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Guidelines for speciation within the *Mycobacterium tuberculosis* complex. Second edition.

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Abstract

There is an increasing need for reliable and standardized techniques for the isolation of *Mycobacterium bovis* from veterinary and human clinical specimens and for its differentiation from other members of the *Mycobacterium tuberculosis* complex; namely, M. *tuberculosis* and M. *africanum*. The techniques used in veterinary laboratories differ from those used in medical laboratories, mainly because strains of M. *bovis* grow poorly or not at all on the glycerol-based media used for the cultivation of M. *tuberculosis*.

Members of the M. *tuberculosis* complex are very closely related genetically, and differentiation between them is not easy. Experience has shown that tests for nitratase activity, oxygen requirements, susceptibility to the isoniazid analogue thiophen-2-carboxylic acid hydrazide (TCH) and either susceptibility to pyrazinamide or pyrazinamidase activity provide a reliable differentiation of M. *tuberculosis*, M. *bovis* and M. *africanum*. Niacin production is the basis of a simpler but less reliable test.

These guidelines, based on the methods used routinely at the Public Health Laboratory Services Mycobacterium Reference Unit, London, UK, and at the Pan American Institute for Food Protection and Zoonosis, Buenos Aires, Argentina, describe isolation methods, identification tests and their interpretation, and the media and reagents required.

1. Introduction

In countries with a low prevalence of animal tuberculosis, where bovine tuberculosis eradication programmes are in progress or have been completed, detection of tuberculous lesions at slaughterhouse inspection should be followed by examination of cattle at the farm of origin in order to identify further cases.[1] In some cases, epidemiological investigations include wildlife sources (such as the hare in Argentina, the opossum in New Zealand and the badger in the UK) and, on occasions, the human attendants. Identification of the isolated organism confirms the provisional diagnosis based on macroscopic and microscopic examination of the lesions.[2] In areas of low incidence of animal tuberculosis, it is important to determine whether isolates are M. *bovis* rather than M. *tuberculosis* or other pathogenic mycobacteria, particularly members of the M. *avium* complex.

The situation in countries with a high prevalence is different, as gross lesions suggestive of tuberculosis are observed in a relatively high proportion of animals at slaughter. Laboratory confirmation in every new case would mean an overwhelming burden of work for the laboratory with no clear benefit if no eradication campaign is under way.[1,3] Furthermore, in regions with a high prevalence, mycobacteria other than M. *bovis* are rarely the cause of gross granulomatous lesions in cattle. On the other hand, in such countries, examination of human clinical specimens for M. *bovis* is required in order to demonstrate the extent of cattle-to-human transmission of disease and thereby to provide incentives for the institution of control measures.[4] Decisions to develop laboratory facilities must be based on considerations of cost, cost-effectiveness, complexity of procedures, safety, availability of trained staff, continuous availability of reagents and power supply and maintenance of equipment.

The *Mycobacterium tuberculosis* complex (the mammalian tubercle bacilli) contains four named species, although taxonomic studies based on phenotypic characteristics, the relatedness of soluble cytoplasmic antigens and DNA homology clearly indicate that they are not merely very closely related but are, in fact, variants of a single species. Thus, the definition of named 'species' within this complex is somewhat artefactual. Certain DNA sequences identify the complex, but even if the rapid progress in nucleic acid technology soon provides probes capable of reliably detecting specific DNA sequences of M. *bovis*, some time would elapse before they be could be available for use in the clinical laboratory.

The main species within this complex are M. *bovis* (the bovine tubercle

bacillus); M. *tuberculosis* (the human tubercle bacillus); M. *africanum* (a somewhat heterogeneous group of strains principally found in equatorial Africa with properties intermediate between the former two) and M. *microti* (a rare pathogen of voles and other small mammals that has not been encountered in recent years). Bacille Calmette-Guérin (BCG vaccine) was derived from a strain presumed to be M. *bovis* but it now possesses features that clearly differentiate it from strains of that species. In addition, members of the M. *tuberculosis* complex not directly conforming to the above species are occasionally isolated. These include strains isolated from water buffalo, seals, cats and the dassie or rock hyrax.

