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Appraisal of state-of-the-art

Methods used in preclinical assessment of anti-Buruli ulcer agents: A global perspective

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ABSTRACT

Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is the third most common chronic mycobacterial infection in humans. Approximately 5000 cases are reported annually from at least 33 countries around the globe, especially in rural African communities. Even though anti-mycobacterial therapy is often effective for early nodular or ulcerative lesions, surgery is sometimes employed for aiding wound healing and correction of deformities. The usefulness of the antibiotherapy nonetheless is challenged by huge restrictive factors such as high cost, surgical scars and loss of income due to loss of man-hours, and in some instances employment. For these reasons, more effective and safer drugs are urgently needed, and research programs into alternative therapeutics including investigation of natural products should be encouraged. There is the need for appropriate susceptibility testing methods for the evaluation of potency. A number of biological assay methodologies are in current use, ranging from the classical agar and broth dilution assay formats, to radiorespirometric, dye-based, and fluorescent/luminescence reporter assays. Mice, rats, armadillo, guinea pigs, monkeys, grass cutters and lizards have been suggested as animal models for Buruli ulcer. This review presents an overview of *in vitro* and *in vivo* susceptibility testing methods developed so far for the determination of anti-Buruli ulcer activity of natural products and derivatives.

1. Background

Buruli ulcer (BU) is the third commonest mycobacterial disease, after tuberculosis and leprosy; the causative organism is *Mycobacterium ulcerans* (WHO, 2011). It largely affects inhabitants of remote rural areas in West and Central Africa, usually along wetlands with limited access to health facilities and is an important cause of human suffering. At least 33 countries within the tropical, subtropical and temperate region have reported BU, with 5000–6000 cases reported annually in 15 of the 33 countries. Most cases occur in tropical and subtropical regions except in Australia, China and Japan. In West Africa, Benin, Côte d'Ivoire and Ghana reported most cases with Côte d'Ivoire reporting almost half of the global cases (WHO, 2013).The exact mode of transmission to humans remains unknown, but in contrast to tuberculosis and leprosy, the infection is largely believed to be acquired directly or indirectly from the environment and not through contact with other patients (Stinear & Johnson, 2008).

BU is among the so-called neglected tropical diseases (NTDs). According to a WHO report (2009), at least 1 billion people, representing about one sixth of the world's population suffer from one or more NTDs. They represent about 17% of the global burden of parasitic and infectious diseases and are endemic in rural communities of Sub-Saharan Africa and poor urban areas in low-income countries of Asia and Latin America. It is estimated that about 534,000 people worldwide die from an NTD each year (WHO, 2009).

There is no vaccine against BU and the main control strategy is early case detection and treatment to reduce the suffering associated with the disease. Although recent experience indicates that combination chemotherapy with streptomycin and rifampin improves cure rates, the utility of this regimen is limited by the 2-months duration of the treatment, potential toxicity and a required parenteral administration of streptomycin, and drug–drug interactions caused by rifampin (Chauty et al., 2007; Johnson, 2010; Zhang et al., 2013). Surgery is, however, necessary for some severe forms of the disease (large ulcerated forms, disseminated forms, and osteomyelitis) (Kibadi et al., 2010). This surgical treatment can only be used in a few medical centers with proper and adequate equipment and is neither affordable nor accessible to a large part of the population (Johnson et al., 2004).

Despite this problem, there is inadequate emphasis on the development of new anti-mycobacterial drugs. The urgent need to alleviate the burden related to this disease led the scientific community to

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investigate various resources, including plant-derived biologically active metabolites against the causative agent (*M. ulcerans*). Discovery and development of new drugs for BU treatment are greatly hampered by the slow-growing rate of *M. ulcerans*, requiring even up to 6 months of incubation on solid media to produce visible colonies.

In an attempt to provide guidance on quality assurance for anti-*M. ulcerans* screening, this review focuses on analytical methods involving primary *in vitro* approaches and *in vivo* animal models. We focused our literature search on publications on Medline PubMed (NCBI) database dealing with the evaluation of activities of natural products against *M. ulcerans* and limited it to papers published in English or in French. Key words used were "antimycobacterial screening", "Buruli ulcer", "*Mycobacterium ulcerans*", "drugs susceptibility test", and "mycobacteria". All retrieved titles and abstracts were analyzed for relevant studies that are discussed here.

2. Requirements for products and growth conditions

2.1. Product management

Water, dimethyl sulfoxide (DMSO), tween 80, methanol, and ethanol are the most frequently used solvents to prepare test products solutions. Given their physico-chemical properties, methanol and ethanol present the challenge of rapid evaporation, which may alter the concentrations of the stock solutions, and as well inhibit mycobacteria growth. Given these limitations, products in 100% DMSO have become the standard (Gad, 2005, chap. 13). Added advantages of stock solutions in 100% DMSO are: (1) elimination of microbial contamination, thereby reducing the need for sterilization by autoclaving which can affect the quality of the test product, and (2) good compatibility with test automation and integrated screening platforms, assuring, for example, good solubility during the serial dilution procedures (Cos et al., 2006). It is however noteworthy that DMSO is potentially cytotoxic to many microorganisms including *M. ulcerans*. It is therefore advisable to keep the in-test concentration of DMSO below 0.625% to avoid any interference with the biological test systems. This requires intermediate dilution step in water. Because of the variability of individual compounds, there are no general storage conditions that guarantee sample integrity (Verkman, 2004).

