



# Traitement des infections à germes intracellulaires

*Pr. Philippe BROUQUI*

*Service des Maladies Infectieuses et Tropicales*

*CHU Nord, Pole MIT AP-HM.*

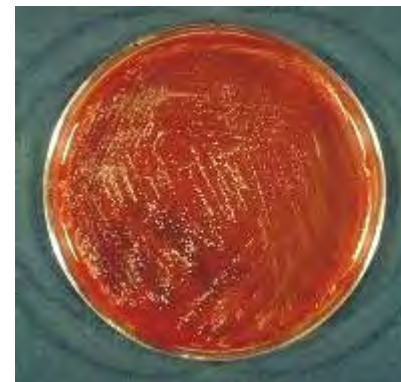
*Unité des Rickettsies CNRS UMR 6020*

*Marseille*



# Introduction

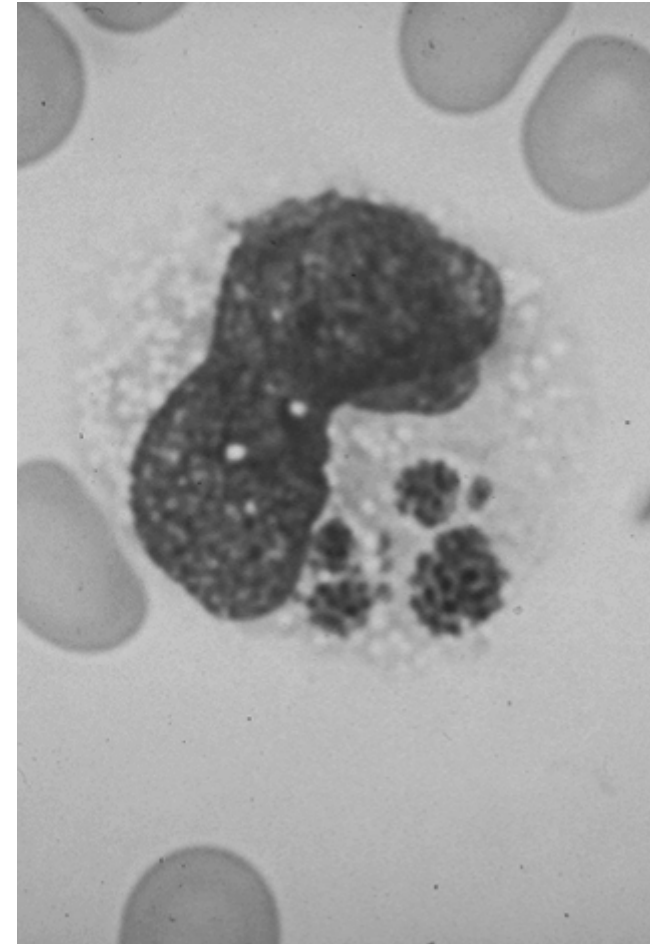
- Concept différent
- Pharmacocinétique
- Antibiogramme
- Identification moléculaire des résistances aux antibiotiques
- Femme enceinte et enfant
- Nouvelles molécules
- Traitements courts
- Traitements alternatifs





# Définition

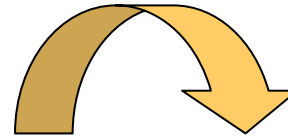
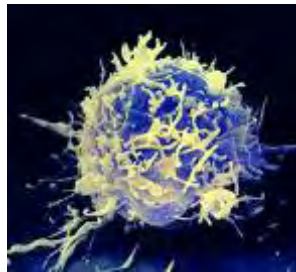
- Bactérie intracellulaire
  - Notion de sanctuaire
  - Pas d'antibiogramme
  - Intérêt de la biologie moléculaire



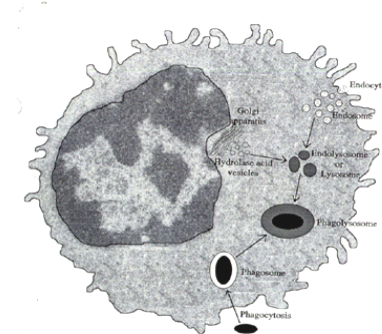
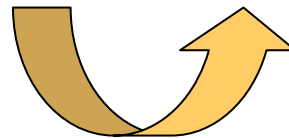
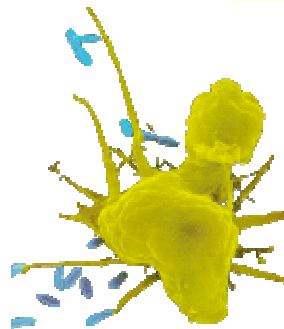


