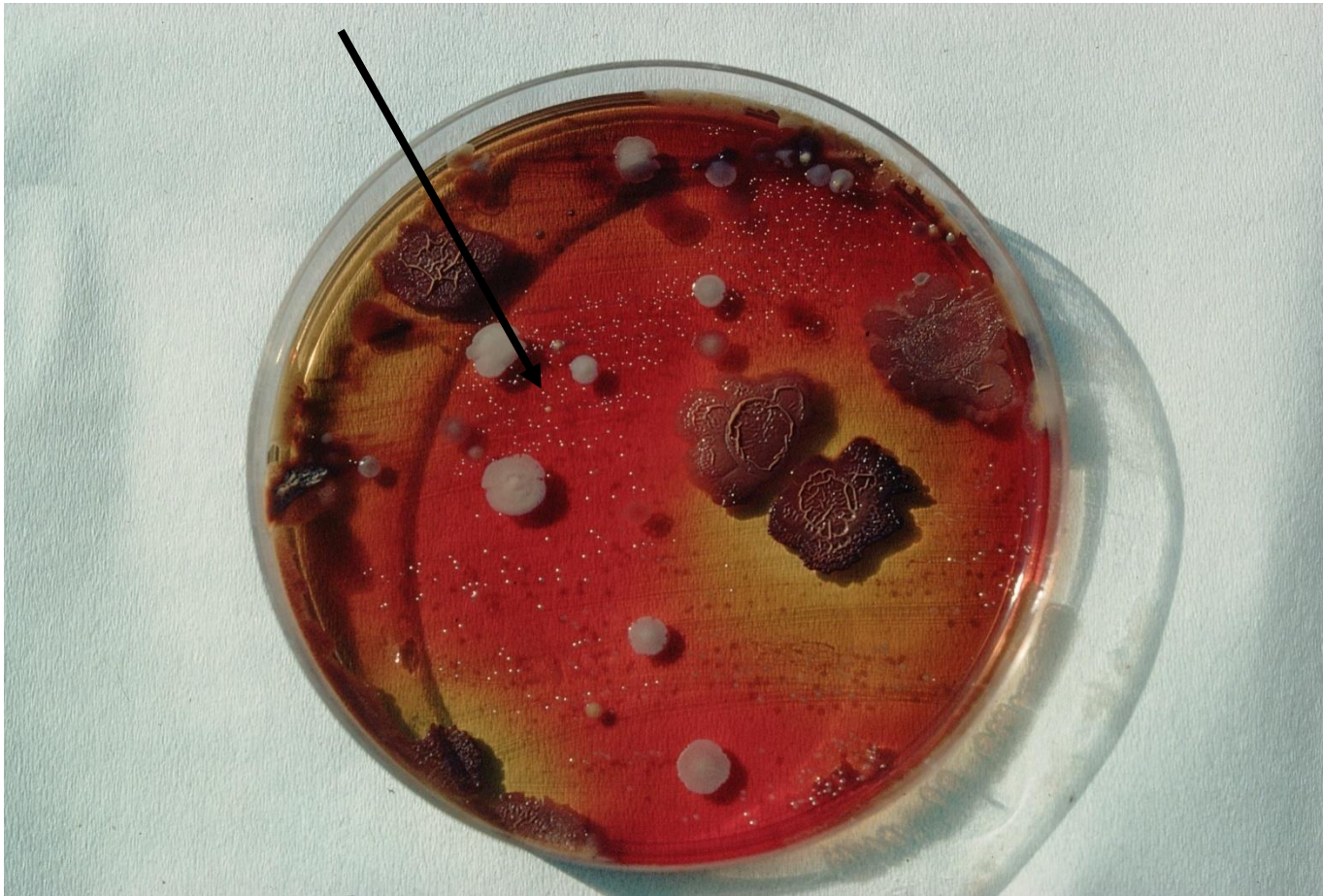
- **Northern-Ireland** (1999-2001) *2 dishes/organ (n=342)*

➤ L.N.	Par	RP	SM	RM
➤ Pos.	60%	81%	66%	82%
➤ Pos. alone	1.7%	6.2%	0.7%	8.9%

Distribution of *Brucella* infection (sheep & goats)

	172 Sheep & goats <i>Blasco et al. 2002</i>	142 Sheep <i>Marín et al. 1996</i>	40 Goats <i>Marín et al. 1996</i>
Cranial L.N.	37.4 %	33.8 %	80.0 %
Scapular L.N.	26.4 %	33.8 %	50.0 %
Prefemoral L.N.	-	36.6 %	47.5 %
Iliac L.N.	46.1 %	51.4 %	65.0 %
Mammary L.N.	69.2 %	81.7 %	82.5 %
Spleen	28.0 %	36.0 %	25.0 %
Uterus	17.6 %	19.7 %	25.0 %
Milk	60.9 %	62.5 %	74.3 %

