

Principles and methods of culturing *Mycobacterium bovis*, isolation and identification

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Identification of the agent

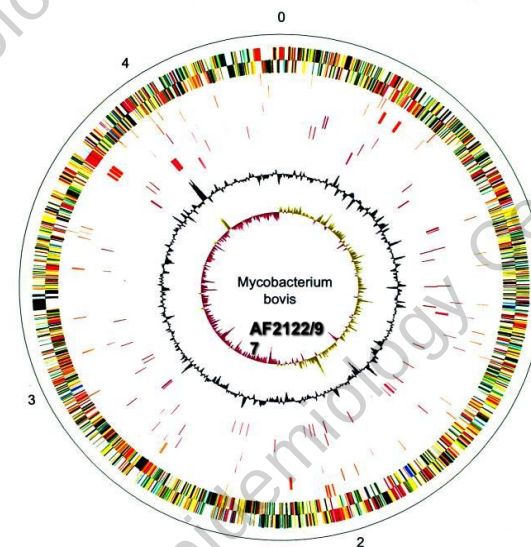
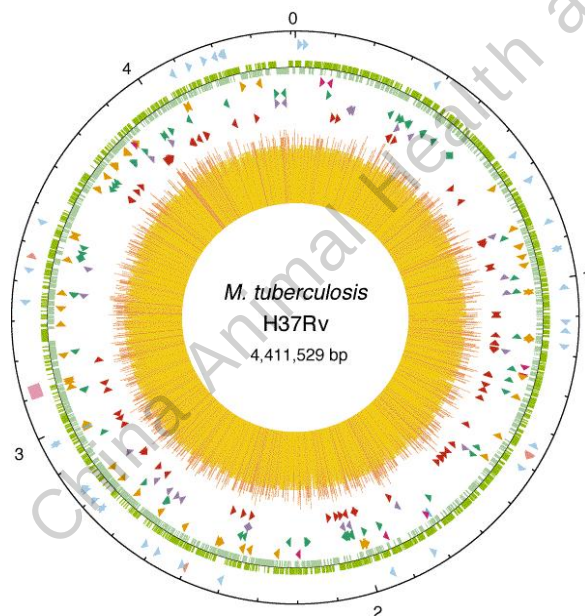
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- ***Mammalian tuberculosis*** is a chronic bacterial disease of animals and humans caused by members of the *Mycobacterium tuberculosis* complex (MTBC)
 - ✓ *M. tuberculosis*
 - ✓ *M. bovis*
 - ✓ *M. caprae*
 - ✓ *M. africanum*
 - ✓ *M. microti*
 - ✓ *M. canettii*
 - ✓ *M. pinnipedii*