Isolation techniques for M. *bovis* differ from those for M. *tuberculosis*. The former grows poorly, or not at all, on standard glycerol-containing Löwenstein-Jensen medium as used for isolation of M. *tuberculosis*, but its growth is stimulated by sodium pyruvate.

The M. *tuberculosis* complex was originally subdivided according to virulence for the rabbit and growth characteristics on egg-based media. The former is expensive, impractical, and carries definite hazards to human health; the latter is subjective and unreliable. Accordingly, four simple *in vitro* cultural and biochemical tests have been selected after careful evaluation and used extensively in epidemiological studies of human and animal tuberculosis.[5-8] These tests are nitratase activity, oxygen preference (aerobic or micro-aerophilic), susceptibility to the isoniazid analogue thiophen-2-carboxylic acid hydrazide and susceptibility to the antituberculosis agent pyrazinamide (PZA) or the pyrazinamidase test. The test for PZA susceptibility, although technically demanding, was included as it forms part of the examination of isolates from patients for drug susceptibility.

Pyrazinamide susceptible strains hydrolyse pyrazinamide and tests for this enzymatic activity have been used to distinguish between the two species. Originally, this test was included in Bönicke's amide row,[9] but a simpler and safer test was subsequently introduced by Wayne.[10] Although there appears to be a close relationship between pyrazinamidase activity and susceptibility to pyrazinamide, the enzyme test should not be used as a substitute for the formal susceptibility test until further data on such a correlation are available. On the other hand, from the point of view of differentiation, the pyrazinamidase test provides similarly useful information as the pyrazinamide susceptibility test and is, moreover, swifter and simpler to perform. The enzymatic method of Wayne is described.

The tests described above divide the M. *tuberculosis* complex into six types as shown in Table 1. One advantage

of this typing scheme is that the variants most often isolated from man and animals (classical M. *tuberculosis* and M. *bovis*) differ in all the tests. Strains of M. *africanum* are divisible into two types. Type I phenotypically resembles M. *bovis* and is principally found in West Africa, while Type II has more in common with M. *tuberculosis* and occurs mainly in East Africa.[11,12] Omission of the test for pyrazinamide susceptibility (or pyrazinamidase activity) could lead to a failure to discriminate between M. *bovis* and Type I strains of M. *africanum*. The few available strains of M. *microti* are identical to M. *africanum* Type I.

2. Technical methods

2.1 Laboratory equipment and design

Ideally, the tuberculosis laboratory should be provided with the following basic equipment: work bench, safety cabinet, incubator, centrifuge, vortex mixer, precision balance, pH meter, drying oven, refrigerator, shaker, microscope, staining rack, sink and a separate basin for washing hands. The safety cabinet should be Class I or II, depending on local policies and regulations.[13,14]

An autoclave and an inspissator (coagulator) for preparation of egg-based media are also required, preferably in a separate room. Purpose-built inspissators are commercially available. Alternatively, water baths and ovens may be adapted for this purpose. If an oven is used, it should be provided with an air convection system and a source of humidity. In either case the equipment should be thermoregulated at 80-85°C (± 1°C) and provided with trays so that tubes can be placed in a sloping position.

The laboratory design should take into account the fact that tuberculosis is mainly transmitted by the airborne route. All work with tuberculous material and cultures should be conducted in containment laboratories, physically separated from other areas. The principle of the containment laboratory is that, during working hours, the air is continually extracted to the outside of the building by, for example, an extractor fan in the wall or window. When the safety cabinet is in use, air is extracted through the cabinet - other extractors must then be switched off.

For a review of the general principles of laboratory design and safety procedures, see references 13-15.