Brucella on Blood Agar



 ***Selective media almost always needed***

Selective media

Farrell

Base:

SDA, BAB or BMB
+ 5 % serum

Nalidixic acid	5 mg
Bacitracin	25 000 UI
Natamycin	50 mg
Polymyxin B (sulf.)	5 000 UI
Nystatin	100 000 UI
Vancomycin	20 mg

(Oxoid SR209A)

Modified Thayer-Martin

(Brown et al. - Marín et al. modification)

Base:

GC medium
Haemoglobin sol. 10 %

Vancomycin	3 mg <i>(20 mg)*</i>
Colistin	7.5 mg
Nystatin	100 000 UI
Nitrofurantoin	10 mg
Amphotericin B	2.5 mg <i>(4 mg)*</i>

**CITA medium*

Comparison of Farrell and m. Thayer-Martin

<i>Brucella</i> species	Medium	mean CFU
23 <i>B. abortus</i>	Farrell	53.86
23 <i>B. abortus</i>	m T-M	63.16
31 <i>B. melitensis</i>	Farrell	74.48
31 <i>B. melitensis</i>	m T-M	99.50

- 182 infected animals

- 172 Farrell +

(Marín *et al* 1996)

- 180 m T-M +

- 182 Farrell + or m T-M +

☞ ***Simultaneous use of Farrell + mT-M media increase the sensitivity of bacteriological diagnosis***

Presumptive identification

- Clinical & Epidemiological context
- Growth on Farrell / mT-M (slow > 3-4 days)
- Morphology of colonies
(smooth, homogenous, glossy, etc.)
- Gram negative coccobacilli
- Agglutination of anti-*Brucella* serum
- Catalase +, Oxidase +, Urease +
- No use of sugars

 ***Typing : expert laboratories***

Direct Diagnosis

- Bacterioscopy
- Isolation & identification of *Brucella*
- *Antigens: Immuno-enzymology - fluorescence*
 - *Not practicable, no standardisation*
 - *Low specificity, low sensitivity*
- PCR

Direct diagnosis by PCR

- *bscp 31 Kd*
 - 16S rRNA
 - IS 711/6501
 - ☞ Specificity: genus *Brucella*
 - ☞ Sensitivity ??
-
- ***No great/long experience in field conditions***
 - ***Real-time PCR under validation***

PCR IS711 : vaginal swabs

Bacteriology			
PCR	+	-	
+	5	21	26
-	0	8	8
	5	29	34

PCR IS711 : organs

Spleen	Bacteriology		Total
PCR	+	-	
+	13	5	18
-	4	10	14
	17	15	32

L.N.	Bacteriology		Total
PCR	+	-	
+	11	18	29
-	0	1	1
	11	19	30

➤ ***PCR is a good complementary test but could not replace bacteriology up to now in all situations....***

Added value of Real time PCR

<i>Brucella</i>	IS711 Nb copies	Conventional single PCR *			PCR RT*		
		IS 711	<i>bcsp</i> 31	<i>per</i>	IS711	<i>bcsp</i> 31	<i>per</i>
<i>B. abortus</i> 544	6 to 8	100	1000	1000	2	2	2
<i>B. melitensis</i> 16M	7 to 10	1000	1000	1000	2	2	2
<i>B. ovis</i> 63/290	> 20	100	1000	1000	0.2	2	2

*Lower limit of detection in fg

Bounaadja *et al.* (2009)

- **Sensitivity** ↗
- **Limits contaminations**

Direct diagnosis (conclusion)

Isolation (or PCR) & Typing of *Brucella*

- **Advantage:** unequivocal diagnosis
- **Disadvantage:** long and expensive, limited to equipped and experienced labs.
- **Not applicable at all stages of an eradication program (too many outbreaks)**
- **Essential in the last stages:**
 - Diagnosis confirmation
 - Trace-back and forward tracing



Diagnostic tools

- **Direct:**
 - Detection of the *Brucella* and/or their specific components
 - **Indirect**
 - Measure of the immune response
- ***Essential in surveillance, control and eradication programmes.***

Immune response of the infected host

Brucella =
Facultative intracellular pathogens



Cell response (DTH)

&

Humoral response (antibodies)

Indirect diagnosis

☞ Serological tests

- Early, sensitive but low specificity (RBT/FPA))
- Sensitive but lower specificity (iELISA – pool possible)
- Late, more specific but less sensitive (CFT)
- Specific \geq but the lowest sensitivity (cELISA)
- Highly sensitive/specific (Milk iELISA > Milk ring test)
 - \neq tests: \neq antibodies detected

☞ Cell tests: Brucellin Skin Test (BST)

highly specific, but not usable in vaccinated animals

- Frequent discrepancies between tests
- Associations usually needed

Immune response of the infected host - Antibodies

- **Foetus**
 - congenital infection – no Ab before 1st gestation
 - **Young**
 - low and transitory response
 - **Adults**
 - Response in 1-2 months, sometimes no or low
 - Persistence 6 months or more
 - Fluctuant (calving/abortions) - **milk**
-
- ☞ **Latent infection - abortion, lambing**
 - ☞ **Great individual variations**
 - ☞ **Tests repetition - Discordance - vaccination**