# Pharmacokinetic

Blood

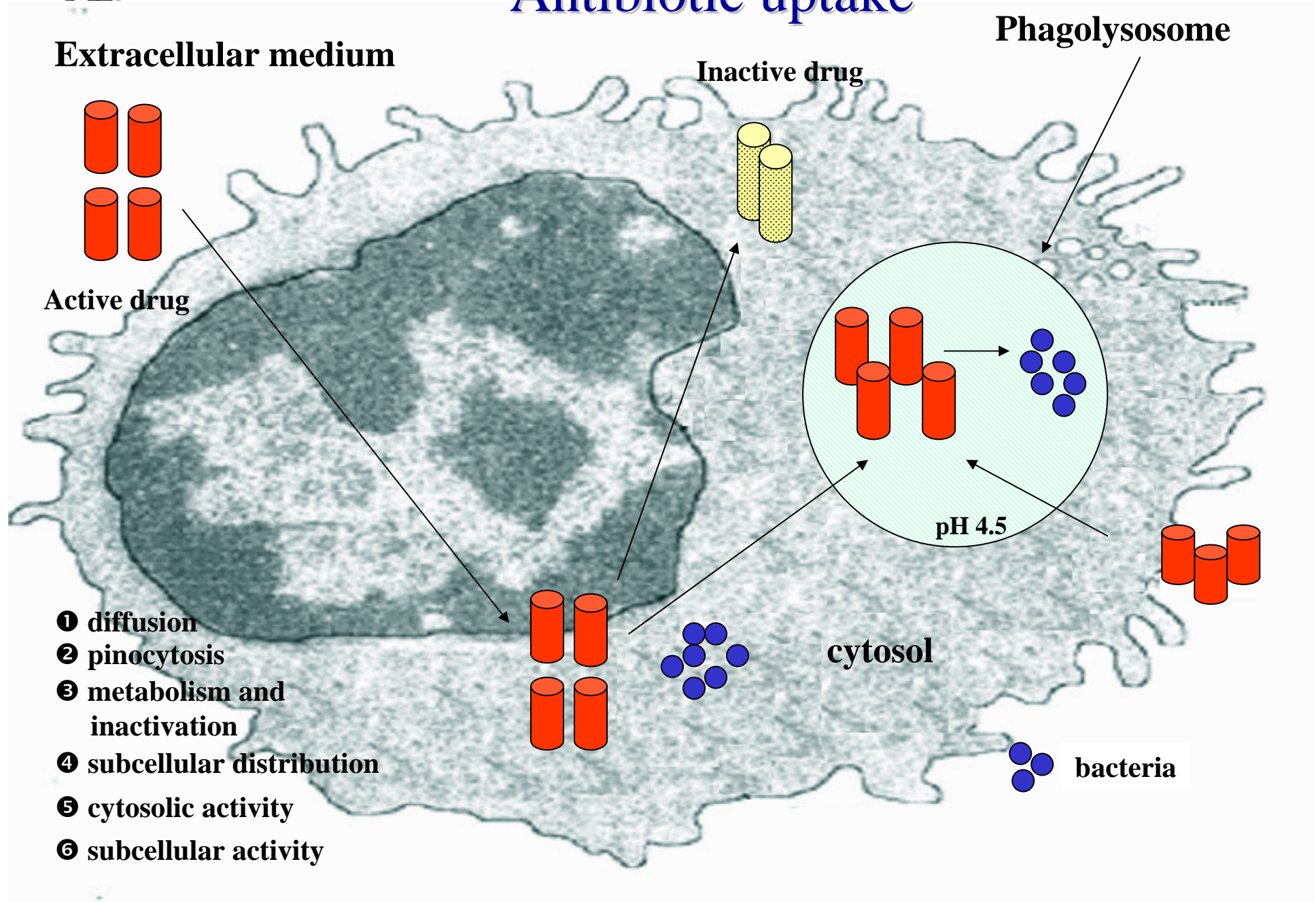


Cell target

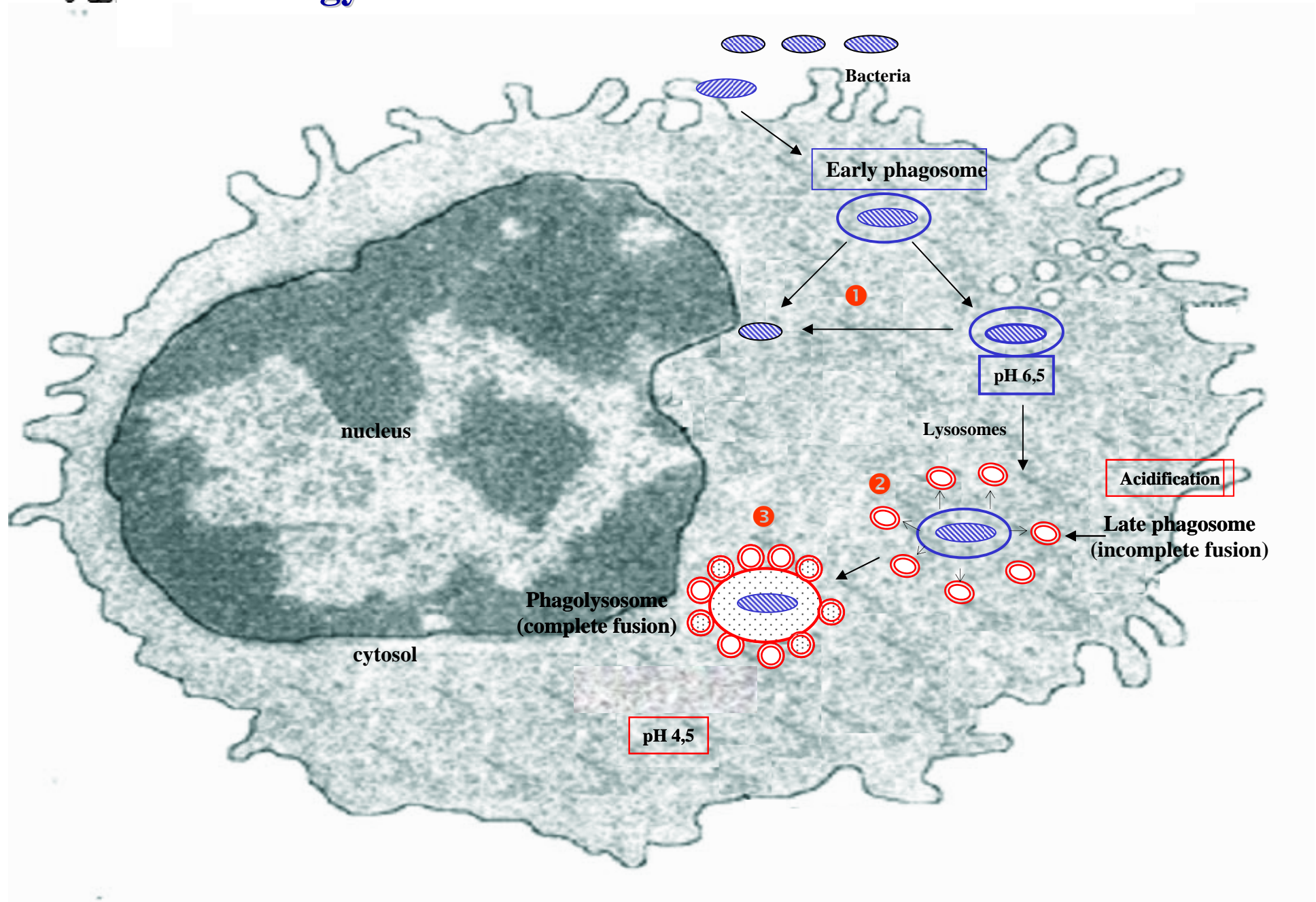


Intracellular  
compartment pH

# Antibiotic uptake



# Strategy for survival of intracellular bacteria in cells





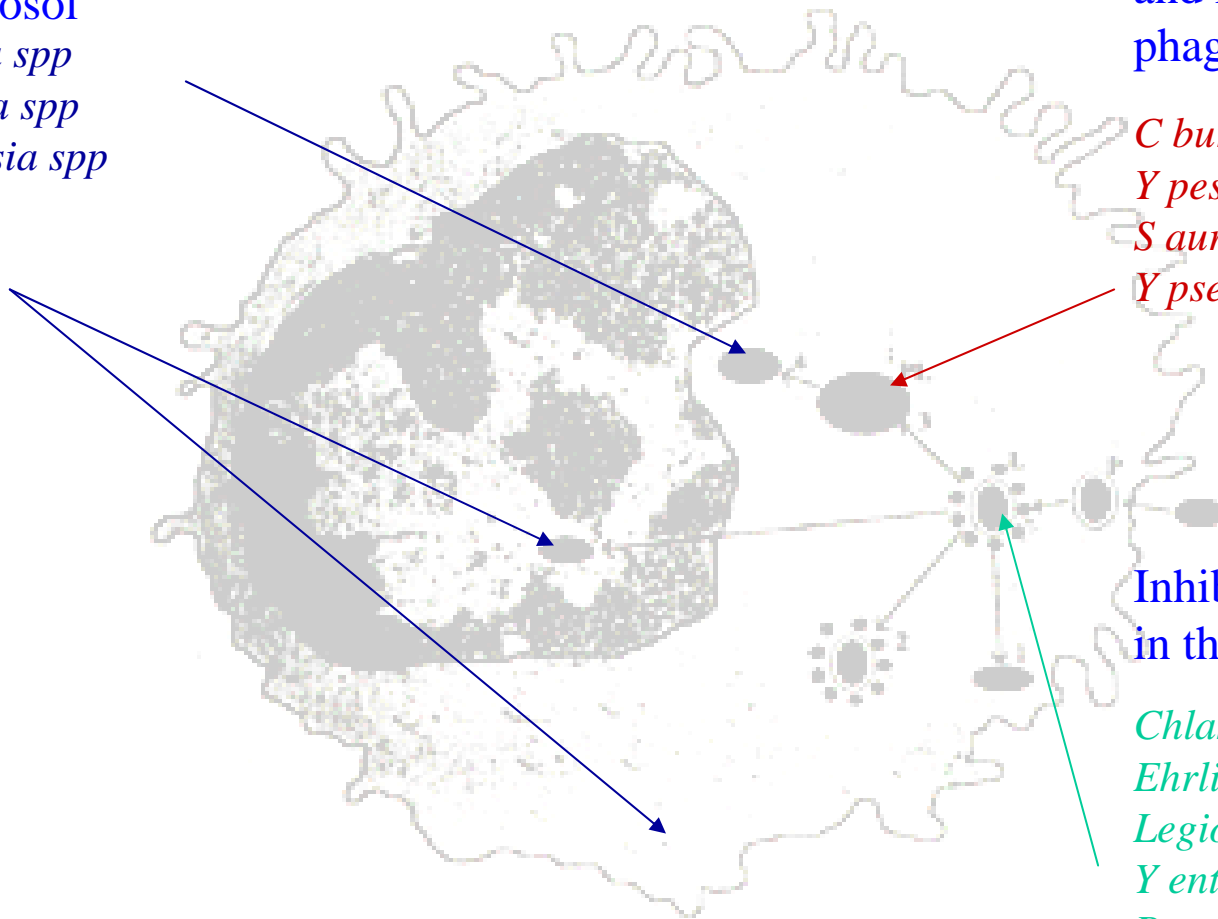
# Survival and Intracellular location of bacteria

Escape phagosome live in the cytosol

*Listeria spp*  
*Shigella spp*  
*Rickettsia spp*

Adapt in acidic environment and live in the phagolysosome

*C burnetii*  
*Y pestis*  
*S aureus*  
*Y pseudotuberculosis*



Inhibits fusion and live in the phagosome

*Chlamydia spp*  
*Ehrlichia*  
*Legionella spp*  
*Y enterocolitica*  
*Brucella spp*



# Facteurs influençant l'activité intracellulaire d'un antibiotique

- Pénétration intracellulaire de l'antibiotique
- Localisation sub-cellulaire de l'antibiotique /microorganisme
- Sensibilité du microorganisme à l'antibiotique
- Influence du milieu intracellulaire sur le couple antibiotique/microorganisme
  - pH, enzymes, fixation protéique...



# Méthodes d'étude de la pénétration intracellulaire des antibiotiques

- Choix de la cellule
- Temps de contact avec l'antibiotique
- Élimination de l'antibiotique extracellulaire
- Dosage de l'antibiotique intracellulaire
  - Autoradiographie
  - Fluorescence (tétracyclines, fluoroquinolones..)
  - Dosage chimique et biologique après lyse cellulaire



# Pénétration intracellulaire des antibiotiques

- Macrophages #Polynucléaires
  - Pénètrent bien : ( $C/E > 4$ )
    - Azythromycine, Roxithromycine, Erythromycine
    - Clindamycine
    - Quinolones
  - Pénètrent : ( $C/E$  1-4)
    - Rifampicine, Doxycycline, Chloramphénicol, Bactrim
    - Aminoglycosides ++
  - Pénètrent pas : ( $C/E < 1$ )
    - Bétalactamines et glycopeptides

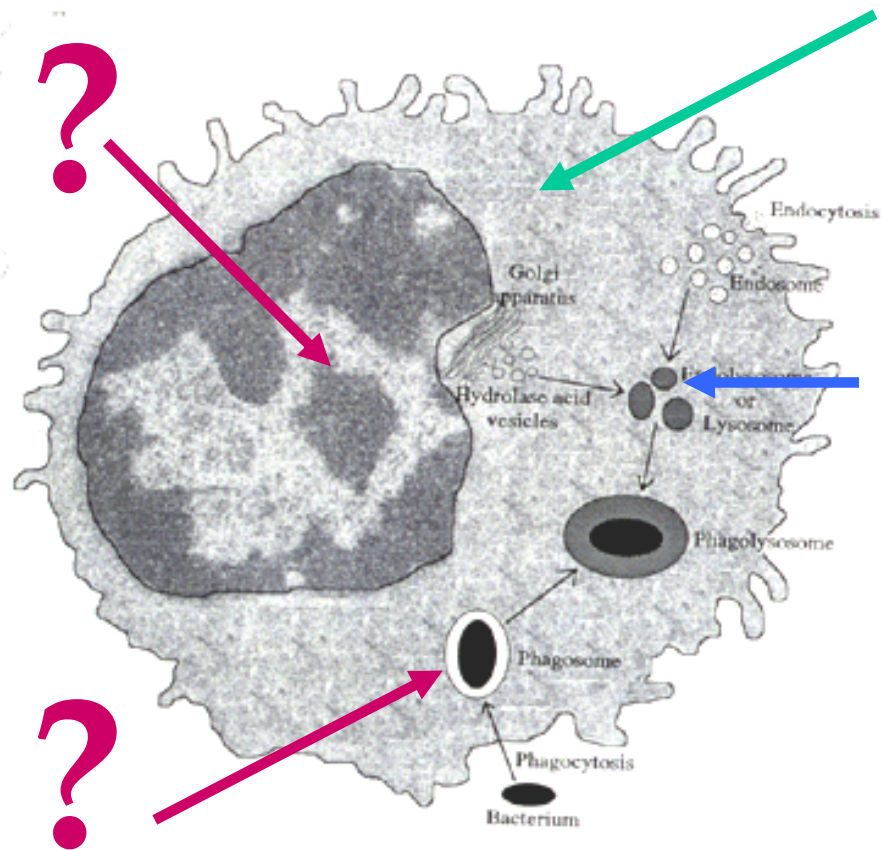


# Méthodes d'étude de la localisation sub-cellulaire des antibiotiques

- Fractionnement cellulaire en gradient de saccharose
  - Dosage biochimique ou biologique des fractions
- Utilisation des traceurs cellulaires (double marquage)
  - Autoradiographie, microscope confocal
- Différenciation lysosome/cytosol