- **Genome sequencing – MTBC**

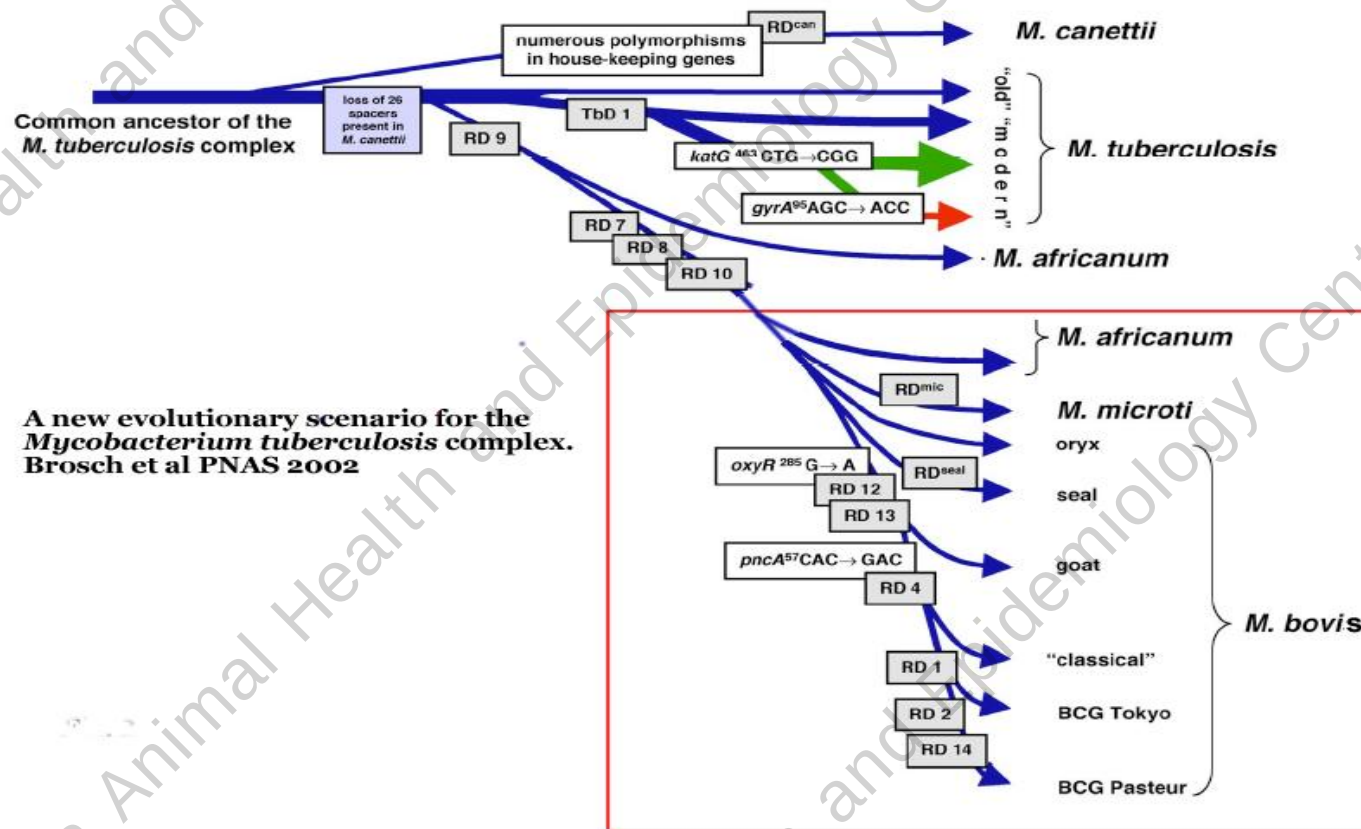
Mycobacterium tuberculosis complex



- **Co-linearity : 99.95% nucleotide identity**
- **Genome deletions major driver in shaping genome and evolution**
- **Unique, unidirectional events**
- **Powerful markers for molecular epidemiology and phylogenetics**



• The new evolutionary scenario of MTBC



Appearance of a wide host range



Test methods available for use in cattle, goats, and camelids and their purpose

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Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post_vaccination
Detection of the agent						
acid-fast staining and microscopy	-	-	-	+	-	-
Bacterial isolation	++	-	++	+++	++	-
Histopathology and antigen detection	+	-	+	+	-	-
Real-time PCR (direct from specimens)	++	-	++	+++	++	-
Detection of immune response						
Delayed hypersensitivity skin test	+++ (+++ / +)	+++ (++ / +)	+++ (++ / +)	++	+++ (++ / +)	-
IGRA	++ (++ / +)	++ (+ / +)	+++ (++ / +)	+ (- / -)	+++ (++ / +)	-
ELISA antibody test	+ (- / ++)	+ (+ / ++)	+ (- / ++)	-	+ (- / ++)	-
Lateral flow antibody test	+	+	+	-	+	-

Key: +++ = recommended for this purpose; ++ recommended but has limitations; + = suitable in very limited circumstances; – = not appropriate for this purpose.



Laboratory diagnostic capacity

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- Biosafety protection level***

Species	bacterial operation	animal infection experiments	Samples Detection	Non infectious materials	Transport packaging	
					A /B	UN NO.
<i>M. tuberculosis</i>	BSL-3	BSL-3	BSL-2	BSL-1	A	UN 2814
<i>M.bovis</i>	BSL-3	BSL-3	BSL-2	BSL-1	A	UN 2814
<i>M.caprae</i>	BSL-2	BSL-2	BSL-2	BSL-1	B	UN 3373
<i>M.africanm</i>	BSL-2	BSL-2	BSL-2	BSL-1	B	UN 3373
<i>M.microti</i>	BSL-2	BSL-2	BSL-2	BSL-1	B	UN 3373

in BSL-3:
Widely drug-resistant
Multidrug-resistant



- *Successful recovery of mycobacteria by bacteriological culture depends on :*
 - ✓ *the sampling technique at the abattoir;*
 - ✓ *the type of tissue specimens collected ;*
 - ✓ *their preservation in transit to the laboratory (refrigeration or freezing);*
 - ✓ *the chemical decontamination ;*
 - ✓ *the type of culture media chosen to grow mycobacteria.*