Immune response of the infected host

Brucella =

Facultative intracellular pathogens

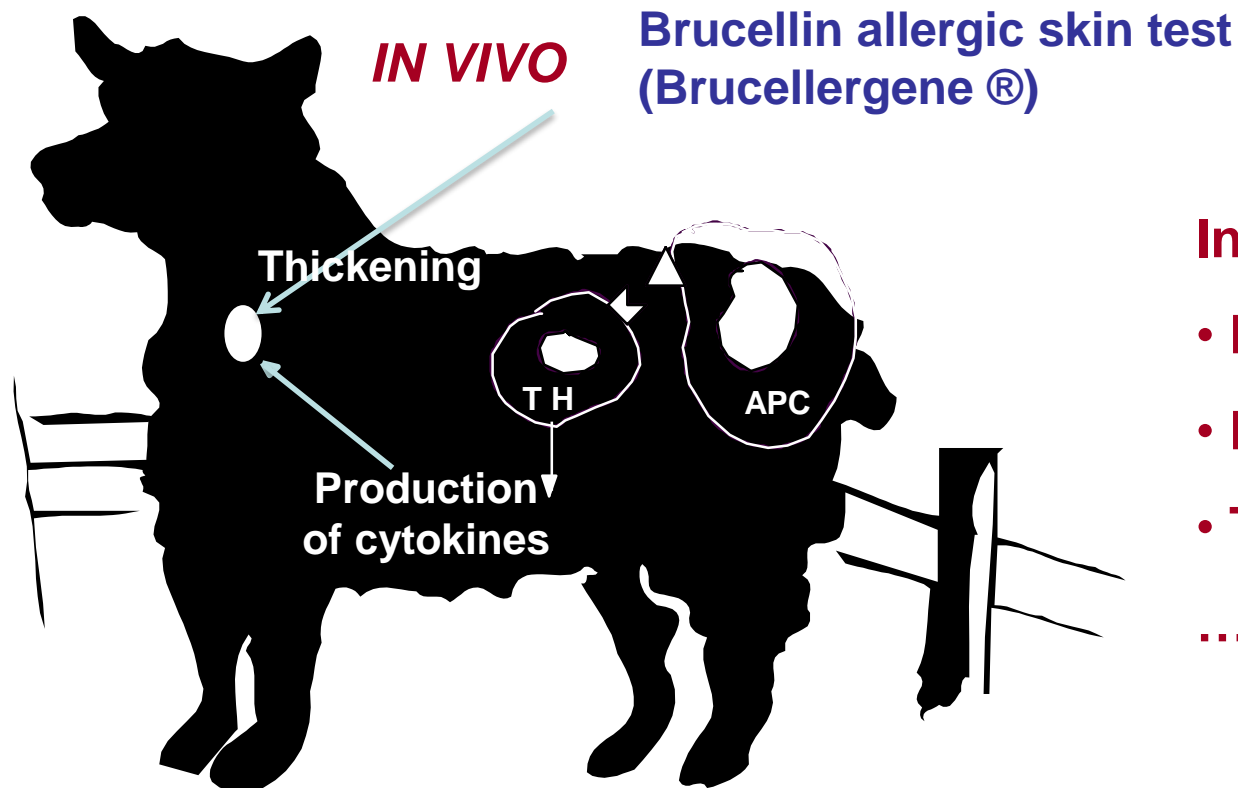


Cell response (DTH)