# Localisation subcellulaire des ATB

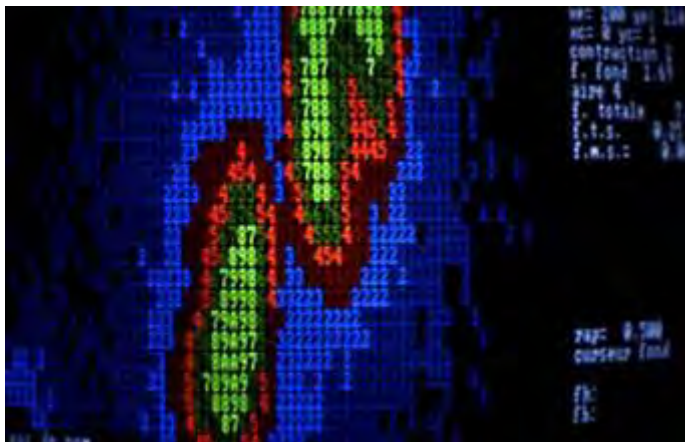
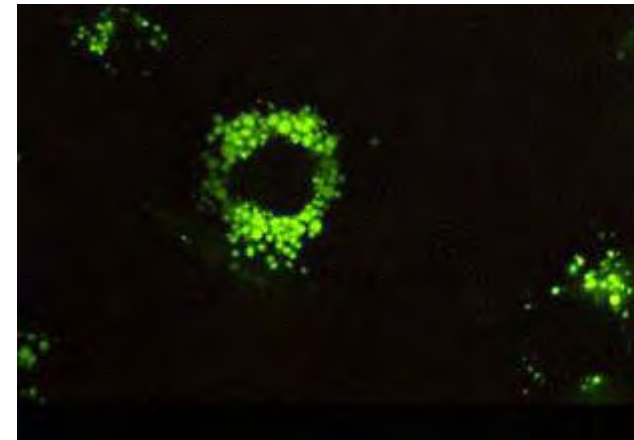


- Cytosol
  - Cyclines
  - Chloramphenicol
  - Rifampicine
  - Quinolones
- Lysosomes
  - Aminosides
  - Erythromycine
  - Clindamycine
  - Quinolones
  - Rifampicine
  - Cyclines et chloramphénicol
  - ???



# Moyens pour la mesure du pH intracellulaire

- Marqueur chimique
  - Dextran ( base faible) , traceurs fluorescents..
- Microscopie confocale





# Rôle du pH dans l'activité des antibiotiques

Antibiotique	PH optimal
Quinolones	8
Erythromycine	7.8
Betalactamines	7
Aminosides	7.5
Ethambutol	7
Cyclines	6.6
Rifampicine	<5
Pyrazinamide	<5



# Antibiotic uptake, subcellular localisation and pH of optimum activity of antibiotics

Antibiotic	Mode of entry	Cytosol	Lysosomes	PH of optimum activity
Aminoglycosides	Pinocytosis		+++	7
Betalactams	Diffusion	+		7
Chloramphenicol	Diffusion	++	Unknow	7
Erythromycin	Transport	+	+++	7.8
Fluoroquinolones	Unknow	++	++	8
Rifampin	Diffusion	++	++	<5
Tetracyclines	Diffusion	++	++	6.6

Symbols : + = low concentration ; ++ = medium concentration ; +++ = high concentration



# Co-localisation ATB-Bactéries

- **Lysosomes et P-L**
  - Antibiotiques
    - Aminoglycosides, erythromycine, quinolones, rifampicine, clindamycine, cyclines ?
  - Bactéries
    - *C burnetii*, *Y pestis*, *S aureus*, *Y pseudotuberculosis*
- **Cytosol**
  - Antibiotiques
    - Cyclines, rifampicine, chloramphénicol, quinolones
  - Bactéries
    - *Rickettsia spp*, *Shigella spp*, *Listeria spp*



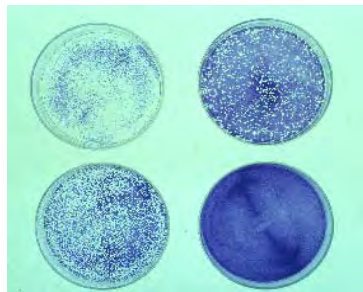
# Co-localisation ATB-Bactéries

- **Phagosome**
  - ATB ?
  - Bactéries
    - *Chlamydia spp*, *Ehrlichia spp*, *Legionella spp*, *Y enterocolitica*, *Brucella spp*.



# In vitro antibiotic susceptibility testing

- guinea pig model
- embryonated eggs
- cell systems
  - plaque assay (*McDade J., Appl.Microbiol. 1969*)
  - dye uptake assay (*Raoult D. et al J. Infect. Dis. 1987*)



*plaque assay*



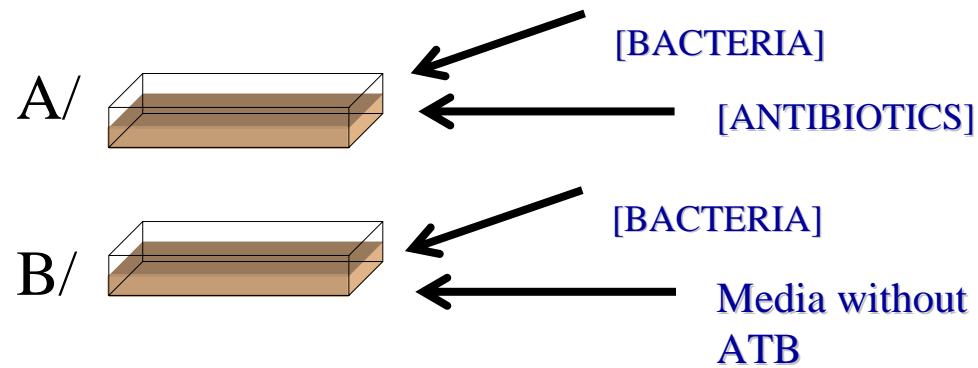
*dye uptake assay*

- IF assay (*Ives T.J. et al Antimicrob. Agents Chemother. 1997*)
- flow cytometry (*Kelly D.J. et al Am.J.Trop.Med.Hyg. 1995*)



# Tissue-Cell culture system

- Methods



*P3 laboratory*

**Incubation at 37°C for several days**



# Dye uptake assay

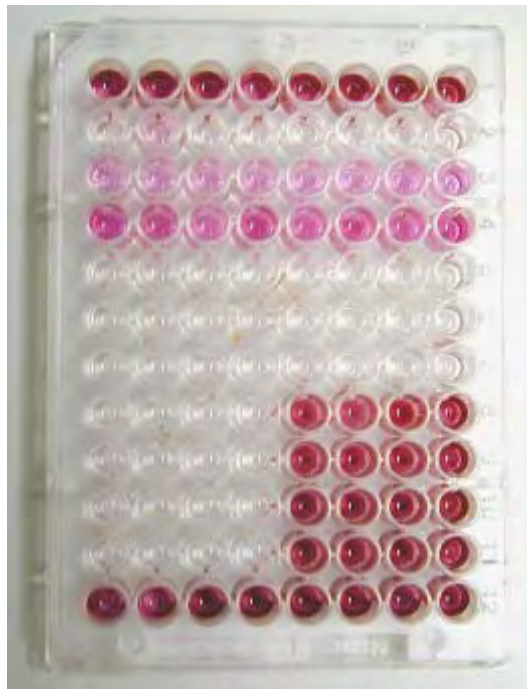
- Distribution of cells in 96-well microplate
- Incubation at 37°C with 5% CO<sub>2</sub> for 24-48 hours to obtain confluent monolayers



- . After incubation, cell monolayers are stained with neutral red dye (vital dye) for 1h at 37°C



# Dye uptake assay



Uninfected cells OD = 1  
Infected cells 2000 PFU OD = 0  
Infected cells 200 PFU OD = 0.150  
Infected cells 20 PFU OD = 0.700

*•The optical density at 492 nm of each well is determined using a spectrophotometer*

**The MIC corresponds to the lowest antibiotic concentration for which the mean OD at 492 nm is higher than that of the 20 PFU controls**



Doxycycline 0.015 µg/ml OD = 0  
Doxycycline 0.03 µg/ml OD = 0  
Doxycycline 0.06 µg/ml OD = 0  
Doxycycline 0.125 µg/ml OD = 0.89 **MIC**  
Doxycycline 0.25 µg/ml OD = 0.95  
Doxycycline 0.5 µg/ml OD = 1  
Doxycycline 1 µg/ml OD = 1



# Plaque assay

*The plaque assay system is currently the recommended technique allowing enumeration of the inoculum (Plaque Forming Unit PFU) and evaluation of the bacteriostatic activity of antibiotics.*

- Cells are prepared in Petri dishes
- Supernatant is discarded by aspiration of the medium

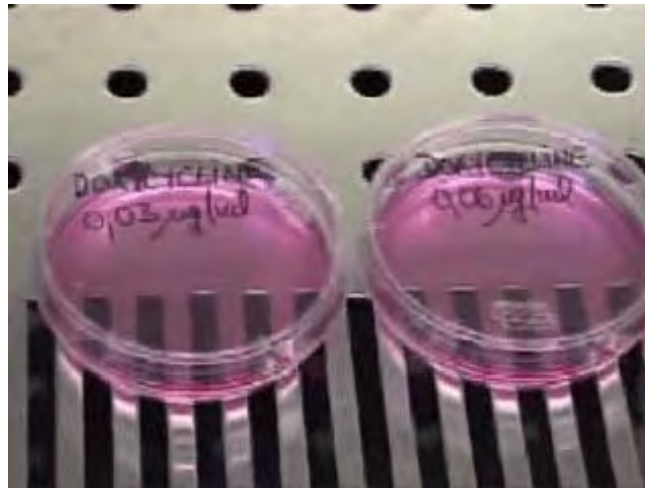


- Cells are infected for 1 hour with a bacterial inoculum.