- ***The best tissues for the isolation of MTBC:***
 - ✓ *collected during post-mortem examination from animals tested positive to tuberculin or interferon gamma tests;*
 - ✓ *TB-compatible lesions;*
 - ✓ *If pathological lesions are not detected, collect specific lymph nodes from the head, respiratory and/or digestive locations .*
 - ✓ *Pets or zoo animals: biopsy specimens from lymph nodes, oral swabs or tracheo-bronchial wash*



- *Sample (tubercles)*

- lymph nodes (bronchial, mediastinal, retropharyngeal and portal)
- lung, liver, spleen

The collection area :

the junction of typical
tuberculosis nodules and
healthy tissues





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- *The tissue samples :*

- ✓ remove extraneous material (e.g. fat);
- ✓ cut into small pieces;
- ✓ *homogenised using a stomacher or tissue grinder (e.g. mortar, pestle, blender, etc.);*
- ✓ *with sterile PBS;*
- ✓ *mandatory decontamination* *Avoid the growth of other bacteria*



- *decontamination :*

!! control the time :10-20 min

- ✓ 0.375–0.75% hexadecylpyridinium-chloride detergent (HPC);
- ✓ or 2–4% sodium hydroxide;
- ✓ or 5% oxalic acid;
- ✓ or 4% sulfuric acid;
- ✓ or among others.

restored to a neutral pH after decontamination

Due to the cell wall characteristic of mycobacteria, they can resist certain detergents or extreme changes in the pH.



- *suspension treatment:*
 - ✓ centrifuged;
 - ✓ the supernatant is discarded;
 - ✓ the sediment is used for culture and microscopic examination



- ***Solid media***

- Modified Lowenstein-Jensen
- Pyruvate L-J medium $C_3H_3NaO_3$
- Middlebrook 7H10/7H11



- ***Liquid culture medium***

- Middlebrook 7H9





- Medium formulation**

Inorganic salt	Modified Lowenstein-Jensen	Pyruvate L-J medium
magnesium sulfate 硫酸镁	1.24 g	1.24 g
Magnesium citrate 枸橼酸镁	0.6 g	0.6 g
L-Asparagine L-天冬酰胺	3.6 g	3.6 g
Or Sodium Glutamate 谷氨酸钠	7.2 g	7.2 g
Glycerol 丙三醇	12 mL	-----
sodium pyruvate 丙酮酸钠	-----	1.6 g
glucose 葡萄糖		4.0 g
Distilled water or deionized water	600 mL	
Egg yolk liquid	1000 mL	
malachite green (2%)	20 mL	

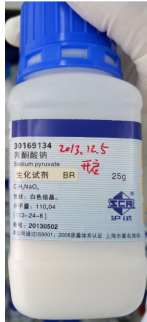


- Medium formulation*

7H11		7H9	
7H11 Agar	21 g	7H9 Broth	4.7 g
deionized water	900 mL	deionized water	900 mL
glycerol	5 mL	Tween 80	0.5 g
121°C 15 min		121°C 10 min	
OADC Enrichment	100 mL	OADC Enrichment	100 mL

Key Process Steps (e.g. Pyruvate L-J medium)

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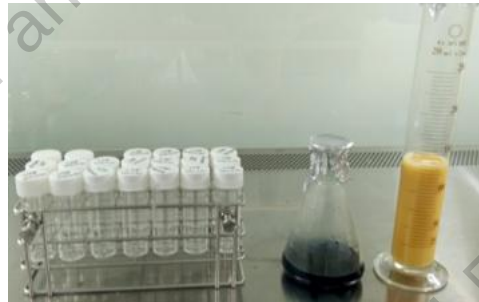


Weighing-Fluidization
(thoroughly dissolved)