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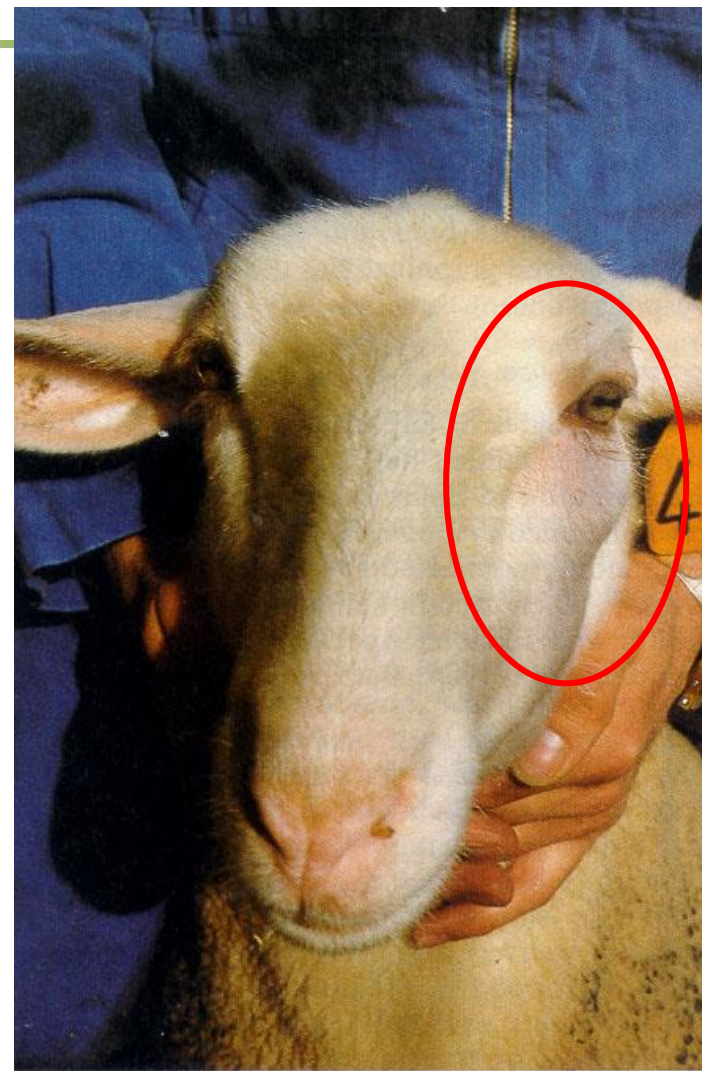
Humoral response (antibodies)

The cell response



In adults:

- Rapid
 - Persistent
