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PREPARATION OF A NEW MEDIUM FOR *IN VITRO* CULTURE OF BORRELIA MICROTTI AND BORRELIA PERSICA

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ABSTRACT

Borrelia microtti and *Borrelia persica* are two Iranian strains of spirochetes whose vectors are *Ornithodorous tholozani* and *Ornithodorous erraticus* which are responsible for relapsing fever. BSK medium has been used for *in vitro* culture of other strains of borrelia, although the two mentioned strains could not be successfully cultured in it. We have modified BSK medium by using guinea pig and fetal calf serum instead of rabbit serum. It was observed that for *in vitro* culture of *Borrelia persica*, guinea pig serum and for *Borrelia microtti*, fetal calf serum show the best growth.
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INTRODUCTION

Borrelia persica (B. persica) and *Borrelia microtti (B. microtti)* are two spirochetes responsible for relapsing fever in Iran whose vectors are *Ornithodorous tholozani (O. tholazoni)* and *Ornithodorus erraticus (O. erraticus)* (small strain) respectively.[1] These two spirochetes have only been maintained *in vivo* as *in vitro* culture methods have not been possible due to the fastidiousness of the organisms. However, the first efforts for *in vitro* cultivation of Borrelia was carried out by Knapp and Navy who cultivated *B. turicatae* in dialysis bags in peritoneum of mice. Later efforts were made to grow it in fertilized eggs.[2,3,4] A Japanese by the name of Nagachi[5] cultivated *B. recurrentis* and *B. duttoni* in ascitic fluid containing rabbit kidney cells with a layer of paraffin.

But, it was only in 1976 that the first synthetic medium was constructed for cultivation of Borrelia by Kelly.[6] It is an enriched medium and some kinds of borrelia grow in it with a generation time from 6 to 12h and 2×10/mL.[1,7,8] It has been used for *B. hermsii*, *B. parker*, *B. turicatae*, *B. hispanica* and *B. recurrentis*.[6] One of the most important constituents in Kelly's medium is N-acetyl glucosamine (NAGA); other aminopolysaccharides cannot replace this constituent. A modified Kelly medium is good for *B. burgdorferi*, responsible for lyme disease,[8] and solid medium containing agarose, on which infected tissues of mice are grown.[8,9] A new medium, Mineral Protein Medium (MPM), containing sucrose and agar has recently been used successfully for growth of L forms (without cell wall) of *B. burgdorferi*.[10]

At present, the best medium for growth of Borrelia is BSK (Barbour, Stoner and Kelly) which is complicated and expensive. It is comprised of an enrichment medium along with albumin and BSA. Albumin added alone or with rabbit serum provides long chain fatty acids as Borrelia are unable to cause elongation of stored fatty acids by beta-oxidation.[11,12,13] The Cholesterol present in cell culture medium is required for cell structure,[12] and glucose outside cells with phospholipids are used for building cells.[14,15]

As this medium is complicated and expensive and is not the best medium for growth of Borrelia, modifications were carried out as an effort for an optimum medium.

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MATERIAL AND METHODS

Bacteria

The strains of *B. persica* (Pasteur Institute of Iran) and *B. microtti* (Pasteur Institute of Iran) were maintained in *O. erraticus* and *O. tholozani*. White mice were infected by *B. microtti* by having infected *O. erraticus* feed on their skins. Guinea pigs were infected in a similar manner by allowing *O. tholozani* to feed on their skins.