2.2 Specimen collection and transport

In veterinary practice, lymph nodes from the respiratory tract, lung and liver tissue, lymph

nodes from the gastrointestinal tract and pus or caseous material from open tuberculous cavities are, in that order, the specimens most frequently submitted for laboratory examination. They are usually selected at veterinary inspection in slaughterhouses when macroscopic lesions suggestive of tuberculosis are detected. In medical practice, the usual specimens are either sputum samples or lymph node biopsies.

Place specimens in sterilized, wide-mouthed, hermetically sealable plastic or heavy glass containers and label them clearly and indelibly on the container itself, not on the lid. When histological examination is also required, divide the specimen in two and immediately put the portion designated for histology in 10% formalin. It is important to mark the two containers clearly so as to avoid the arrival of formalin-preserved specimens in the bacteriology laboratory: a not infrequent occurrence.

Laboratories are usually situated in urban areas, sometimes far from slaughterhouses and rural clinics. If transport is not immediately available, specimens should be kept cold by placing them in a +4°C refrigerator, a container with ice or (if available) a freezer at -20 to -30°C. The latter prevents the growth of fungi. Alternatively, specimens may be preserved by adding a 3% weight/volume (w/v) solution of sodium borate so that the container is two-thirds full. Such specimens may be stored in the dark at room temperature for up to four days before processing.

2.3 Initial inspection and decontamination of specimens

Note: all operations involved in inspection, decontamination and inoculation of culture media should be performed in a Class I or II safety cabinet or under other forms of protection according to local safety recommendations and regulations. It is very important that staff are adequately trained in general safety measures. Carefully examine tissue specimens macroscopically and remove small pieces of lesions by means of sterilized scissors and forceps. Cut selected tissues aseptically into smaller pieces with scissors and further homogenize them in a mortar and pestle or in a thick walled 'universal' bottle containing glass beads (an equal amount of 1-2 mm and 3-4 mm beads) which is then vortexed. (In some laboratories Griffith tubes are used. These should always be held in a thick wad of paper tissue to avoid injury if the tube breaks.) Remove about 3 ml

of homogenised tissue by means of a wide-bore pipette and add to an equal amount of autoclaved 1*N* (4%) sodium hydroxide (NaOH) in a screw cap tube. Mix well on a vortex mixer, leave at 37°C for 20 minutes, neutralize by adding a 14% w/v solution of monopotassium phosphate (KH_2PO_4) containing enough phenol red indicator, around 40 mg/litre, to impart a yellow colour (this turns red when neutralization is complete) and centrifuge for 20 minutes at 2000-3000 G. Decant the supernatant, and inoculate the deposit on to suitable media. Treat sputum and pus in the same manner. If an adequate centrifuge is not available, stand the neutralised material overnight at 4°C and then inoculate media with the sediment in the bottom of the bottle. In this respect, the use of centrifuges which do not attain 2000 G gives no better sensitivity than methods not employing centrifuges. An alternative 'softer' decontamination method is to add an equal volume of a 23% solution of anhydrous trisodium phosphate (Na_3PO_4), leaving overnight at 37°C and either inoculating the sediment at the bottom of the bottle or a centrifuged deposit.

If contamination is a recurrent problem, increase the duration of the NaOH treatment, or the temperature at which the treatment is carried out, or both; bearing in mind, however, that such modifications will kill more of the mycobacteria and that treatment with 4% NaOH for 60 minutes at 37°C will kill most of them. Oxalic acid (3%) instead of NaOH is a useful decontaminating agent if specimens are heavily contaminated with *Pseudomonas* species. Add 1 ml of specimen to 3 ml of 3% oxalic acid in a bottle. Mix well on a vortex mixer for 2 minutes and stand for 10-15 minutes, but no longer. Add 16 ml of sterile distilled water, centrifuge and culture the deposit.

Mycobacteria may be concentrated by the flocculation technique. Add a few drops of the flocculating solution composed of 1.5% w/v calcium chloride ($CaCl_2.2H_2O$) and 1.5% w/v barium chloride ($BaCl_2.2H_2O$), mixed immediately before use, to 10 ml amounts of the specimen/ decontaminating solution and leave to stand overnight. Remove the flocculated sediment with a pipette and inoculate the media.