# Plaque assay

- *Antibiotics are added at different concentrations at the same time, whereas no antibiotics are added in drug-free controls*
- *Infected cells are then overlaid with Eagle MEM with 2% fetal calf serum and 0.5 % agar.*

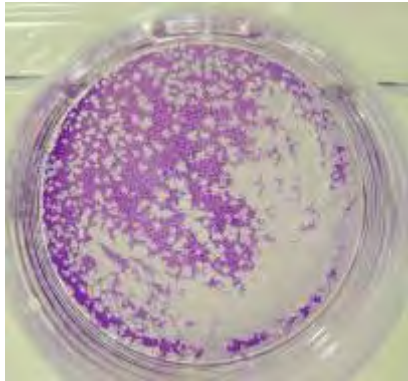


**Petri dishes are incubated 7 to 10 days at 37°C in a 5% CO<sub>2</sub> atmosphere.**

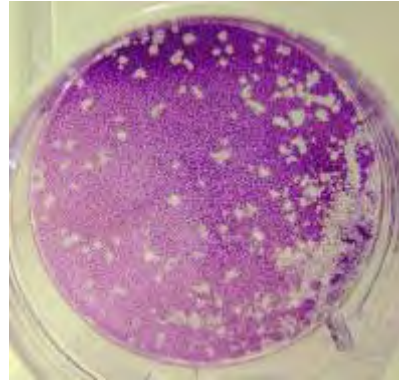


# Plaque assay: results

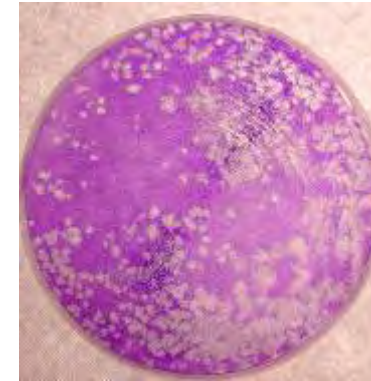
**Doxycycline 0.015  $\mu\text{g/ml}$**



**Doxycycline 0.03  $\mu\text{g/ml}$**



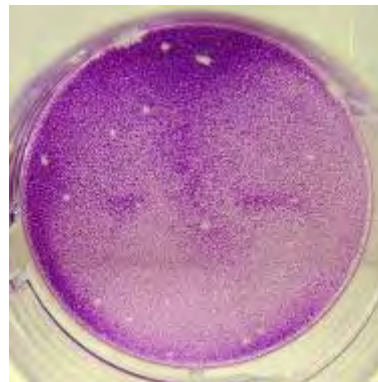
**Doxycycline 0.06  $\mu\text{g/ml}$**



**Doxycycline 0.125  $\mu\text{g/ml}$**



**Doxycycline 0.25  $\mu\text{g/ml}$**



**Doxycycline 0.5  $\mu\text{g/ml}$**

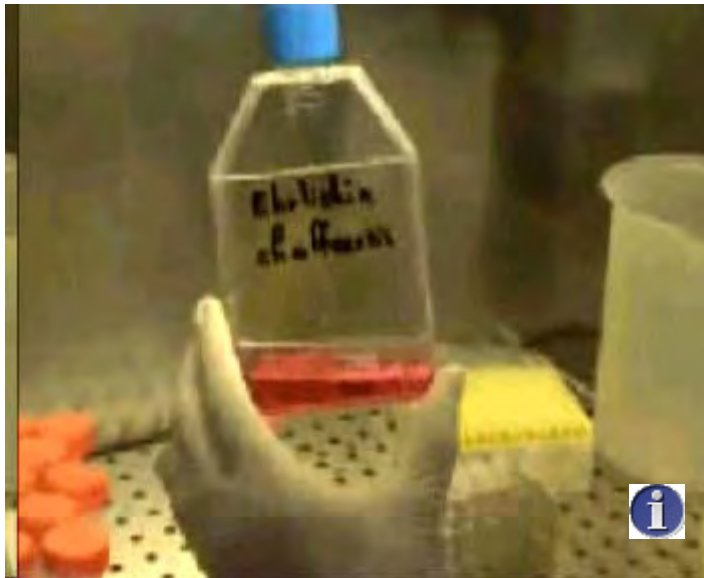


*The MICs are defined as the lowest antibiotic concentration allowing complete inhibition of plaque formation, as compared to a drug-free control*



# Diff-quick assay

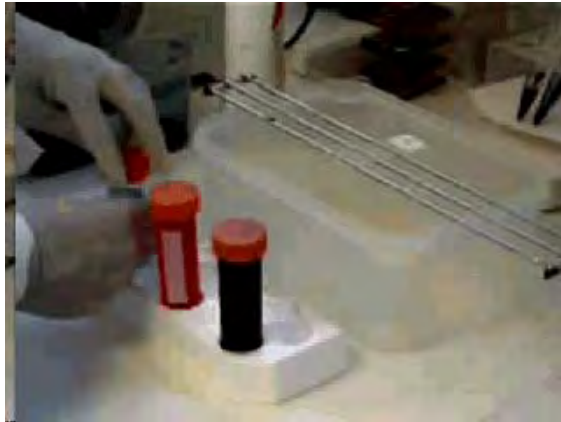
**Evaluation of the growth of bacteria (as determined by counting the percentage of infected cells after Diff Quick staining) with or without antibiotics as compared to a primary inoculum**



- **Grow bacteria in cell culture in 96-well plates until > 50% of cells are infected**
- **Add antibiotics (three wells for each antibiotic concentration tested)**
- **Incubation of the plates at 37°C for 10 days**
- **Harvest cell cultures each day during 10 days**
- **Preparation of slides by cytopspin centrifugation**



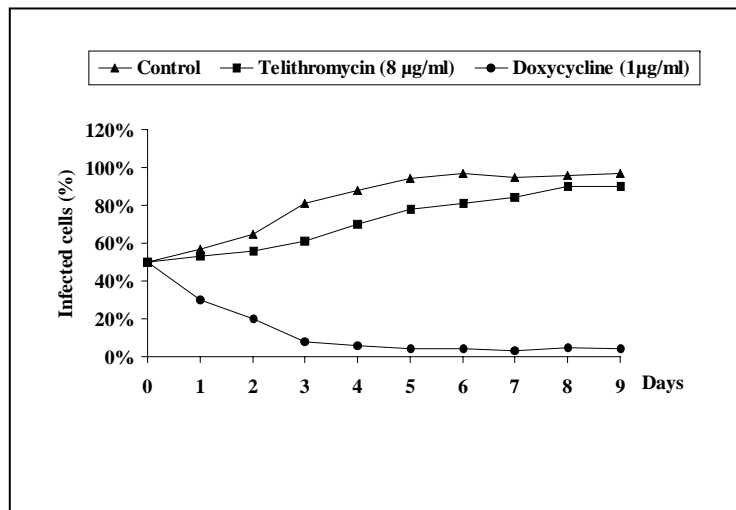
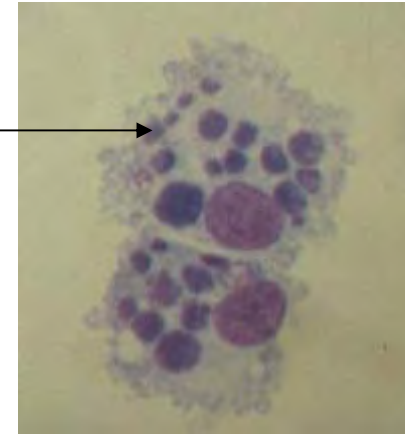
# Diff-quick assay



