MICROBIAL PATHOGENICITY

Evaluation of liquid culture media to support growth of *Mobiluncus* species

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Mobiluncus curtisii and *M. mulieris* are anaerobic, gram-negative, motile curved rods isolated commonly from the vagina of women with bacterial vaginosis. Hitherto, there has been difficulty in isolating and growing these bacteria and little attention has been paid to growth in liquid media. Reasons for establishing the means of attaining optimal growth in such media include production of antigens for diagnostic and immunological studies and production of the soluble cytotoxin. In this study the efficacy of 12 liquid culture media in supporting growth was examined. *M. mulieris* (strain A198) multiplied ≥ 10 -fold in only five media – Schaedler broth, Columbia blood broth (CBB), peptone-starch-dextrose (PSD) broth, brain-heart infusion plus arginine and spent tissue-culture medium. Similarly, *M. curtisii* (strain A98) multiplied ≥ 10 -fold in only three media – Schaedler broth, CBB and PSD. Some strains of both bacterial species grew very poorly or not at all, in all the media tested. With an inoculum of $\geq 10^5$ /ml, CBB, or PSD plus 10% horse serum, supported the growth of some strains of both bacterial species to 10^9 organisms/ml within 48 h, and viable bacteria persisted longer in some media (e.g., CBB) than in others. While variation in growth of *Mobiluncus* spp. may occur between one laboratory and another, these observations provide the basis for optimisation of a universal liquid culture medium that should facilitate production of antigens and cytotoxin.

Introduction

Mobiluncus curtisii and *M. mulieris* are anaerobic, gram-negative motile curved rods isolated from the vagina, particularly of women with bacterial vaginosis (BV) [1]. Microscopic detection of motile curved rods in a wet smear has been used as a criterion for the presence of *Mobiluncus* spp. in a vaginal specimen [2], but this procedure may also detect other motile bacteria unrelated to the curved rods. Therefore, it was important to find a means of isolating *Mobiluncus* spp. selectively in primary culture, a difficult task because the anaerobic flora of the vagina is profuse (10^{12} bacteria/g of secretion). Approaches to this objective have concentrated on attempts to inhibit the growth of other more rapidly growing bacteria present in vaginal specimens by using antibiotics and other agents in solid media [3–5]. This may be successful but is laborious and time-consuming, perhaps taking up to 6 weeks for isolation and definite identification. The

use of liquid media, particularly if prolific growth were attainable, would provide the opportunity to selectively enhance the growth of *Mobiluncus* spp. In turn, this would not only enable the dynamics of growth to be determined more easily but also facilitate preparation of antigens for diagnostic and immunological use and the production and examination of diffusible extracellular metabolites, such as the cytotoxin [6] which may be an important pathogenicity factor. It has been suggested recently [7] that *M. curtisii*, in particular, is associated with BV and is of possible importance in its aetiology. This gave further impetus to determining the efficiency of various liquid media to support growth of *Mobiluncus* spp., possibly as a prelude to pathogenicity studies, and was the purpose of the current investigation.

Materials and methods

Bacterial strains and culture media

Three strains each of *M. curtisii* and of *M. mulieris* isolated from the vagina of women with BV were received from four laboratories in the UK and Sweden

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[6]. They were subcultured every 5–7 days on Columbia Blood Agar (CBA) (Difco) and incubated anaerobically at 37°C. Nine media were evaluated: Schaedler broth (BBL), Columbia Blood Broth (CBB; Oxoid), Peptone Starch Dextrose (PSD) broth (Oxoid), *Helicobacter pylori* medium (Oxoid), fresh tissue-culture medium (FTCM) (Flow), spent tissue-culture medium (STCM) (Flow), *Brucella* broth (Oxoid), Brain-Heart Infusion (BHI) broth (Oxoid) and Cooked Meat Carbohydrate (CMC) medium (Southern Group Laboratories). In addition, in attempts to enhance growth, several agents were added as single supplements to BHI broth as follows: arginine-free base (Sigma) 0.3%, sodium hippurate (Sigma) 1.0%, sodium salts of formate and fumarate 0.3%, and bovine serum 2.5%. Also, heat-inactivated horse serum 10% and *Helicobacter* growth supplement (Oxoid) were added as single supplements to PSD broth and to *Brucella* broth, respectively.

Pre-reduction and inoculation of media

Agar and broth media were placed in an anaerobic cabinet (Forma Scientific) for 24–48 h before use to allow for displacement of oxygen. For each of the media tested, a 3-ml sample was inoculated in triplicate with a bacterial strain, as described below. An uninoculated 3-ml sample was kept as a negative control. A suspension of each of the bacterial strains (*c.* 10^5 – 10^6 cfu/ml) was prepared for each medium by harvesting the growth occurring on a single CBA agar plate into 2 ml of BHI broth. Each 3-ml sample of medium was inoculated with 0.1 ml of the bacterial suspension and incubated under anaerobic conditions (N_2 80%, H_2 10%, CO_2 10%) at 37°C for up to 7 days.

Quantification of *Mobiluncus* spp. in broth culture

A 'micromethod' was used [8] that allowed the simultaneous dilution of up to 12 broth cultures in 96-well plates (12×8 rows) with a multichannel pipette. Serial 10-fold dilutions were made by transferring 20- μ l volumes into successive wells each containing 180 μ l of BHI broth, up to a dilution of 10^8 . Then 10- μ l samples of each dilution were pipetted in triplicate on to pre-reduced CBA. The plates were incubated anaerobically at 37°C for 5 days, after which the small *Mobiluncus* colonies (<1 mm diameter) were counted with the aid of an electronic colony counter (Jencons). The number of *Mobiluncus* organisms/ml of broth sample was calculated by a modification [8] of the method developed by Miles *et al.* [9].