2.4 Culture media and their inoculation

Most laboratories use solid, egg-based media. Löwenstein-Jensen (LJ) medium, which contains

glycerol, is used for the isolation of M. *tuberculosis* but glycerol is inhibitory for most strains of M. *bovis*. For this reason media containing sodium pyruvate instead of glycerol should be used for the isolation of M. *bovis*. These media are also better than LJ for the isolation of M. *avium*. A suitable pyruvate-based medium is that of Stonebrink, the composition of which is shown in the Appendix. Alternatively, LJ medium with sodium pyruvate in place of glycerol (LJP) may be used.

After homogenizing and decontaminating the specimen, inoculate the media by means of a pipette delivering around 0.2 ml. It is recommended that both Stonebrink and LJ media be inoculated; ideally two slopes of each or, for veterinary work, at least two of Stonebrink or LJP and one of LJ. Strains of M. *bovis* grow best at slightly reduced oxygen tension: tightening the screw caps of the culture bottles will ensure that, as oxygen is consumed, the atmosphere becomes microaerophilic. Incubate the slopes at 35-37°C.

2.5 Reading of cultures

Examine the slopes after 48 hours incubation for contamination by other organisms; this may be evident as visible growth or as a discolouration or softening of the medium. Discard any contaminated slopes. Subsequently, examine the slopes weekly for up to 8 weeks. Examine any bacterial growth microscopically for acid fastness by the Ziehl-Neelsen method or a related technique.

Visible growth of M. *bovis* is rare before 3 weeks and usually occurs after 4 or 5 weeks incubation. Colonies of tubercle bacilli are off-white (buff) in colour. They are never yellow: such a pigmentation indicates that the acid fast bacilli belong to another species. Some strains of M. *bovis* grow exclusively on pyruvate media, some grow better on pyruvate than on glycerol media, but strains showing no substrate preference are occasionally encountered. Thus differential growth on the two media may suggest, but not confirm, that the isolate is M. *bovis*, especially as other species, e.g. members of the M. *avium* complex, may also exhibit this differential growth. If M. *bovis* grows on LJ medium, the colonies are small, flat and smooth ('dysgonic' growth) while those of M. *tuberculosis* are usually rougher and more heaped-up ('eugonic' growth), sometimes resembling bread-crumbs; too much reliance should not, however, be placed

on gross appearance. Further tests are required to identify the organism to species level.

3. Identification tests

3.1 Suspension bottles

Take small iron nails, or pieces of stout stainless steel wire, about 10 mm in length, wash them in acetone and then in ether to remove grease. Wash glass beads, 3 mm in diameter, in weak (approx. 0.5%) hydrochloric acid (HCl) to remove soda. Place one nail and a few glass beads in a small screw-capped bottle containing 1 ml of phosphate buffer pH 7.4 (anhydrous disodium hydrogen phosphate (Na_2HPO_4) 6.6 g, and monopotassium phosphate (KH_2PO_4) 1.75 g, in 1 litre of distilled water; or prepare buffer from commercially available tablets ('Dulbecco A' phosphate buffered saline). Sterilise by autoclaving.

3.2 Preparation of bacterial suspensions

Place a 10 µl loopful of growth from the primary culture into a suspension bottle. Place the bottle on a magnetic stirrer so that the iron nail and the glass beads break up the clumps of bacteria. Allow the bottles to stand for 5 minutes to allow the larger residual clumps of bacteria to settle. This suspension contains approximately 10^5 colony-forming units/ml. Alternatively, place the bottle on a vortex mixer for about 10 seconds, and allow to stand for longer, 10 to 15 minutes, as more aerosol is generated by this technique.

3.3 Inoculation of the test media

Media are inoculated either by means of a pipette delivering the stated amount of suspension or by use of a bacteriological loop. Disposable plastic loops are preferred. Withdraw loops from the suspension edgewise so that large convex drops are not transferred.