 - To any *Brucella*
-including vaccines



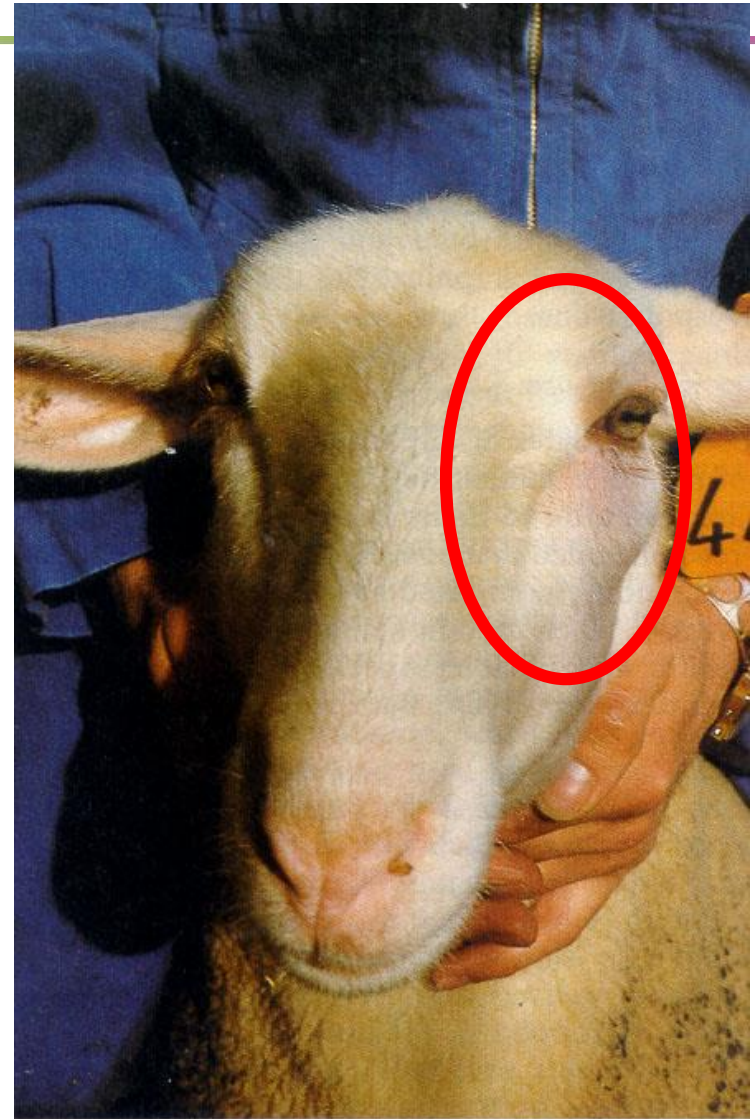
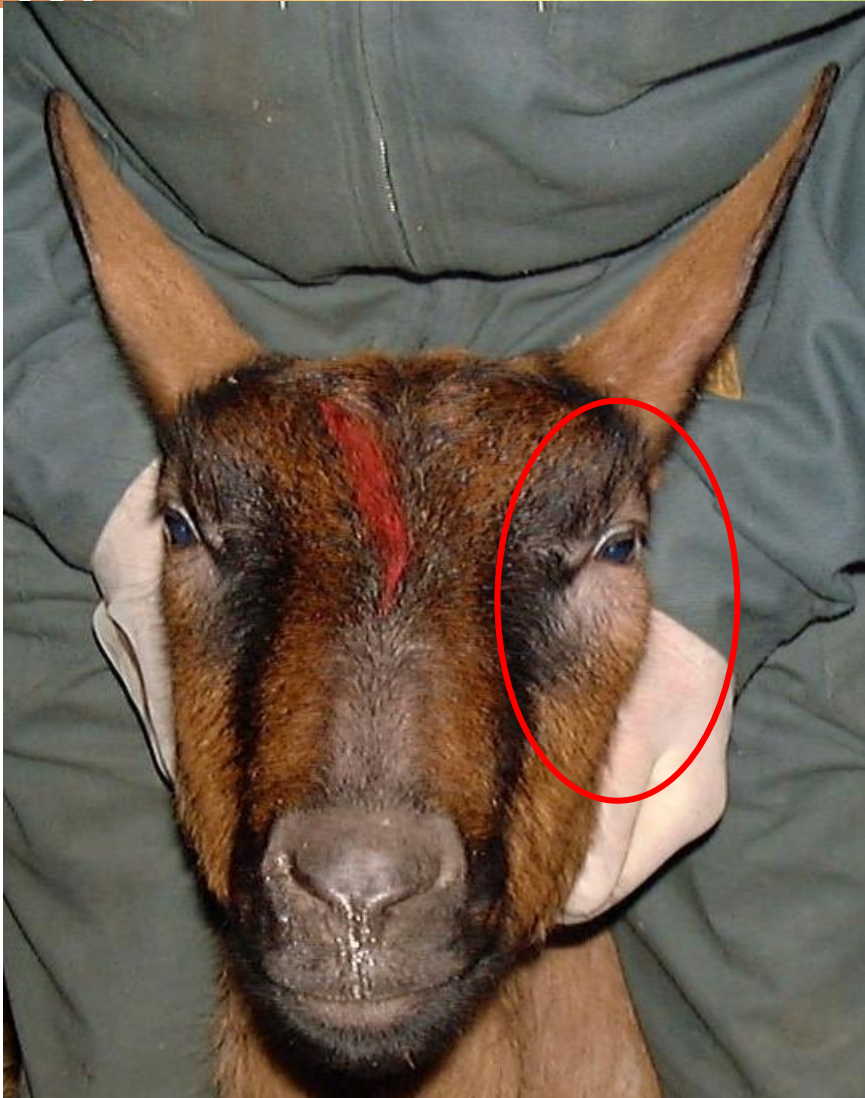
Intradermic
Measure of skin thickening
Reading at J+72h

