Chapter 3

Basic microbiology

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M. ulcerans belongs to a group of mycobacteria that are potentially pathogenic for humans or animals. These are sometimes called "opportunistic mycobacteria" or "occasional pathogens". Most species belonging to this group are found almost everywhere in nature, and may become pathogenic under special circumstances. Some of them have rarely (e.g. *M. malmoense*) or never (e.g. *M. ulcerans*) been isolated from the environment. The epidemiological profiles of the diseases they cause, however, suggest that they are present in nature (*23*).

Recently, *M. ulcerans* was detected by molecular biological techniques in water samples collected in Australia (*42, 43*) and in bugs collected from roots of aquatic plants in swamps in endemic regions of Benin and Ghana (*33*). *M. ulcerans* was, however, not recovered by culture from these environmental samples. Isolation of *M. ulcerans* in primary culture from clinical specimens is possible but not easy.

Isolation in primary culture

Several authors have discussed the difficulties of isolating *M. ulcerans* in primary culture from clinical specimens (*44*,*45*). Despite numerous attempts, *M. ulcerans* has never been cultivated from environmental samples, although a large variety of other mycobacterial species have been isolated (*43*). Several reasons may explain the difficulty or the inability to cultivate *M. ulcerans* from clinical or environmental specimens.

Sampling

Sampling is often inadequate. Clinical specimens should be collected from sites usually rich in bacilli, e.g. the necrotic base of the lesion and the undermined edges of the ulcer, including subcutaneous tissue. Environmental specimens should be collected from sites where *M. ulcerans* is suspected to be concentrated, e.g. by filtering organisms, and from sites where *M. ulcerans* is demonstrably best able to survive. In tropical regions, surface samples are subjected to high temperatures and ultraviolet light, which in the laboratory have been shown to affect the viability of the bacilli. In deeper sites, such as at the bottom of swamps, ultraviolet rays do not penetrate and the temperature is lower and more stable. Moreover, in deeper parts of swamps oxygen concentration is reduced: *in vitro*, microaerophilic conditions favour the multiplication of *M. ulcerans* (*46*).

Transportation to the laboratory

M. ulcerans grows optimally on conventional mycobacteriological media at 32 °C, and is very sensitive to higher temperature. One day at 41 °C kills more than 90% of the bacilli and, for some strains, one day at 37 °C also kills more than 90% of the bacilli (*33*). Meyers et al. also observed that growth at 32 °C was retarded after exposure to 37°C for one day, and was completely inhibited after exposure to 40 °C for 10 days (*47*). The temperature of transportation to the laboratory is therefore critical, especially

for specimens collected in tropical countries where the temperature may exceed 37 °C for long periods.

In many studies, primary cultures were set up days or weeks after collection of the specimens. Ideally, the specimens should be processed on the day of collection to obtain a maximum of positive primary cultures. When this is not feasible, specimens may be kept at +4 °C or in transport media. Freezing is not advisable because *M. ulcerans*, like other mycobacteria (e.g. *M. leprae, M. lepraemurium*), is highly sensitive to freezing–thawing cycles (*48*).

Transport media

Three transport media (S, P and P5) have been developed in the Mycobacteriology Unit of the Institute of Medicine, Antwerp, Belgium. Transport medium S is a selective Dubos medium supplemented with antibiotics, as described by Saxegaard for the isolation of *M. paratuberculosis* from the intestinal tissues of goats (*49*). Transport medium P is a Dubos medium supplemented with PANTA[a] as used for the isolation of *M. tuberculosis* in the BACTEC system. Culture rates from specimens transported in S or P media are identical (*50*). Given the preference of *M. ulcerans* for low oxygen concentrations, a new semi-solid transport medium (P5) was developed by the addition of 0.5% agar to transport medium P. The three transport media produce comparable results (about 40% positive primary cultures). Successful primary culture is not related to the period of elapsed time in the transport medium but is dependent on the number of viable acid-fast bacilli present in the inoculum. Transport medium P5 is, however, superior to S and P because positive cultures can be obtained even after 7 weeks of storage compared to 3 weeks with S and P. Some 90% of primary cultures are positive after less than 3 months' incubation at 32 °C. These results do not depend on the type of transport medium used.

Decontamination methods

M. ulcerans is susceptible to decontamination methods. All of the decontamination methods currently used for the isolation of *M. ulcerans* from clinical specimens (Petroff, NALC-NaOH) or for the isolation of mycobacteria from environmental specimens (Petroff, oxalic acid) (*51*) have a detrimental impact on the viability of *M. ulcerans* (*52*). This explains, at least in part, the difficulty often experienced in cultivating this organism from clinical specimens and the failure to cultivate *M. ulcerans* from environmental specimens that, by definition, are heavily contaminated with other microorganisms and thus require drastic methods for decontamination. Moreover, it is likely that environmental samples are less rich in bacilli than clinical specimens. Indeed, smears from some clinical specimens stained by the Ziehl-Neelsen method usually reveal clumps of acid-fast bacilli, (4+ according to the scale of the American Thoracic Society) (*53*), while environmental specimens are infrequently smear-positive (Portaels F, unpublished data, 1998). The application of drastic methods on scanty positive specimens may therefore be detrimental to the successful culture of *M. ulcerans*.

[a] *A mixture of five antibiotics (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin).*

Culture media

Löwenstein-Jensen medium is the most appropriate of the common conventional solid media for mycobacterial cultivation. Ogawa and Middlebrook media are less appropriate. The optimal pH for growth of *M. ulcerans* lies between 5.4 and 7.4 (*54*).

Incubation conditions

Incubation at 32 °C is essential for the isolation of *M. ulcerans* in primary culture. Another important factor is the oxygen concentration. It has recently been demonstrated that reduced oxygen concentration enhances the growth of *M. ulcerans,* suggesting a preference of this organism for microaerophilic environments (*46*).

In vitro characteristics of M. ulcerans

M. ulcerans is a slow-growing mycobacterium. Its generation time is about 20 hours, similar to that of other slow-growing mycobacterial species (*55*). Primary cultures may take between 6 and 8 weeks, similar to tubercle bacilli, but subcultures are generally positive within 2 weeks depending on the number of acid-fast bacilli in the inoculum.

M. ulcerans can be easily identified by classical identification schemes (*56)*. Several phenotypic characteristics differentiate *M. ulcerans* from the other slow-growing mycobacterial species. Very few strains grow at 37 °C. The organism is resistant to isoniazid, but most of the strains are inhibited by hydroxylamine and *p*-nitrobenzoate. The other slow-growing species that are susceptible to hydroxilamine and *p*-nitrobenzoate are also susceptible to isoniazid (*54)*. Some phenotypic characteristics seem to differentiate African, Australian and North American strains (*57*). Acid phosphatase activity is in general positive for African strains, but negative for strains from other origins.