3.4 Screening tests to confirm that an isolate is a member of the *M. tuberculosis* complex

Inoculate two slopes of LJ medium (containing glycerol or pyruvate, as indicated by growth of the primary isolate) and one tube of LJ medium containing *p*-nitrobenzoic acid (PNB), 500 mg/litre. Incubate one LJ slope and the PNB slope at 37°C in an internally illuminated incubator and the other LJ slope at 25°C and examine at 3, 7, 14 and 21 days. When growth is

evident on the LJ slope incubated at 37°C, examine it for pigment. [If an internally illuminated incubator is not available, remove the slopes from the dark incubator as soon as growth is evident, loosen the caps to admit some oxygen and expose them to daylight (but not direct sunlight), or place 1 metre from a laboratory bench lamp, for 1 hour. Reincubate in the dark incubator and examine for pigment the following day.]

Members of the *M. tuberculosis* complex do not grow within three days at 37°C, they do not grow at all at 25°C or 45°C or on PNB medium and do not produce yellow to orange pigment in the light or dark (Table 2). Note: problems in maintaining the incubation temperature at 25°C may be encountered in tropical regions where the ambient temperature is above 25°C. Site the incubator in a cool area or use a refrigerated incubator (if available) or a water bath within a refrigerator or cold room.

4. Differentiation of *M. bovis* from other members of the *M. tuberculosis* complex

As discussed above and shown in Table 1, members of this complex may be differentiated by nitratase activity, oxygen preference, susceptibility to thiophen-2-carboxylic acid hydrazide (TCH) and pyrazinamide (PZA) and pyrazinamidase activity. An additional test is for niacin production which, although less specific than those in Table 1, is widely used because of its simplicity.

4.1 Nitratase test

Several methods have been described. The three described below give consistent results.

1. Inoculate 2 ml of Middlebrook 7H9 broth (see Appendix) and incubate at 37°C until heavy growth is evident, usually for about 18 days. Add 0.05 ml of a 4 % w/v solution of potassium nitrate (KNO_3), sterilised by autoclaving, and incubate for a further 4 hours at 37°C. Add, in sequence, 0.05 ml (or two drops) of *N* hydrochloric acid (HCl), 0.2 % w/v sulphanilic acid in water and 0.1 % w/v N-1-naphthyl-diethylene-diamine dihydrochloride in water. (Prepare the latter two reagents freshly each month and store in the dark at 4°C.)

   A positive reaction is indicated by a pink colour, showing that nitrate has been reduced to nitrite. Some nitratase positive strains also reduce nitrite, leading to false negative results. To differentiate true negative from false negative results, add a very small amount of zinc dust to reduce nitrate to nitrite. If the

strain is nitratase positive, and has therefore already reduced all the nitrate, the solution will not change colour. If the strain is nitratase negative, nitrate will be reduced to nitrite and a pink colour will appear. Note: 'false positive' nitratase reactions may occur if the culture is contaminated by certain other bacteria.

2. Grow the organisms on a LJ slope (not containing pyruvate), transfer a few colonies to a 0.1% w/v solution of sodium nitrate, incubate at 37°C for 4 hours and then proceed as above.

3. As an alternative to the reagent used in the two above methods, a powder, Lampe reagent, may be used and makes the test easier to perform while giving similar results.[16] Prepare the reagent by placing one part of N-1-naphthyl-diethylene-diamine dihydrochloride, one part of sulphanilic acid and 10 parts of L(+) tartaric acid in a dark bottle and mix by shaking vigorously. The mixture is stable for at least 6 months if stored in the dark. For the test, transfer a heavy loopful of a recently grown culture on solid egg media (LJG, LJP or Stonebrink) to a tube containing an aqueous 0.01M (0.085%) solution of sodium nitrate and incubate for 3 hours at 37°C. Add a small quantity of Lampe reagent (such as a spatula full: the quantity is not critical). Read and interpret as in 1. above.