Results

Growth of *M. mulieris* and *M. curtisii* in different media

The multiplication of *M. mulieris* strain A198 in seven

different media is shown in Fig. 1. Multiplication of ≥ 10 -fold was recorded in five media (Schaedler broth, CBB, PSD broth, STCM and BHI + arginine-free base), but not in FTCM or BHI broth + formate/fumarate. Multiplication did not occur in *H. pylori* medium or CMC medium (not depicted in Fig. 1). Maximal growth (100-fold increase) was shown in Schaedler broth, but viable organisms persisted longer in CBB and, in other experiments, maximal growth in CBB was similar to that in Schaedler broth.

The multiplication of *M. curtisii* strain A98 in the same nine media showed a similar pattern to that described for *M. mulieris* in Fig. 1, although in this case maximal growth was seen in CBB.

Growth of different strains of *M. mulieris* and *M. curtisii* in CBB

The growth of two strains of *M. mulieris* (A198 and L1000) and two strains of *M. curtisii* (A98 and A345) in CBB is shown in Fig. 2. The patterns of growth of *M. mulieris* strain 114.9 and *M. curtisii* strain A228 (not shown) were similar to that of *M. curtisii* strain A345. It is clear that there are differences in the growth of strains within and between the two species, the greatest difference (>10-fold in maximal growth) being observed between *M. curtisii* strain A98 and *M. mulieris* strain L1000, multiplication of the latter being the least of all the six strains.

Growth of different strains of *M. mulieris* and *M. curtisii* in supplemented media

None of the putative growth factors (see *Materials and*

Fig. 1. Growth curves for *M. mulieris* strain A198 in broth media. A, CBB; B, Schaedler broth; C, PSD broth; D, STCM; E, BHI broth + arginine-free base; F, BHI broth + formate/fumarate; G, FTCM.

Fig. 2. Growth curves for four strains of *Mobiluncus* spp. in CBB medium. **A**, *M. curtisii* strain A98; **C**, *M. curtisii* strain A345; **B**, *M. mulieris* strain A198; **D**, *M. mulieris* strain L1000.

methods) that were added to BHI broth, with the possible exception of arginine-free base (Fig. 1), had any significant effect on growth of the four strains shown in Fig. 2, or the other two strains mentioned above. However, in PSD medium supplemented with heat-inactivated horse serum 10% (Fig. 3), after 48 h there was a 1000-fold increase in the number of organisms of *M. mulieris* strain A198 and *M. curtisii* strain A98, respectively, a titre of 10^9 cfu/ml being attained. This was at least 10-fold more than in

Fig. 3. Growth curves for four strains of *Mobiluncus* spp. in PSD broth containing heat-inactivated horse serum 10%. **A**, *M. mulieris* strain A198; **B**, *M. mulieris* strain L1000; **C**, *M. curtisii* strain A98; **D**, *M. curtisii* strain A345.

unsupplemented PSD medium. It is noteworthy that, consistent with growth in other media, the growth of strains L1000 and C345 was 10–100-fold less than that of the aforementioned two strains. Furthermore, in the serum-supplemented medium the number of viable organisms of all strains declined rapidly, in comparison with more sustained viability in CBB.

The effect of inoculum size

In all previous experiments, an inoculum size of 10^5 – 10^6 organisms/ml had been used. This had resulted in a titre of 10^9 organisms/ml of *M. mulieris* strain A198 and *M. curtisii* strain A98 in, for example, serum-supplemented PSD medium after 48 h. When the inoculum size was reduced to $(1-2) \times 10^3$ organisms/ml, multiplication in this medium was approximately the same, i.e., 1000-fold, so that the maximum titre of strain A198 reached only 10^5 organisms/ml and of strain A98 10^6 organisms/ml after 144 h.

Discussion

Mobiluncus spp. are fastidious in their growth requirements and most routine laboratory media either do not contain or have insufficient growth factors to support their multiplication. In the past, the emphasis was on the development of solid media for colony production, thus enabling a distinction to be made between the two species and between strains through colony cloning. For this purpose, providing colony size is acceptable, colony density, which reflects medium sensitivity, is not of over-riding importance. However, the sensitivity of solid medium is important if it is used for primary isolation, where colony development for *Mobiluncus* spp. may prove difficult. Nevertheless, growth in liquid medium may be even more difficult. Thus, in one study [10] all strains of *Mobiluncus* spp. tested gave better growth on solid than in liquid medium. This is emphasised in the current study by the fact that most of the liquid media studied were incapable of supporting acceptable levels of growth of either bacterial species, a finding consistent with that in another study [11]. Furthermore, several investigators have shown that even the addition of several putative growth factors did not improve growth in liquid medium [5, 10, 11], although supplementation with serum proved beneficial, as noted in the current study. The present study also demonstrated that liquid media that supported maximal growth of the *Mobiluncus* spp. were mainly counterparts of solid media that had been deemed optimal for colony production [5]. Indeed, these observations indicate that investigators in the field need not consider more than a few commercial liquid media, perhaps only one (CBB), for excellent growth. This is based on the fact that the key to high levels of growth in liquid media was found to be: (a) the use of media comprising CBB or PSD plus horse serum 10%, (b) the selection of a bacterial strain

known to multiply to high titre as some strains, whatever the medium, consistently reached maximal titres greater than attained by others, and (c) an initial inoculum of 10^5 – 10^6 organisms/ml. Under these circumstances, a viable count of 10^9 organisms/ml could be attained consistently in 48 h, maintenance of a high titre being a feature of some media (e.g., CBB), but not others. Such maintenance of viable organisms in liquid medium may be particularly important in the production of soluble metabolic products, such as the cytotoxin. The latter was not demonstrable previously in a centrifuged deposit of organisms or in colonies from solid medium [6].

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