Brucellin AST

Intradermic/subcutaneous
Reading at J+48h



Brucellin AST



Brucellin AST

Immune response of the infected host

Brucella =

Facultative intracellular pathogens



Cell response (DTH)

&

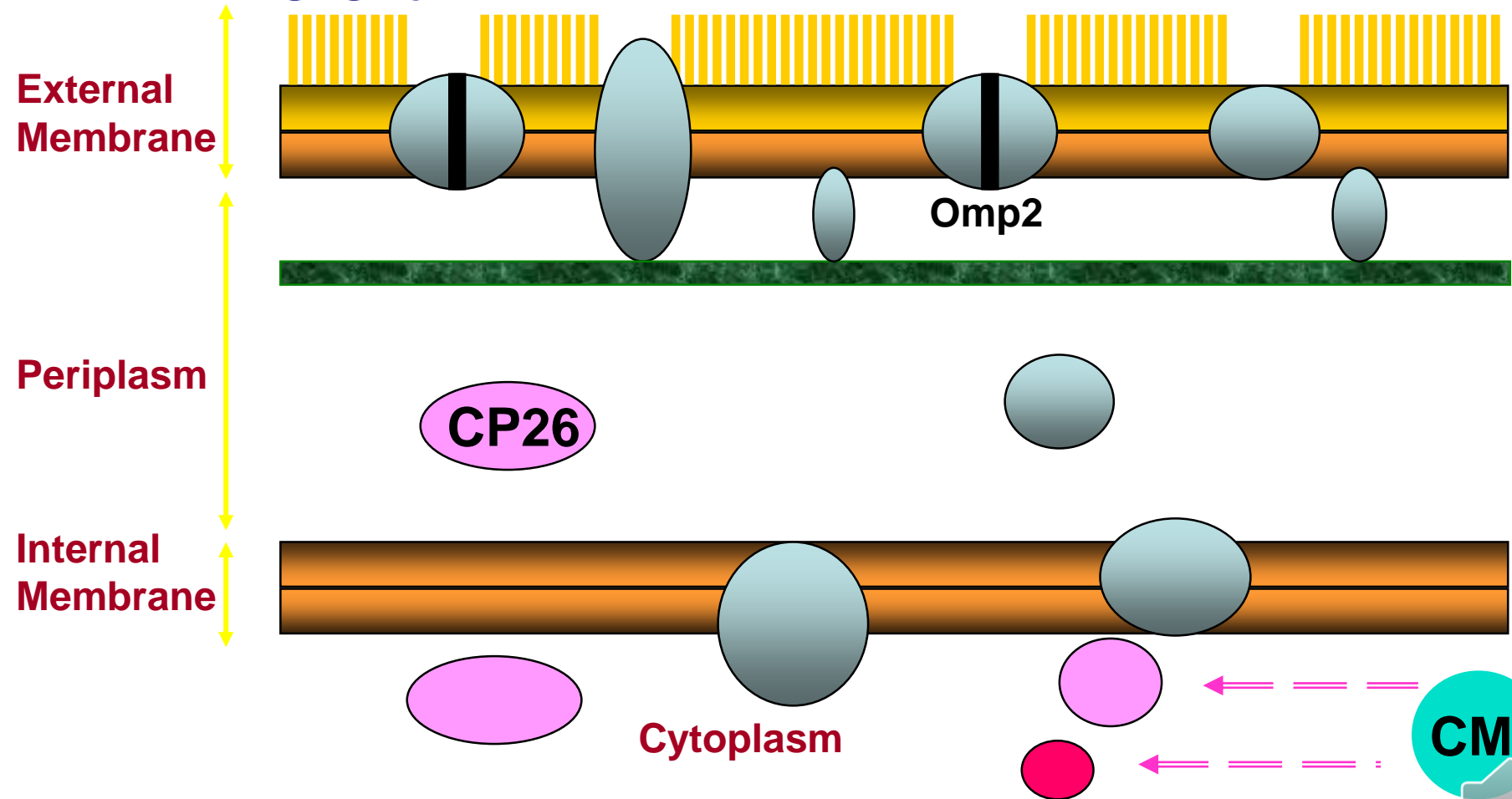
Humoral response (antibodies)