4.2 Oxygen preference

The medium is Middlebrook 7H9 or Kirchner broth (see Appendix) rendered semisolid by the addition of 0.1% pure agar and dispensed in 10 ml amounts in screw-capped bottles. Pipette 0.2 ml of the bacterial suspension about 1 cm below the surface of the medium and mix carefully, avoiding bubbles and aeration. Incubate at 37°C for 18 days. Aerobic strains grow at or near the surface, while microaerophilic strains grow as a band 10-20 mm below the surface, sometimes extending upwards.

4.3 Susceptibility to thiophen-2-carboxylic acid hydrazide (TCH)

Prepare slopes of LJ containing TCH, 5 mg/litre. Inoculate, incubate at 37°C for 2-3 weeks and compare growth with that on TCH-free control medium. Note: 1) some strains of *M. bovis* grow feebly or not at all on LJ containing glycerol and thus give false negative results if this medium is used. 2) Pyruvate inhibits the activity of isoniazid and could have a similar effect on TCH, which is an analogue of isoniazid, and thus give false positive results.[17]

Therefore those strains of M. *bovis* that only grow on media containing pyruvate may not be testable by this method. 3) Some strains of M. *tuberculosis* are susceptible to TCH: these are known as the South Indian or Asian strains and are found mainly in South India but also in Asian immigrant communities in other countries. Thus tests for susceptibility to TCH must be supplemented by the other identification tests.

4.4 Susceptibility to pyrazinamide (PZA)

The results are critically dependent on pH. If the pH is too high, the agent fails to inhibit bacterial growth and if it is too low the acidity inhibits bacterial growth. Several methods of varying complexity have been described but that of Yates gives consistent results.[1,13] Four tubes of media are used, two control and two containing PZA (see Appendix). Inoculate one test and one control tube with 20 µl of suspension and the other pair of tubes with 20 µl of a 1:10 dilution of the suspension. Incubate for 21 days at 37°C. PZA susceptibility is indicated by growth in the control tubes only or in the PZA tube receiving the heavier inoculum but not in that receiving the lighter inoculum, provided that there is growth in the corresponding control tube.

Note: 1) Strains other than M. *bovis* may acquire resistance to pyrazinamide by mutation. 2) Some recent isolates of M. *bovis* do not grow in the acidified medium. Although this has not been a problem in the United Kingdom, it could be so in other regions where use of the pyrazinamidase test (see next section) may be more suitable.

4.5 Detection of pyrazinamidase activity (Wayne's method)[10]

Inoculate butts of Wayne's medium (see Appendix) heavily, so that the inoculum is visible to the naked eye. Incubate at 37°C for 7 days and add 1 ml of freshly prepared ferrous ammonium sulphate ($Fe(NH_4)_2(SO_4)_2$, $6H_2O$), 1% w/v in distilled water. Refrigerate at 4°C for 4 hours. A positive reaction is indicated by a pink band in the upper part of the agar butt. Include positive and negative control slopes inoculated with M. *tuberculosis* and M. *bovis* respectively. A few strains of M. *bovis* produce a very faint pink band which is scored as negative.

Pyrazinamidase is one of a series of enzymes detected in the amide row of Bönicke,[9] but that method is more complex and hazardous as it involves washing by means of centrifugation and the use of hazardous chemicals to detect ammonia production.

4.6 The niacin test

The niacin test, available as a simple paper strip test (Difco), is usually positive with M. *tuberculosis* and negative with M. *bovis*. Exceptions occur, such as occasional niacin negative M. *tuberculosis*, and M. *africanum* gives variable results. Thus it is not as discriminative as the four tests mentioned above but is favoured in many laboratories on account of its speed and simplicity. Isoniazid test strips, used for detecting isoniazid in urine, are also suitable for this purpose as isoniazid is an analogue of niacin and gives an identical colour reaction. In some laboratories it has been found that the isoniazid test strip gives more reliable results than the niacin test strip. Some workers therefore consider the former to be the method of choice. Other techniques for the detection of niacin have been described,[3,13] but as they involve the use of toxic and carcinogenic chemicals, they are best avoided.