2.2. Growth conditions

Mycobacterium ulcerans differs from most other pathogenic mycobacteria in that it grows optimally at 30–33 °C and not at 37 °C (Eddyani & Portaels, 2007). The generation time (36 h) of *M. ulcerans* is longer compared to that of other slow growing mycobacterial species such as *M. smegmatis* (Pattyn, 1965). Using the radiometric BACTEC 460 system, a generation time of 23 h was determined for *M. ulcerans* (Portaels et al., 2001). The media commonly used to culture slowly growing mycobacteria (e.g. Löwenstein-Jensen, Ogawa and Middlebrook media) are also suitable for *M. ulcerans*. Löwenstein-Jensen (LJ) is a conventional culture medium for *M. ulcerans*. Middlebrook 7H11 agar, Middlebrook 7H9, BacT/Alert medium, BACTEC medium and Middlebrook 7H12 liquid media are also convenient for culturing *M. ulcerans* in vitro. The incubation period varies according to the type of culture medium used and is generally longer on solid medium. Cultures are read within 2 to 3 weeks with Middlebrook 7H12, 4 to 8 weeks with Middlebrook 7H11 agar, and 6 to 8 weeks or more with LJ while 8 to 14 days are required when Middlebrook 7H9 broth is used (Scherr, Röltgen, Witschel, & Pluschke, 2012; Yemoa et al., 2011).

2.3. Bacterial load

The bacterial load to be used for susceptibility testing is critical for accuracy and reproducibility of results. In fact, inoculum concentration can have a profound influence on the antibacterial potency of a product, endorsing the need for standardization of inoculates (Anon., 2003; Gautam, Saklani, & Jachak, 2007). For dilution methods, Hadacek and Greger, (2000) recommend an inoculum size of about 10^5 CFU/ml for most bacteria. A false-positive activity can be obtained with a much smaller inoculum size (e.g.10^2 CFU/ml), whereas the risk for false-negatives are increased and endpoint readings hampered with a much higher inoculum size (e.g. 10^7 CFU/ml). Bacterial inoculates can be prepared from cultures or from existing biofrozen stocks. To prevent the selection of an atypical variant, sample should be collected from culture during the exponential growth phase and should consist of four or five colonies of a pure culture on agar (Anon., 2003). The bacterial load to be considered also depends mainly on the growth rate of the bacteria under consideration. The inoculum size of fast growing species should be smaller than that of slow growers.

For the preparation of *M. ulcerans* inoculum, many strategies have been used so far. The *M. ulcerans* isolates are conventionally subcultured on Lowenstein-Jensen slants at 32 °C, incubated for 8 weeks and examined during that period for growth and purity. A loopful of culture is then added to sterile glass beads and distilled water in a screw-cap tube and homogenized with a vortex to fragment the colonies. The turbidity of the suspension is then adjusted to that of a No. 1 McFarland and serially diluted 10-fold to yield 10^{-2} and 10^{-4} suspensions for the susceptibility testing. Preparation of inoculum is usually done on ice to prevent aggregation of *M. ulcerans* due to the high lipid content, which allows them to stick together at room temperature. Smears of the resultant *M. ulcerans* suspensions should be stained with Ziehl Neelsen (ZN) stain to reveal acid-fast bacilli (AFB) and to check for microbiological purity (Addo et al., 2007). Indeed, for the preparation of *M. ulcerans* inoculum, bacteria can be diluted in phosphate buffered saline (PBS) instead of water.

In addition to the turbidity determination, the inoculum size can also be determined using microscopy such as the ZN staining according to the method described by Shepard and McRae (1968). Even though the acid-fast staining techniques are the most rapid way to verify the presence of mycobacteria, accurately quantifying mycobacteria by smearing cells onto a glass slide is difficult (Treuer & Haydel, 2011). The most reliable procedure is deemed through the enumeration of bacteria colony forming unit (CFU) after culture, which is a very slow process due to its slow growing nature. Thus this approach for inoculum size determination is difficult to obtain and usually not practicable. Treuer and Haydel, (2011) proposed the use of a modified Kinyoun acid-fast staining method adapted for use with a Petroff-Hausser sperm and bacteria cell counting chamber by using a liquid suspension staining technique. The result achieved by this method accurately correlates the viable cell counts by agar plate counting.

The use of weight to determine the inoculum size has also been proposed. Portaels, Traore, de Ridder, and Meyers (1998), proposed that fresh colonies of *M. ulcerans* are collected from the LJ medium and suspended either in distilled water or culture medium. The turbidity of the resulting suspension is then adjusted with distilled water to be equivalent to that of a standard 1 mg/ml suspension of *M. bovis* BCG (containing approximately 10^8 CFU per ml), from which further dilution is performed to 10^{-1} and 10^{-2} mg/ml. The inocula are 0.1 ml of diluted solutions (Yemoa et al., 2011).

Therefore in preparing bacteria suspension for susceptibility testing, *M. ulcerans* growth patterns such as slow growing and a low optimum growth temperature between 28 and 32 °C; as well as ability to aggregate in broth, should be considered in designing a suitable testing system. The medium supplemented with tween 80 or in BacT/Alert culture bottles supplemented with enrichment medium can be effectively used to prevent aggregation in liquid medium and reduce incubation time. Usually, *M. ulcerans* is grown for 6 to 8 weeks at 30 °C until the log phase is reached. The test inoculum is prepared by diluting the culture with BacT/Alert medium to a final optical density at 600 nm (OD600) of about 0.02 (Scherr et al., 2012).