The S-LPS of *Brucella* – The Major antigen

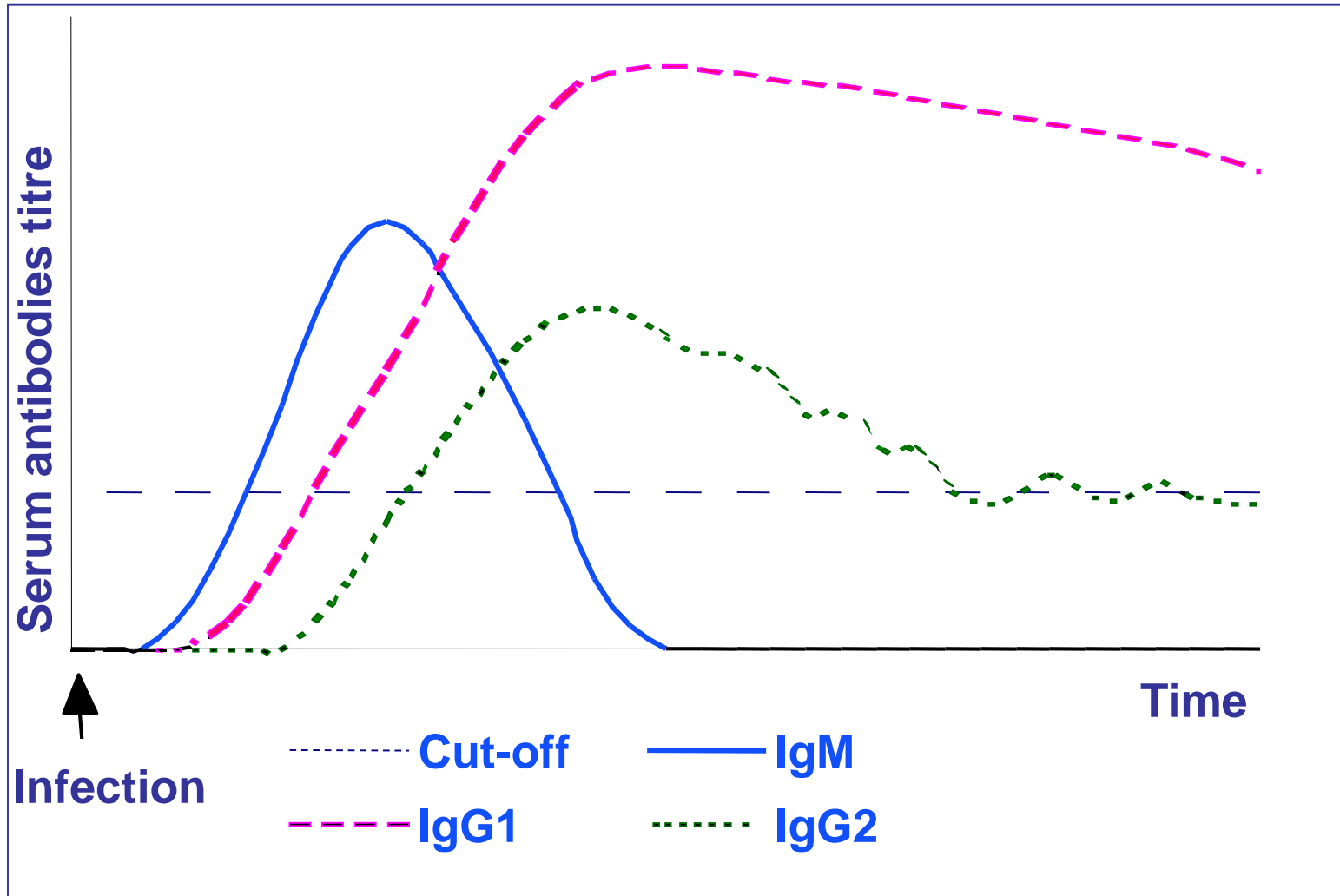
Ab

➤ The main cause of cross-reactions!!

O-Chain



Serological tests – old tools



Anti-*Brucella* post-infection antibodies
Schematic evolution curve

Immune response effectors in brucellosis

Immune mechanisms

Immunoglobulins

Tests	Immunoglobulins				Sensitised T-cells
	IgG1	IgG2	IgM	IgA	
SAT	-	+	+	-	-
RBT	+	-	+	-	-
CFT	+	-	+/-	-	-
iELISA	+	+	+/-	+/-	-
MRT	+/-	+/-	++	++	-
CMI	-	-	-	-	+

Immune response: great individual variability

Possible situations (*Plommet, 1984*)

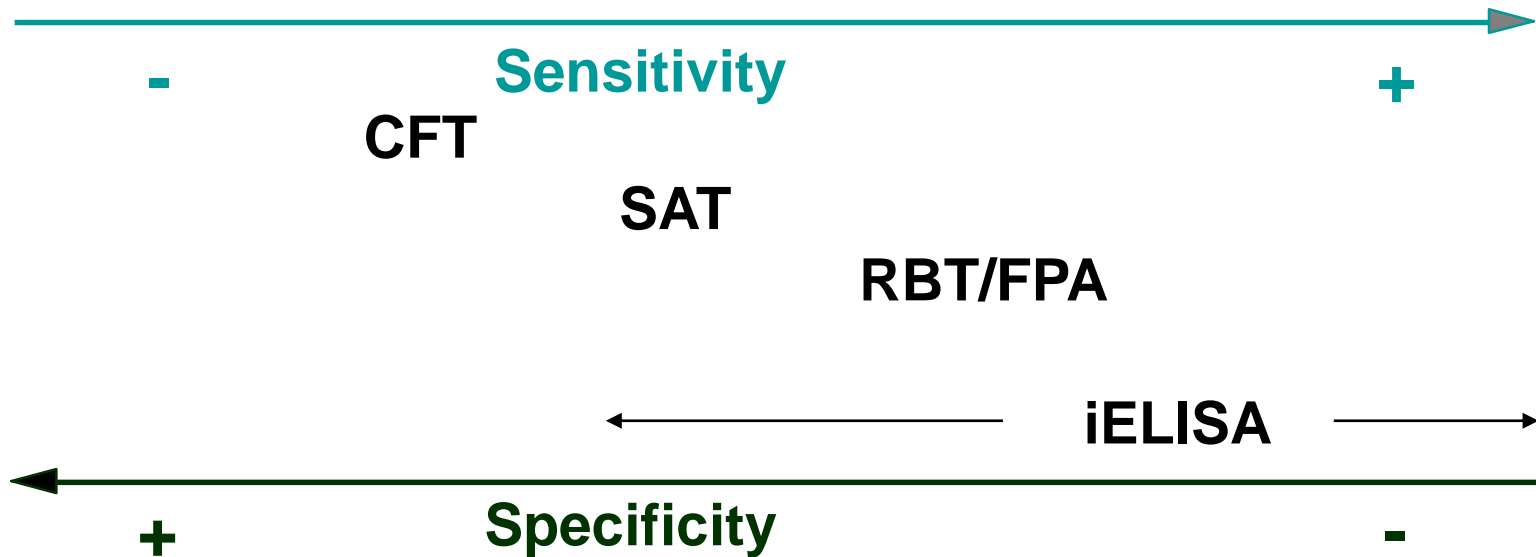
Test	1	2	3	4	5	6	7	8
Serology	+	-	-	+	+	+	-	-
Brucellin	+	-	-	-	+	-	+	+
Ring-Test	+	-	+	-	-	+	-	+
Culture (milk)	+	-	+	-	-	+	-	+
Culture (L.N.)		+			+	+		
Interpretation	+	+	+	?	+	+	+	+

Sensitivity & specificity

- Ability to detect anti-S-LPS antibodies +

- Brucellosis outbreaks detected +

- *FPSR herds/flocks detected* +



Serological tests – “old” tools

- *SAT (cattle)*
 - Rose Bengal (RBT)
 - iELISA (serum & bovine milk)
 - Milk ring-Test (bovine milk)
 - Generally used as “*screening*” tests
 - Complement fixation (CFT)
 - Generally used as a “*confirmatory*” test
- ☞ RB, CF & iELISA = the only OIE official tests in S&G