•*Diff Quick staining of the cytospin smears*

•*Determination of the percentage of infected cells*

Morulae

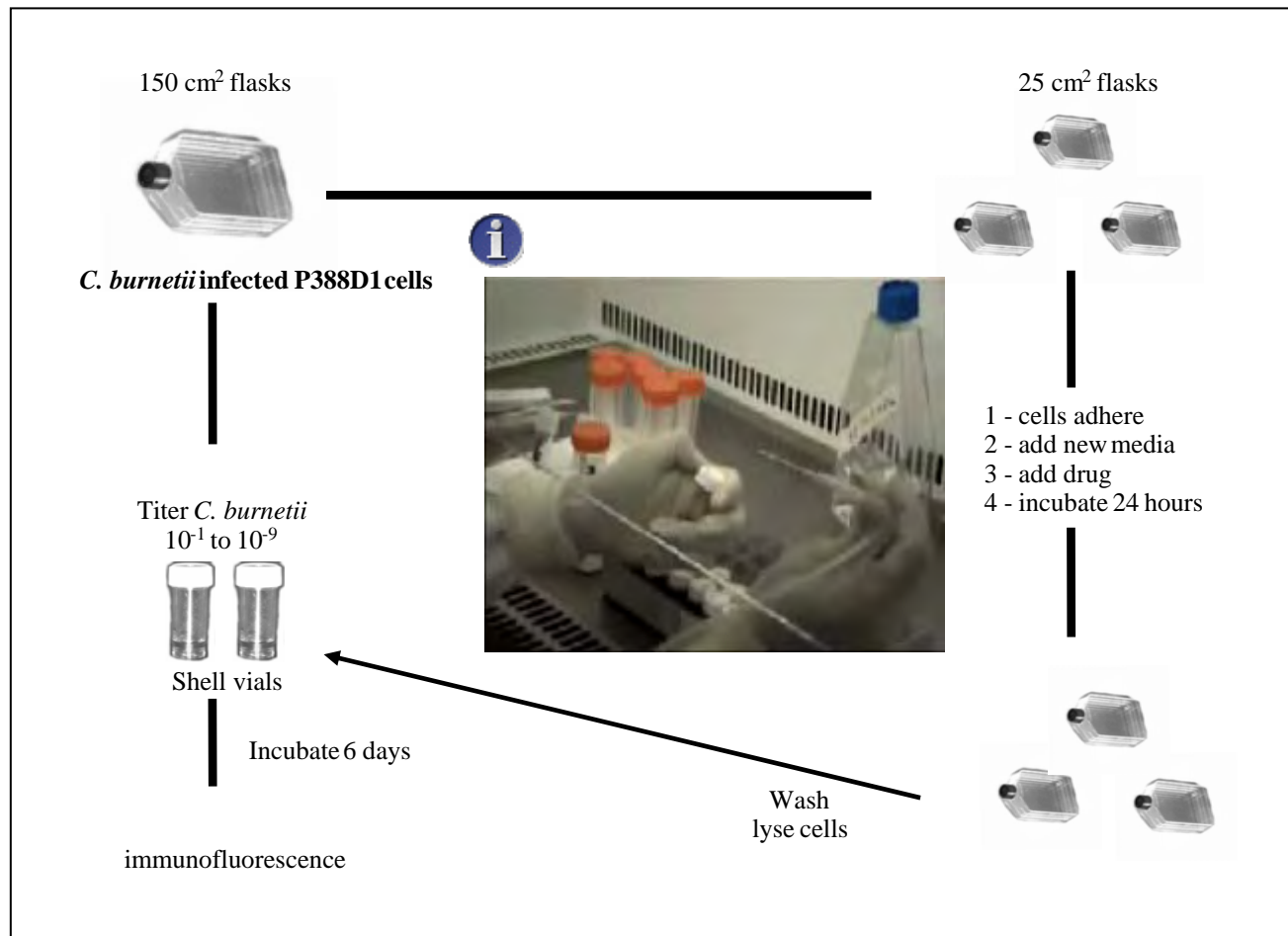


**The MIC is the lowest antibiotic concentration allowing reduction of the percentage of infected cells to < 10% after 10 days incubation of cultures with the antibiotic**



# Bactericidal assay

- Antibiotic exposure of infected cells for 24 hours
- Cell monolayers are harvested and lysed by thermal shock
- Titration of each bacterial suspension onto uninfected cells and incubation for 6 days.
- Cells are then stained by immunofluorescence

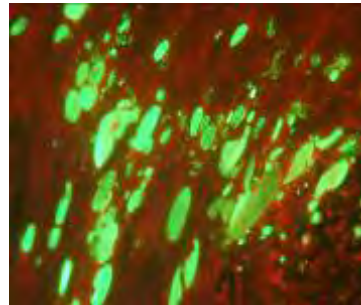




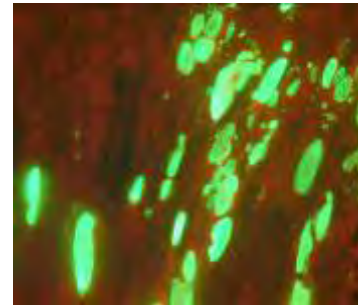
# Bacteriocidal assay: results

*Bacteriocidal activity was deduced from the reduction of the Vacuole Forming Unit (VFU) in cultures receiving antibiotics as compared with drug-free controls*

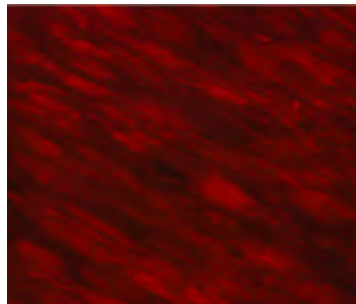
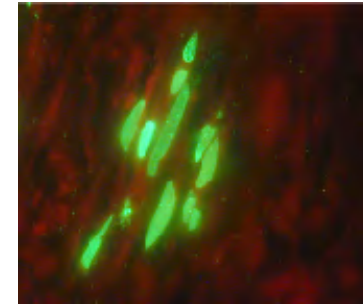
Pure



$10^{-1}$  VFU



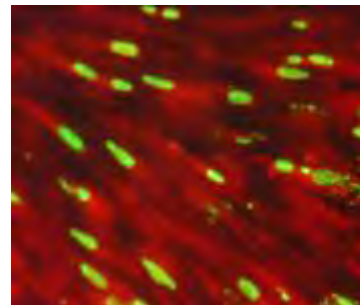
$10^{-2}$  VFU



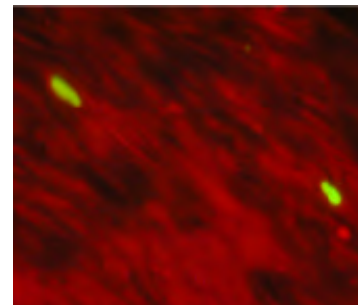
Negative control

Doxycycline + hydroxychloroquine

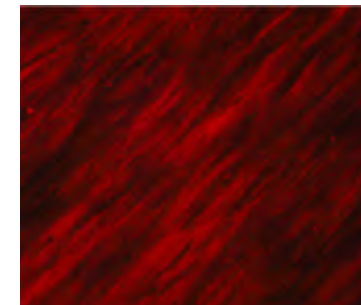
$10^{-3}$  VFU



$10^{-4}$  VFU



$10^{-5}$  VFU

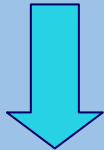




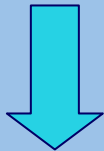
# Light-Cycler assay

*Evaluation of the growth of bacteria (as determined by the quantification of DNA copies) with or without antibiotics as compared to a primary inoculum*

Assay in 24-well microplates

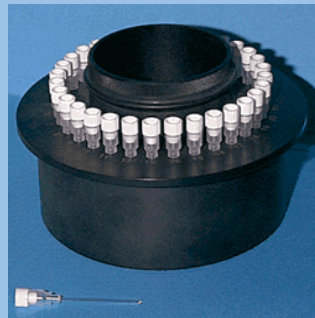


Cells are harvested  
Extraction of DNA



PCR mix

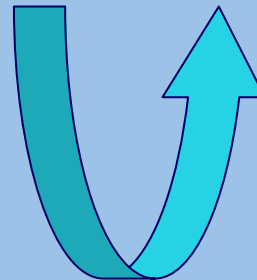
Disposition of samples in the carousel



The Lightcycler machine



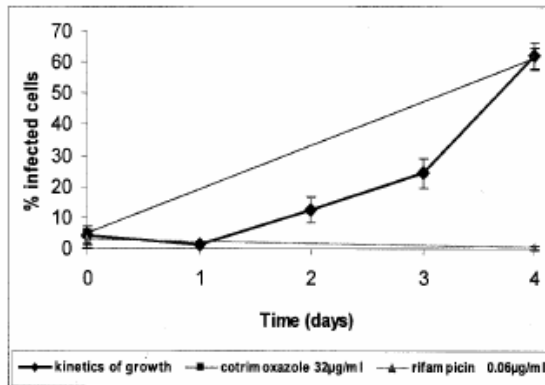
Software analysis of results





# Light-Cycler assay

**a**



Average [CI95%]

day4 = 60% [54-66]

day0 = 5.7% [4.4-7]

*MIC was defined as the first antibiotic concentration allowing the inhibition of growth of bacteria as compared to the number of DNA copies at day 0.*

Average [CI95%]

day4 = 4702 copies [3935-5469]

day0 = 298 copies [236-360]

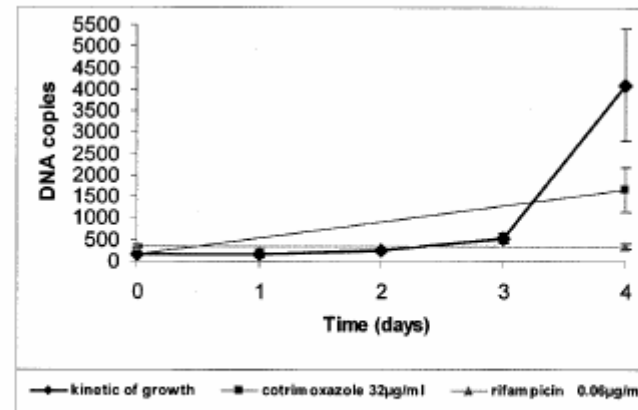
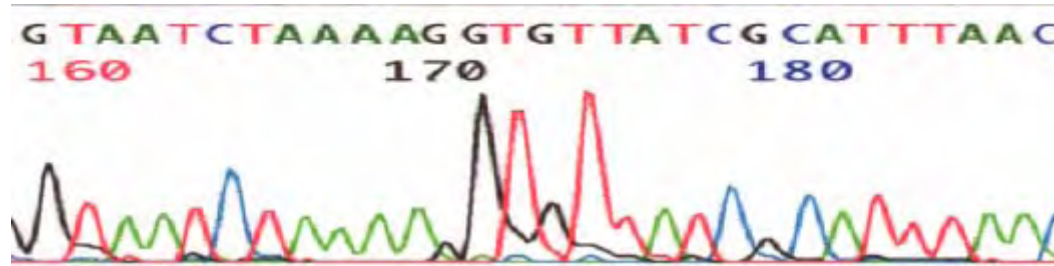
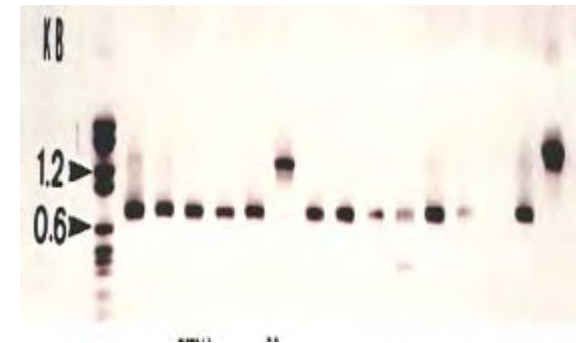


FIG. 1. Susceptibilities of *A. phagocytophilum* to cotrimoxazole (32 µg/ml) and rifampin (0.06 µg/ml) as determined by enumeration of infected cells after Diff-Quick staining (a) or by quantification of DNA copies using the LightCycler instrument (b).