Sterilization
121°C, 15 min

Cooling down to 45-60°C



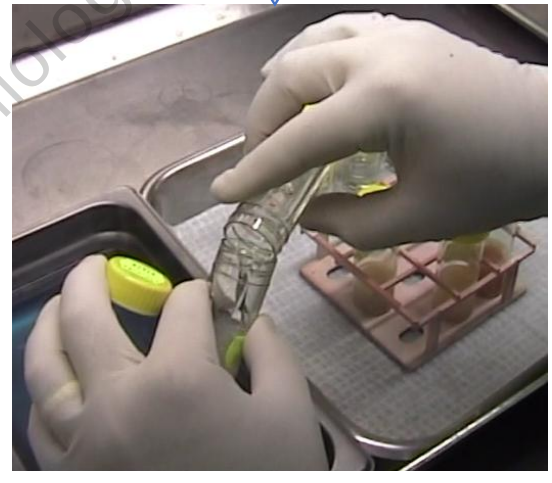
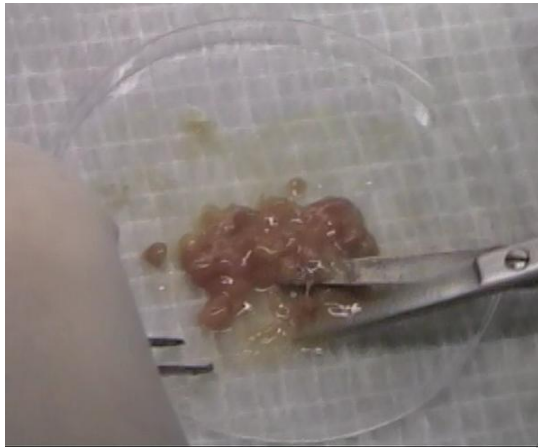
dispensing

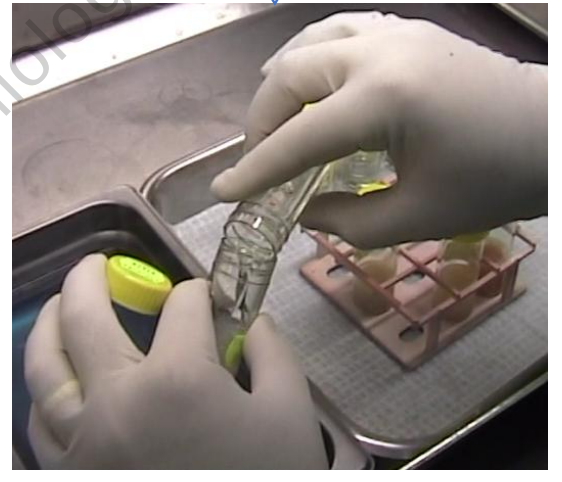
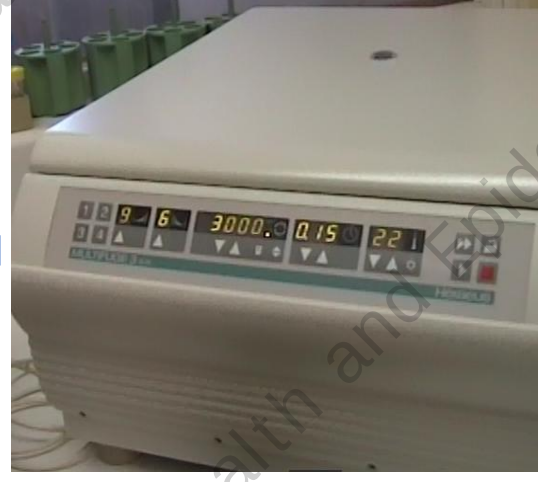
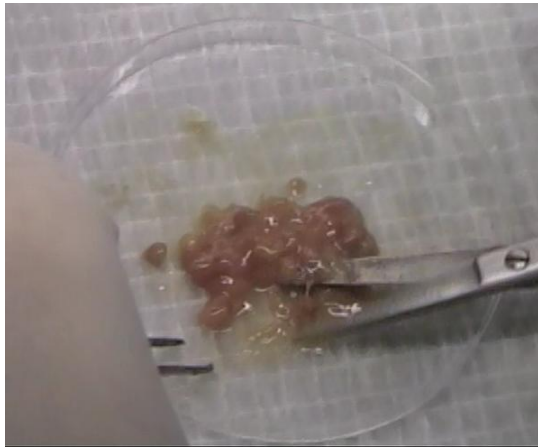


Adding filtered egg mixture



- Cleaning eggs
- Disinfect: the eggs in 75% alcohol
- Break the eggs into a sterile beaker and stir
- Filter with sterile gauze (12 layers)







- *Culture conditions*

- 37°C for 6–12 weeks.

The media should be in tightly closed tubes to avoid desiccation; solid media are used slopes are examined for macroscopic growth at weekly.