TABLE 1. Speciation within the *M. tuberculosis* complex

Species and variant	Nitratase activity	Oxygen preference	Pyrazinmide susceptibility*	Pyrazin-amidase	TCH susceptibility*
M. tuberculosis					
Classical	Positive	Aerobic	Sensitive	Positive	Resistant
Asian	Positive	Aerobic	Sensitive	Positive	Sensitive
M. africanum					
Type I	Negative	Microaerophilic	Sensitive	Positive	Sensitive
Type II	Positive	Microaerophilic	Sensitive	Positive	Sensitive
M. bovis					
Classical	Negative	Microaerophilic	Resistant	Negative	Sensitive
BCG**	Negative	Aerobic	Resistant	Negative	Sensitive

TCH = thiophen-2-carboxylic acid hydrazide;
* These tests are not applicable to all strains of *M. bovis* as some fail to grow on the media used.
** A confirmatory test for BCG is resistance to cycloserine, 20mg/l in Löwenstein Jensen medium.

TABLE 2. Differentiation of members of the *M. tuberculosis* complex from other cultivable mycobacteria.

Growth on PNB Medium*	Growth at 25°C	Growth within 3 days	Pigment production	Identification
-	-	-	None	*M. tuberculosis* complex
+	+	-	Light only	Photochromogen
+	+	-	In dark and light	Scotochromogen
+	+ or -	-	None	Nonchromogen
+	+	+	Variable	Rapid grower

* Löwenstein-Jensen medium containing *p*-nitrobenzoic acid (PNB), 500 mg/litre.

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Appendix - Media

(Powder for Middlebrook 7H9 broth and ready-for-use OADC enrichment are commercially available. In some regions Löwenstein-Jensen medium is commercially available.)

i) Löwenstein-Jensen medium

There are various formulae. Some include potato starch which makes the medium slightly firmer and reduces water of condensation. Others include asparagine as an additional nitrogen source. Neither is essential and they are not included in media used at the UK Mycobacterium Reference Unit or in the PAHO/WHO recommended procedures. Containers and other equipment must be sterilised as the process of inspissation (coagulation) cannot be relied upon to kill all contaminating micro-organisms, especially not spores.

Monopotassium phosphate (KH_2PO_4), anhydrous	4.0 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.4 g
Magnesium citrate	1.0 g
Glycerol (analytical grade)	20 ml
Distilled water, to	1000 ml
Whole fresh eggs	1600 ml
Malachite green, 1% w/v aqueous solution	50 ml

Dissolve the salts and glycerol in the distilled water and steam in autoclave or steamer at atmospheric pressure for 2 hours. Wash the eggs with soap and water and rinse by wiping them with 70% alcohol. Break the eggs into a graduated cylinder until the 1600 ml mark is reached. Transfer to a screw-capped jar containing a few large (about 10 mm diameter) glass beads and shake to break the yolks and homogenise the material. Alternatively use a sterilizable homogenizer such as a Waring blender, if available. Filter through sterile gauze and add to the steamed salt solution. Add the malachite green solution and mix well. (As a further precaution against contamination, 200,000 units of penicillin may also be added.) Dispense as, for example, 7 ml amounts in 28 ml screw-capped bottles, place on sloped racks and inspissate at 80-85°C until hard (about 50-90 minutes). The time required depends on the thickness of the glass of the bottles.

ii) Stonebrink's medium

Monopotassium phosphate (KH_2PO_4), anhydrous	3.5 g
Disodium phosphate ($Na_2HPO_4.12H_2O$)	2.0 g
Sodium pyruvate	6.3 g
Distilled water, to	500 ml
Whole fresh eggs	1000 ml
Malachite green, 2% w/v aqueous solution	20 ml

The medium is prepared in the same manner as Löwenstein-Jensen medium. An alternative medium is the Löwenstein-Jensen medium as described above but with 12 g of sodium pyruvate instead of the glycerol.