# L'évaluation moléculaire de l'activité des antibiotiques

- Quinolones
  - (DNA gyrase de *C burnetii*, et de *M tuberculosis*)
- Rifampicine
  - (Rpo B de *M tuberculosis* et des rickettsies )
- Izionazide
  - ( katG de *M tuberculosis* : Rinder H: *Mol Diagn* 1999)
- Tetracyclines
  - ( Aminov RI Appl Environ Microbiol 2000)

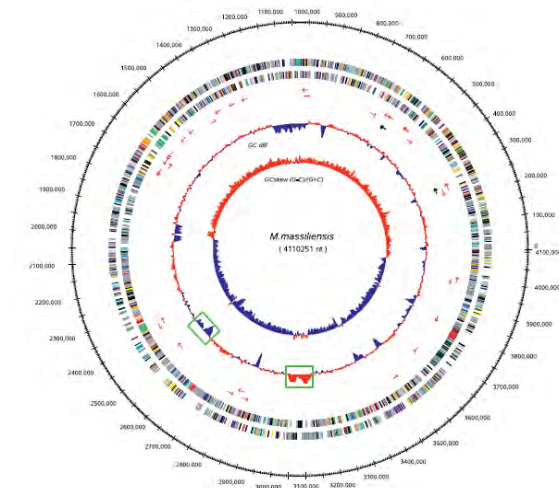




# Le génome bactérien :

## *evaluation des résistances in silico*

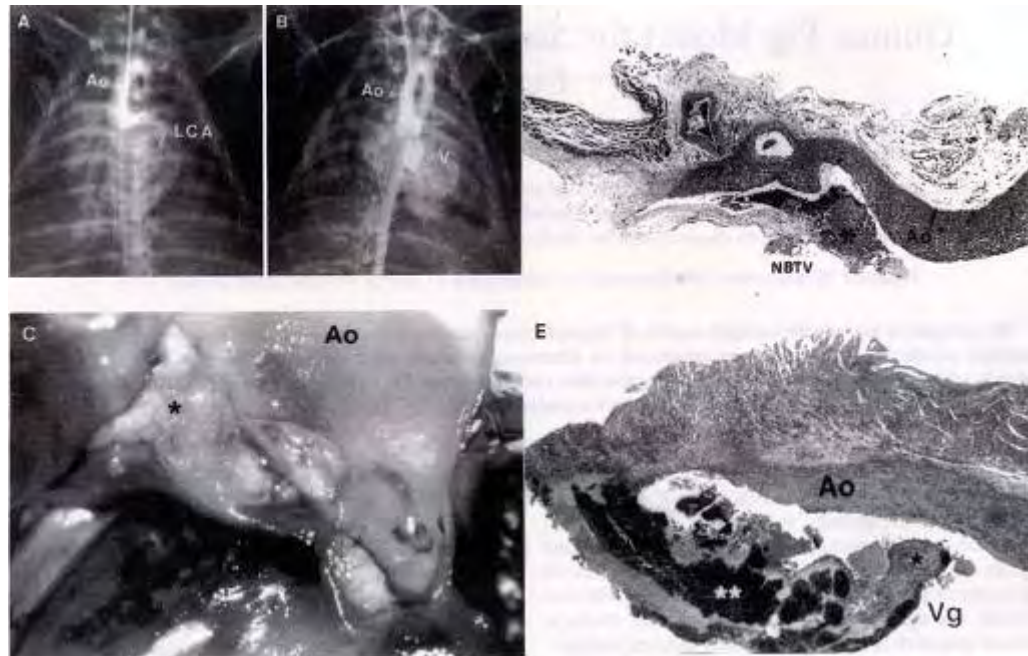
- Permet de comprendre les mécanismes de la résistance aux antibiotiques
  - Anomalie dans les voies métaboliques
    - *T. whiplei*
  - Mutations sur les gènes cibles
    - 23 s pour les macrolides
- Prévoir la résistance
  - *F tularensis* et macrolides





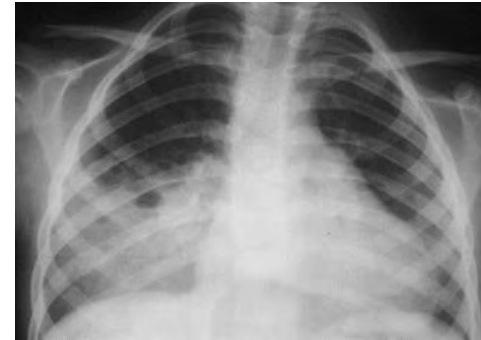
# L'évaluation *in vivo* de l'activité des antibiotiques

- Modèles animaux
  - Endocardites à *S aureus* (Maurin AAC 97)
  - Endocardites à *C burnetii* (LaScola JID 98)
- Essai cliniques
  - Irremplaçable
  - Discordance
    - Brucellose, FBM

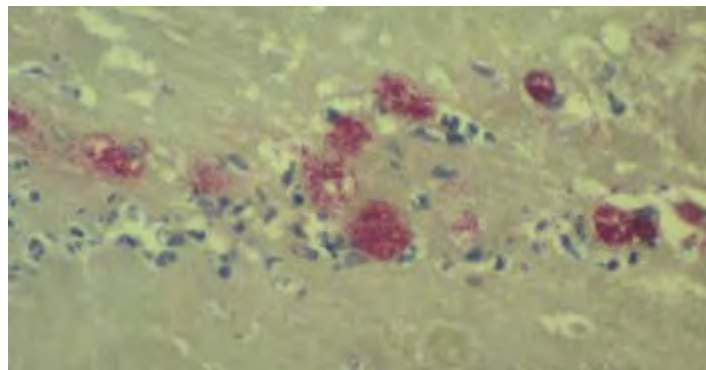




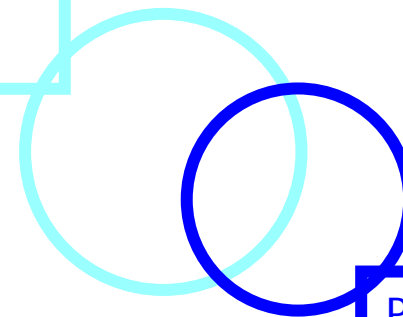
# Fièvre Q



- *Coxiella burnetii*
  - Forme aiguë : pneumonie +/- hépatite +/- fièvre
  - Forme chronique : endocardites ou infections vasculaires



Transaminases  
élevées  
90



Pneumopathies  
45



# Fièvre Q : traitement

- *In vitro* sensible à :

- Doxycycline
- Bactrim
- Sparfloxacin
- Ofloxacin
- Moxifloxacin
- Roxithromycine
- Clarithromycine
- Telithromycine
- Rifampicine
- Ciprofloxacine/Erythromycine variable

- Traitement

- Forme aiguë

- Doxycycline 200 mg/J 15 jours (A)
- Bactrim ? ( E et FE )

- Endocardites

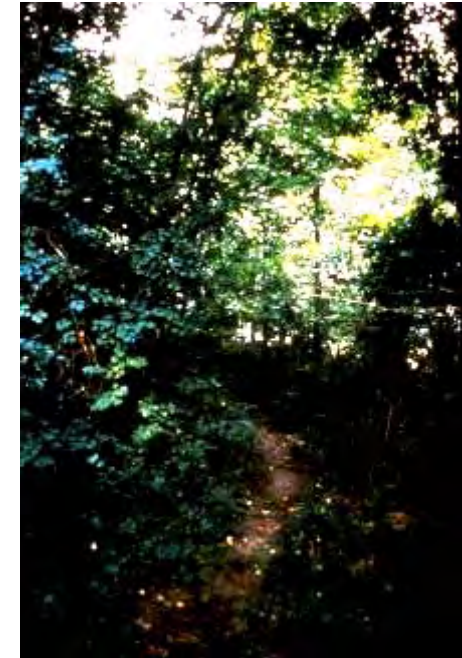
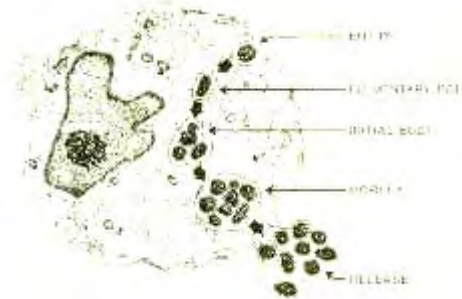
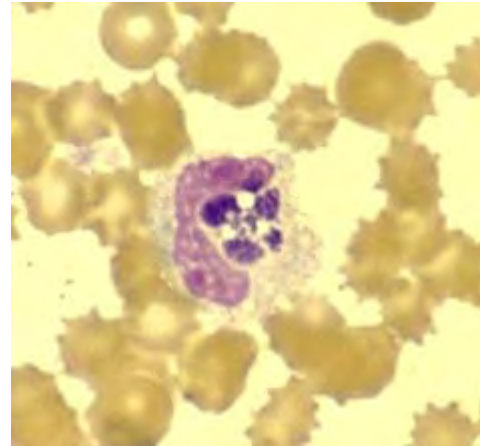
- Doxycycline 200 mg /J et Plaquenil® 600mg/J pendant 18mois ( A )

**Chloroquinémie # 1-2 micro litre/ml**



# Ehrlichioses humaines

- Ehrlichioses monocytiques
  - *Amblyomma americanum*
  - USA
  - *E chaffeensis*
- Ehrlichioses granulocytaire
  - *Ixodes spp*
  - USA et Europe
  - *E phagocytophila*



Syndrome grippal après piqûre de tique



# Ehrlichioses traitement

- In vitro sensible à :
  - Doxycycline
  - Rifampicine
  - Quinolones
    - *E phagocytophila* seulement
- In vitro résistant à :
  - Chloramphénicol, Bactrim pénicilline et érythromycine
  - Modérément sensible à Aminoglycosides

- Traitement
  - Doxycycline 7-14 jours (A et E)
  - Rifampicine ?