- *Automation Equipment* (Based on liquid culture)

- BACTEC MGIT 960
- BacT/ALERT 3D
- Versa TREK/ESP II (Versa)

- Advantage: Separation rate > L-J medium ,

Fully automated, high capacity, quick test

- Disadvantage: False positives, specialized infrastructure , Ups



video: <https://www.thermofisher.cn/cn/zh/home/clinical/clinical-microbiology/blood-culture/mycobacteria-detection-systems.html>



- ✓ Approximately 2 g of tissue were collected, homogenized in 15 mL of 0.85% physiological saline and vortexed at high speed for 1 minute.
- ✓ 15 mL of homogenate was transferred to a centrifuge tube, after which 15 mL of hexadecylpyridinium chloride monohydrate (HPC) 1.5% (w/v) was added and mixed at room temperature for 30 minutes.
- ✓ The mixture was centrifuged at $3,000 \times g$ for 20 minutes at 22°C, and the pellets were resuspended in 10 mL of 0.85% physiological saline as inoculum. Milk samples were similarly treated. 20 mL of milk were centrifuged at $3,000 \times g$ for 10 minutes, and pellets were decontaminated in 20 mL of HPC 0.75% (w/v) for 20 minutes.
- ✓ After centrifugation and suspension, 100 μ L of tissue or milk samples were inoculated on to Lowenstein-Jensen medium supplemented with pyruvate (BD, USA) or Lowenstein-Jensen medium (BD, USA), at 37°C for 4–10 weeks. The growth of bacteria was monitored weekly.



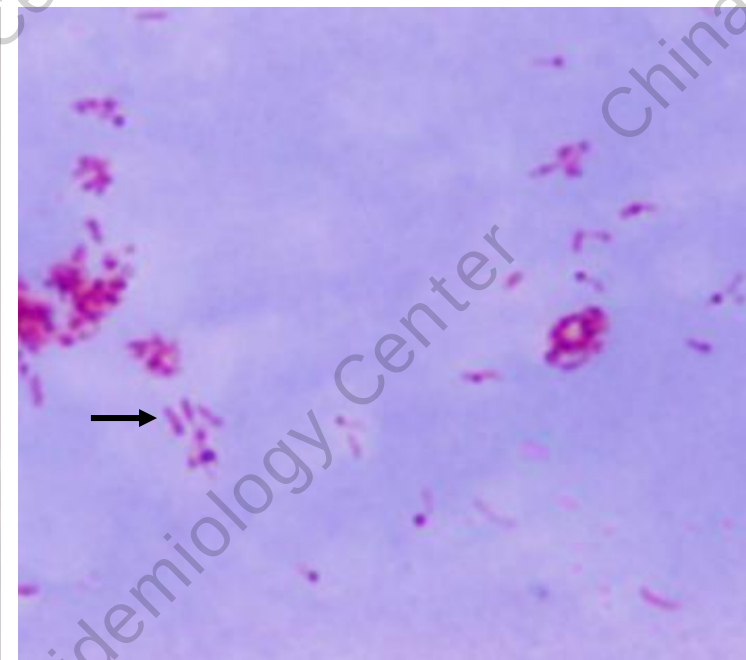
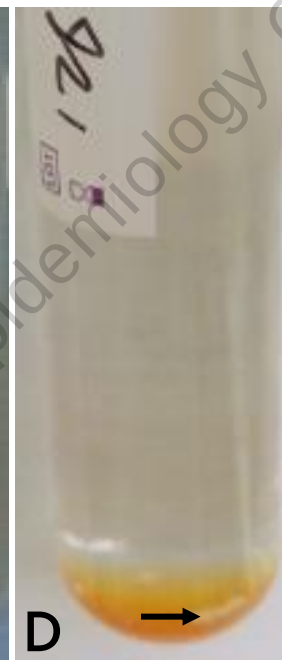
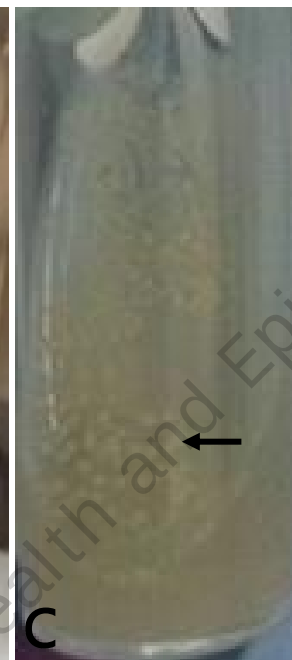
- Colony morphology*

Pyruvate L-J medium

L-J medium

7H11

7H9



M. tuberculosis

M. bovis

grows slowly at 37°C, but does not grow at 22°C or 45°C

Colonies of *M. bovis*: smooth or rough, off-white (buff)

Thank you!

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