iii) Middlebrook 7H9 broth

Ammonium sulphate ($(NH_4)_2SO_4$)	0.5 g
Sodium glutamate	0.5 g
Disodium phosphate ($Na_2HPO_4.12H_20$)	2.5 g
Monopotassium phosphate (KH_2PO_4), anhydrous	1.0 g
Trisodium citrate	100 mg
Ferric ammonium citrate	40 mg
Magnesium sulphate ($MgSO_4.7H_2O$)	50 mg
Zinc sulphate ($ZnSO_4.7H_2O$)	1.0 mg
Cupric sulphate ($CuSO_4.5H_2O$)	1.0 mg
Pyridoxine	1.0 mg
Calcium chloride ($CaCl_2$)	0.5 mg
Biotin	0.5 mg
Tween 80	0.5 ml
Distilled water, to	900 ml

Dissolve by steaming. Distribute as required and autoclave at 115°C for 15 minutes. When cool add 1 volume of Middlebrook OADC enrichment (see below), or 1 volume of sterile horse serum, to 9 volumes of medium.

iv) Middlebrook OADC (oleic acid-albumin-dextrose-catalase) enrichment

Bovine serum albumin, fraction V	50.0 g
Glucose (dextrose)	20.0 g
Sodium chloride (NaCl)	8.0 g
Sodium hydroxide solution (NaOH), 0.1N	25 ml
Oleic acid	0.6 ml
Citric acid 10% W/V aqueous solution	0.4 ml
Catalase (crude)	0.02 ml
Distilled water	975 ml

Dissolve the albumin in the distilled water and then add the other ingredients with thorough mixing. Sterilise by membrane filtration and dispense in 20 ml amounts. Incubate at 37°C overnight to test for sterility and store at 4°C.

v) Kirchner medium

Disodium phosphate ($Na_2HPO_4.12H_20$)	19 g
Monopotassium phosphate (KH_2PO_4), anhydrous	2.5 g
Trisodium citrate	2.5 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.6 g
Asparagine	5 g
Glycerol	20 ml
Phenol red, 0.4% w/v aqueous solution	3 ml
Distilled water, to	1000 ml

Dissolve by steaming. Check that pH is in the range 7.4-7.6 and adjust if necessary. Dispense as required and sterilise by autoclaving at 115°C for 10 minutes. When cool add 1 volume of sterile horse serum (or Middlebrook OADC enrichment if indicated) to 9 volumes of medium.

vi) Medium for pyrazinamide susceptibility tests

This is a two-phase medium, with solid and semisolid layers.

Solid phase. Prepare Löwenstein-Jensen medium with glycerol and adjust electrometrically to pH 5.2 with *N* hydrochloric acid. (Use of a pH meter is essential for accuracy.) Add 9 ml of a 0.22% w/v solution of pyrazinamide, sterilised by membrane filtration, to 300 ml of medium. Add 9 ml of sterile water to a further 300 ml of medium for the control. Place 1 ml amounts of the media in small screw- capped bottles and inspissate (85°C for 1 hour) in a vertical position.

Semisolid phase. Prepare 1 litre of Kirchner medium and add 1 g of agar and 3 g of sodium pyruvate and adjust electrometrically to pH 5.2 with *N* hydrochloric acid. Dissolve agar by steaming. Divide into two 500 ml batches and add 15 ml of the pyrazinamide solution (see above) to one and 15 ml of sterile water to the other. Sterilise by autoclaving and, when cool, add 30 ml of Middlebrook OADC enrichment (see above) to both.

Final medium. Add 2 ml amounts of test and control semisolid medium to the corresponding bottles containing the solid medium. Store at 4°C and use within three weeks of preparation.

vii) Medium for detection of pyrazinamidase activity

Middlebrook 7H9 broth (see above)	1000 ml
Pyrazinamide	100 mg
Sodium pyruvate	2 g
Agar	15 g

Dissolve by steaming. Dispense in 5 ml amounts in screw-cap bottles and sterilise by autoclaving at 115°C for 15 minutes. Allow to cool in the upright position so that butts rather than slopes are formed.