Table 3. Treatments and outcomes for pregnant women with human granulocytic anaplasmosis (HGA) and their neonates

Patient, study	Mothers			Neonates				
	Treatment	Duration of pregnancy	Delivery	Presentation	Test result	Perinatal transmission	Treatment	Follow-up duration
1 [9]	Dox, 100 mg twice daily for 5 days <sup>a</sup>	Full term	Fetal tachycardia, AROM, vaginal	Fever (day 9 after delivery), leukopenia, thrombocytopenia	PCR, buffy coat smear, culture, serologic analysis (all positive)	Yes	Dox, 5 days	5 years
2, PR	Rif, 300 mg twice daily for 7 days <sup>b</sup>	Full term	Normal vaginal	Healthy	Not available	No	None	2 years
3, PR	Rif, 600 mg daily for 7 days	Full term	Not available	Asthma, pneumonia	Blood PCR (negative)	No	None	2 years
4, PR	Dox, 100 mg twice daily for 10 days	PRM at 34 weeks	Fetal tachycardia, cesarean section	CHD, <sup>c</sup> otherwise healthy	Blood PCR (negative)	No	None	1 year
5, PR	Rif, 600 mg daily for 7 days	Full term	Normal vaginal	Healthy	Not available	No	None	Lost to follow-up after 1 month
6, PR	None <sup>d</sup>	Full term	Normal vaginal	Healthy	Not available	No	None	5 months
7 [13]	Rif, 600 mg daily for 7 days <sup>e</sup>	Full term	Not available	Healthy	Not available	No	None	Not available
8 [13]	Rif, 600 mg daily for 7 days	Full term	Normal vaginal	Healthy	Blood PCR (negative)	No	None	Not available
9 [12]	Dox, 2 weeks	Induction of labor at 36 weeks	Normal vaginal	Healthy	Blood PCR (negative)	No	None	Not available

NOTE. AROM, artificial rupture of membranes; CHD, congenital heart disease; Dox, doxycycline; PR, present report; PRM, premature rupture of membranes; Rif, rifampin.

<sup>a</sup> Received after delivery.

<sup>b</sup> Treated for suspected Lyme disease with  $\beta$ -lactam agents, which are not known to be effective treatment against HGA.

<sup>c</sup> Diagnosis made prior to presentation.

<sup>d</sup> Diagnosis made prior to presentation.



# Rickettsioses boutonneuses

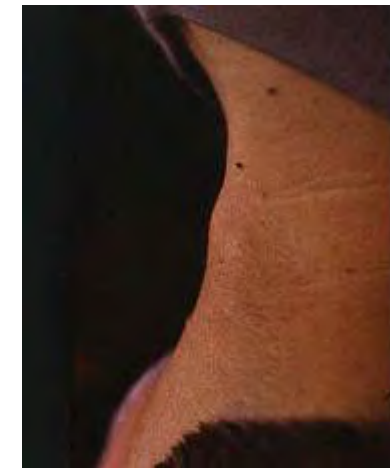
- Fièvre boutonneuse méditerranéenne

- *R conorii*
- *Ubiquitaire*



- TIBOLA

- *R slovaca*
- *Dermacentor marginatus*
- *Europe*



- Fièvre Africaine à tique

- *R africae*
- *Afrique du Sud*





# Rickettsioses : traitement

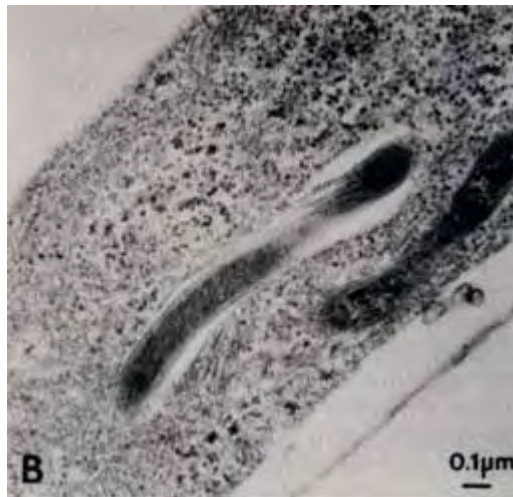
- *In vitro* sensible à :
  - Doxycycline
  - Quinolones
  - Josamycine
  - Roxithromycine
  - Clarithromycine
  - Telithromycine
  - Rifampicine ?
- Traitement
  - Forme simple
    - Doxycycline 200mgune fois (A,E)
    - Josamycine 50 mg/kg 10 jours
  - Forme Malignes
    - Doxycycline 200 mg / J pendant 10 j

**Souches résistantes à la Rifampicine**

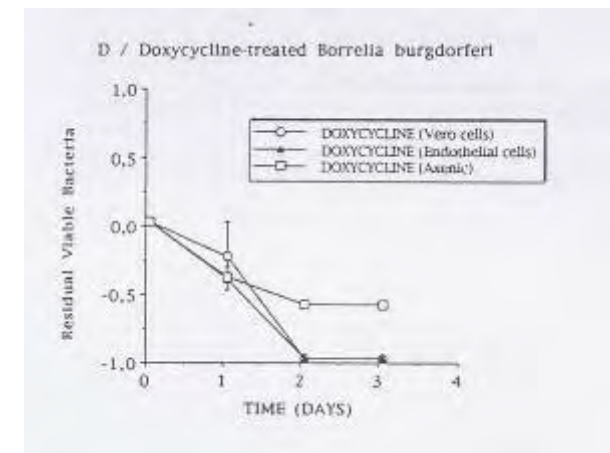
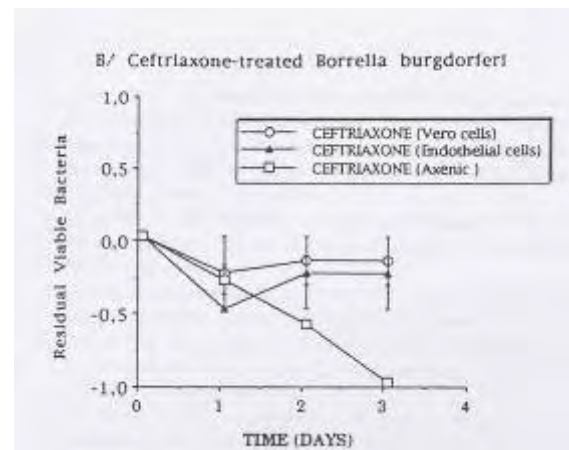


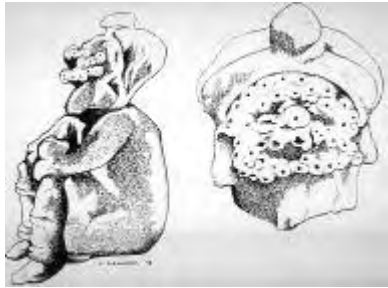
# Maladie de Lyme

- Agent infectieux
  - *B.burgdorferi strico sensu*
  - *B garinii*
  - *B afzelii*
- Localisation intracellulaire



- *In vitro sensible à:*
  - Pénicilline
  - Erythromycine
  - Doxycycline
  - Ceftriaxone
- *In cellulo sensible à*
  - Erythromicine
  - Doxycycline

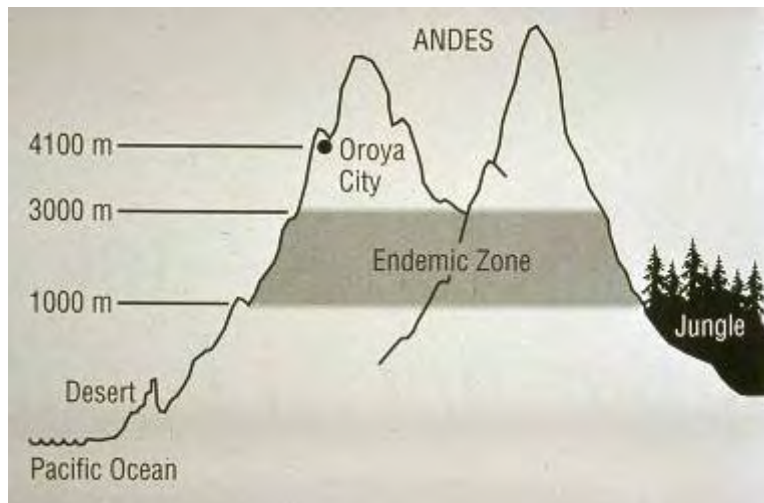




# Bartonelloses



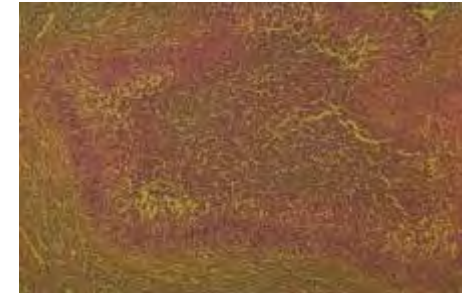
- *Bartonella bacilliformis*
  - Fièvre d'Oroya ou maladie de Carrion
  - Verruga peruana





# Bartonelloses

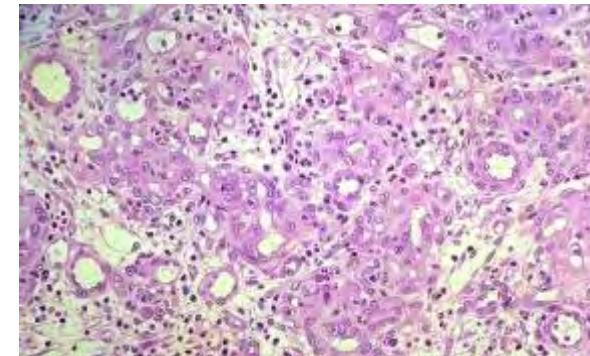
- *Bartonella henselae*
  - Maladie des griffes du chat



- *Bartonella quintana*
  - Fièvre des tranchés et bactériémie chronique chez les SDF