Serological tests – “old” tools (bovine milk)

- MRT
 - Sensitivity & specificity if repeated (cattle only)
 - false negative : udder infection needed, large tank bulk samples, non-milking animals
 - false positive : colostrum, mastitis, dried-off cows
- Milk iELISA
 - Good sensitivity & specificity

Serological tests – old tools (serum)

- All tests
 - Sensitive to antibodies induced by all *S-Brucella* species and biovars (*abortus*, *melitensis* and *suis*)
- RBT
 - Early detection
 - Lacks sensitivity (in sheep particularly)
 - Lacks specificity (in low prevalence or free areas)
 - Sensitive to vaccine-induced antibodies
- CFT
 - Later but prolonged detection
 - Lacks sensitivity (in recently infected animals)
 - Lacks specificity (but less than RBT)
 - Sensitive (less) to vaccine-induced antibodies

Serological old tools - *How to minimise failings?*

- Modification of RBT (75/25 vs. 25/25)
➤➤ the sensitivity
- Use of complementary tools
 - NH-GDT, less sensitive but more specific of the infection (sub-cutaneously vaccinated flocks)
 - AST, in unvaccinated flocks
 - Culture/PCR in vaccinated flocks in low prevalence or free areas
- Use of epidemiology-based strategy of:
 - performing tests: frequency
 - interpreting tests results (in parallel vs. in series)

Serological old tools - *Despite these failings?*

- In infected flocks/areas

- The predictive value of positive results in either test is close to 100%
- RBT has a very high flock sensitivity
- The use of both tests in parallel ↗↗ greatly the individual sensitivity
- Antibodies due to vaccination avoided by the use of the conjunctival route in replacement animals

- In low prevalence or free areas

FPSR (*Y. ent.* O:9) could be identified by:

- The very low proportion of positive results per flock/herd
- The low levels and duration of antibodies
- The use of the brucellin skin test

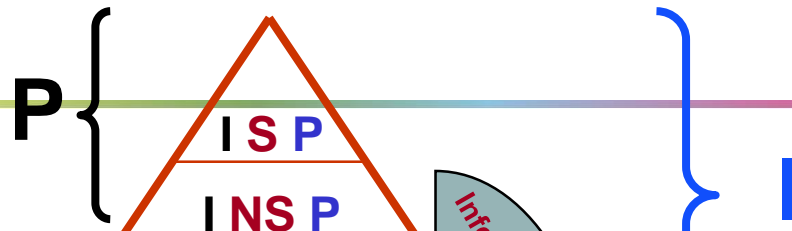
☞ **Eradication in cattle reached in many countries**

☞ **Eradication in S. & G. reached in France (2003), Cyprus and Northern Italy & Spain**

Serological tests – « new » tools

- *Protein-iELISA: very low sensitivity and specificity*
 - **S-LPS iELISA:** *sens. > RBT & CFT, but spe. < RBT/CFT*
 - Standardised in cattle and in S&G
 - And highly sensitive to vaccine induced antibodies
 - Could be used in pools of 10 sera in cattle
 - No validation at large scale in field conditions in S&G
 - Approved in cattle in bulk serum or milk samples
 - ☞ **Promising as replacing RB**
 - **C-ELISA:**
 - low sensitivity and specificity in cattle
 - In sheep & goats? First results disappointing
 - **Fluorescence polarisation Assay:**
 - OIE & EU official test in cattle (very sensitive but expensive)
 - In sheep and goats?
- ☞ **Pigs ????? Associations of tests needed for increasing sensitivity and/or specificity**

INFECTED UNIT
(herd / flock / area)



Infected/ Not infected
Shedding / Not shedding
Test Positive / Negative

I /NI
S/NS
P/N

Relative rates of each category depends on:

- *Outbreak history*
- *Control measures*

NI NS N

NI NS P

FPSR

Control means:

- ☞ **To protect naïve animals (vaccination)**
- ☞ **To identify and eradicate infection more rapidly than it spreads**



Control, surveillance & Eradication of animal Brucellosis...

👉 **Diagnosis is a critical key...**

- *Appropriate standardised and controlled biologicals (OIE)*
- *Appropriate performance (SOPs, ISO 17025)*
- *Tests associations (series or parallel)*
 - 👉 to increase the result predictive values
- *Test result interpretation...always in relation with:*
 - risk-factors
 - status of the herd, the area, the country

New tools needed but....

....epidemiology-based strategy essential for sound testing regime design & result interpretation.

« In some cases, it would be more profitable to make better use of existing procedures than to continue to develop new ones.»

R.J. Chappel, Surveillance, 1989, 16, 3.



Merci de votre attention

Dankie vir jou aandag

Thank you