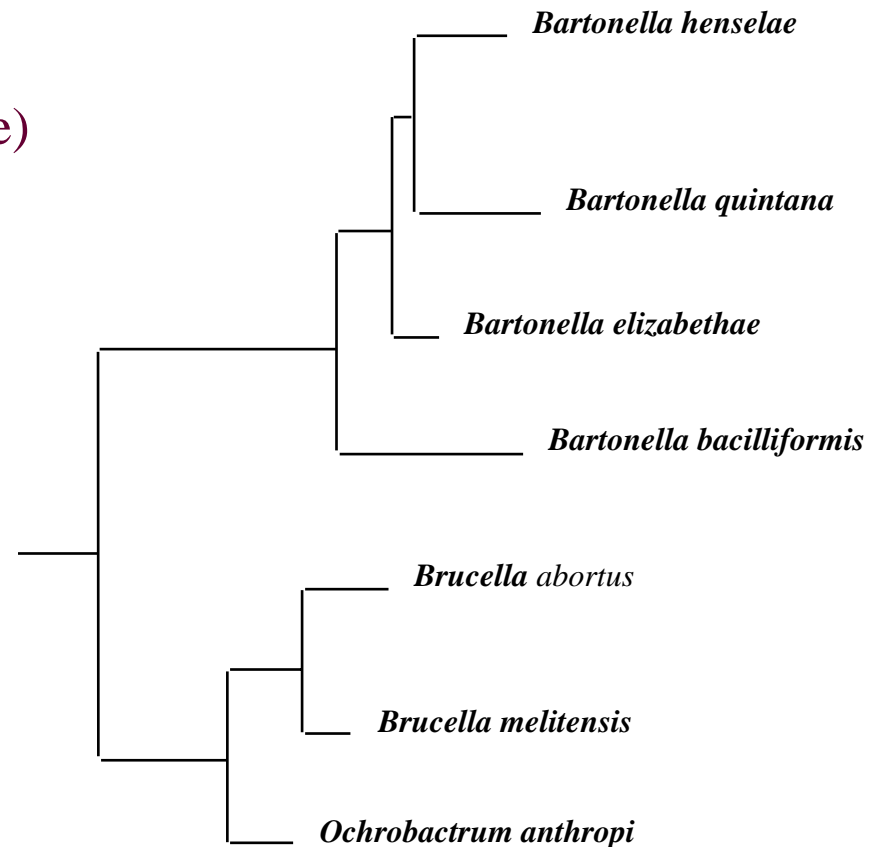
- *B henselae* et *B quintana*
  - Angiomatose Bacillaire ( ID)
  - Péliose hépatique et splénique (ID)
  - Endocardites





# Bartonelloses: traitement

- In cellulo sensible à:
  - Aminoglycosides ( bactéricide)
  - Doxycycline
  - Quinolones
  - Rifampicine
  - Erythromycine





# Traitement des bartonelloses

Disease	Regimen for		Strength of recommendation	Reference
	Adults	Children		
Typical CSD	No recommendation	No recommendation		(56)
	OR for patients with extensive lymphadenopathy, consider: Azithromycin 500 mg PO the first day and 250 mg PO on days 2 to 5 as a single daily dose	OR for patients with extensive lymphadenopathy, consider: Azithromycin 10 mg/kg PO on day 1, and 5 mg/kg PO on days 2 to 5 as a single daily dose	BI	(8; 86)
Complicated CSD	Doxycycline 100 mg PO BID for 4 wks AND rifampin 300 mg PO BID for 3 wks	Unknown	CIII	(56)
Trench fever OR Chronic bacteremia with <i>B. quintana</i> BA*	Doxycycline 200 mg PO QD for 4 wks AND Gentamicin 3 mg/kg IV QD for 2 wks	Unknown	BII AI	(36)
	Erythromycin 500 mg PO QID for 3 months	Erythromycin ethylsuccinate 40 mg/kg PO total/day, in four divided doses (maximum total daily dose of 2 g/d) for 3 months	AII	(58)
	OR doxycycline 100 mg PO BID for 3 months		AII	(58)
PH*	Erythromycin 500 mg PO QID for 4 months	Erythromycin ethylsuccinate 40 mg/kg PO total/day, in four divided doses (maximum total daily dose of 2 g/d) for 4 months	AII	(58)
	OR doxycycline 100 mg PO BID for 4 months		AII	(58)



# Traitement des bartonelloses

---

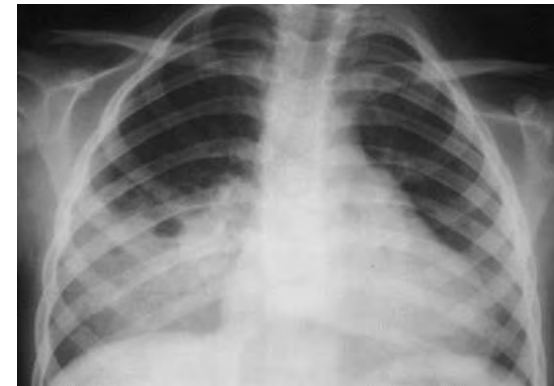
Endocarditis	Suspected <i>Bartonella</i> , culture-negative: Gentamicin 3 mg/kg/d IV for 14 days AND ceftriaxone 2 g IV or IM QD for 6 weeks +/- doxycycline 100 mg PO or IV BID for 6 weeks	Unknown	AII	(85)
			BII	(85)
			BII	(85)
Endocarditis	Documented <i>Bartonella</i> , culture-positive: Gentamicin 3 mg/kg/d IV for 14 days AND doxycycline 100 mg PO BID for 6 wks		BII	(85)
			BII	(85)
Carrion's disease Oroya fever	Chloramphenicol 500 mg PO or IV QID for 14 days AND another antibiotic (beta lactam preferred)  OR ciprofloxacin 500 mg PO BID for 10d	Chloramphenicol 50-75mg/kg/d PO or IV divided into four doses for 14 days AND another antibiotic (beta lactam preferred) OR ciprofloxacin in children 7-12 years 250 mg PO BID for 10d	AII	(85)
			BIII	**
Verruga peruana	Rifampin 10 mg/kg/d PO for 14d OR Streptomycin 15-20 mg/kg/d IM for 10d	Rifampin 10 mg/kg/d PO for 14d (maximum total daily dose of 600 mg/day)	AII	(68)
			AII	(68)

---



# Legionelloses (*Legionella pneumophila*)

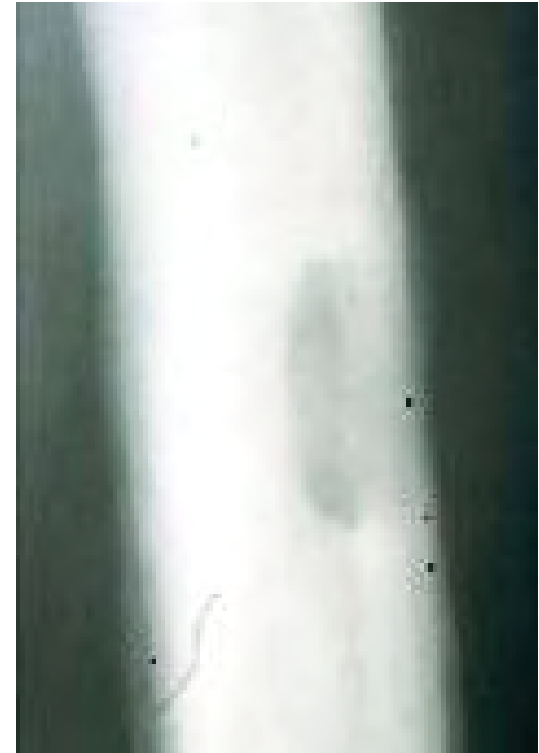
- *In vitro* sur cellules
  - Doxycycline
  - Bactrim
  - ofloxacine, ciprofloxacine, sparfloxacine,
  - rifampicine
  - érythromycine, roxithromycine, azithromycine, clarithromycine
- Traitement de référence ( Durée 14-21 jours )
  - Azithromycine IV ( non disponible en France)
  - Levofloxacine IV ( Tavanic® 250-750 mg / jour )
  - ERYTHROMYCINE 2g per os /j ou 4 g IV par J pd 10-14 J
  - et Rifampicine
  - Autres traitements possibles mais non référencés
    - bactrim, doxycycline, ciprofloxacine, azithromycine, clarithromycine





# Brucellose

- *In vitro* sur cellules
  - Aminoglycosides +++, Bactrim, doxycycline, **quinolones**, rifampicine
- Traitement
  - Doxycycline et / rifampicine ou streptomycine 6 semaines (A)
  - ( Enfant , Femme enceinte) ???





# Mycobactéries atypiques

- *In vitro* sur cellules
  - Clarythromycine, azythromycine, sparfloxacin avec
    - éthambutol et/ou amikacine et /ou rifampicine
    - Hétérogénéité des souches ++++
- Traitement des formes disséminées ++
  - Clarythromycine **ou** azythromycine **et** éthambutol **et au moins 1 parmi**
    - Clofazimine, rifampicine, rifabutine, ciprofloxacine, amikacine



# *Chlamydia* spp.( hors urétrites)

- *In vitro*
  - Doxycycline, ciprofloxacin, ofloxacin, sparfloxacin, rifampicin, erythromycin, azithromycin, clarithromycin, roxithromycin
- Traitement
  - Pneumonies adultes ( *C pneumoniae* et *C psittaci*)
    - Doxycycline 10-21 jours
    - érythromycine ou azythromycine
  - Pneumonie de l'enfant
    - Erythromycine ou azythromycine
  - LGV ou maladie de Nicolas Favre ( *C trachomatis*)
    - Doxycycline 21 jours ou erythromycine





# Traitement courts

- Urétrite à *C trachomatis*
  - Azythromycine 1g en une prise unique ou
    - Doxycycline 200 mg/j pd 7 jours ou érythromycine 2g/j pd 7 j ou 1g/j pd 14 J
- Fièvre typhoïde
  - *In vitro* sur cellules :  
chloramphénicol,quinolones,(ceftriaxone et ampicilline ?)
  - Traitement : Ceftriaxone 3g/j 5 jours ou Quinolones 5 jours
- Rickettsioses
  - FBM, typhus



Merci pour votre attention