Animals thus infected suffered from two to three bouts of 5 day fever interspersed with four days of well being, a typical picture of recurrent fever. Guinea pigs' blood was stained with Giemsa and when the number of borrelia per field reached 10/field (40×magnification) blood was collected and used either for a) infecting other guinea pigs by injection of 1 mL of infected blood intradermally or b) collection of bacteria by centrifugation of blood collected at 1000 rpm for 10 min. In the latter case, the suspension was centrifuged once more at 4000 rpm for 20 min and 5×10 borrelia was obtained. In mice, when the number of borrelia reached 5 per field ×40, blood was collected and injected into other mice for maintenance of culture. But, as the amount of blood for preparation of *B. microtti* from mice is very small, the mice were immunosuppressed by injecting with 0.3 mg of cyclophosphamide intra-peritoneally two hours before infection with *B. microtti*. As the number of bacteria was very high, blood was collected and serum separated in a similar manner.[6,16-20]

In vitro cell culture

This was carried out with BSK medium which contains a number of ingredients, including bovine albumin fraction V, N-acetyl glucosamine and gelatine including HEPES buffer, neopterin glucose, sodium pyruvate and an enriching medium like CMRL-1066 (Sigma) or TCM-199 (Sigma). An important component is rabbit serum, which was replaced with either fetal calf serum or guinea pig serum. The cells were cultured in tubes 18×80 cms with teflon lids, filled to 2 cm from the top of the tube.

Cultures were seeded with different dilutions of Borrelia-rich serum in BSK medium containing different sera. The cells were incubated at 35°C for 5 days. Counts were carried out at different times, and the generation period for each one calculated.

RESULTS

B. microtti was grown in BSK medium with three different animal sera. The best results were obtained with BSK medium and fetal calf serum (Fig. 1). In all three media, it was observed that the lag phase of *B. microtti* was 15-16h followed by log phase from 10-60h, a stationary phase of 65-95h which after that cell death occurred. The number of cells obtained in this medium was 10/mL with a generation time of 7.43h. (Fig. 3)

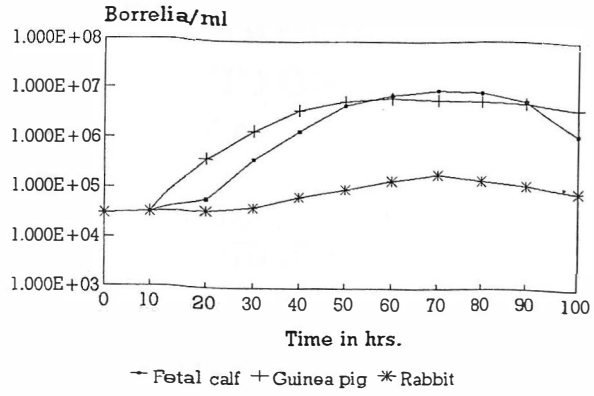

Fig. 1. Growth curve of *B. microtti* in BSK medium containing different sera.

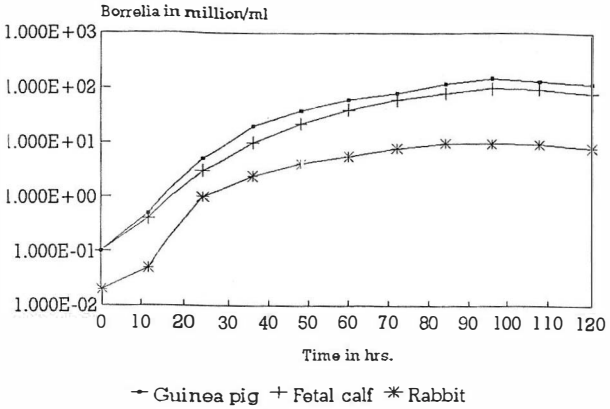

Fig. 2. Growth curve of *B. persica* in BSK medium containing different sera.

For *B. persica*, the best cell growth was seen with BSK medium in guinea pig serum (Fig. 2). The lag phase of *B. persica* ranged from 18-24h and was followed by a log phase of 24-96h and then a stationary phase of 96-112h which was followed by cell death. The number of cells obtained in this medium was 1.5 × (Fig. 4) and generation time was 10.97h.

DISCUSSION

As a modification of BSK medium, different animals' sera were used. The best results for *B. microtti* were obtained with FCS, whereas in rabbit serum no growth was observed. For *B. persica*, best growth was observed in guinea pig serum and not with the others. NAGA is the most important component in the medium which cannot be replaced by any other sugar as it is required for

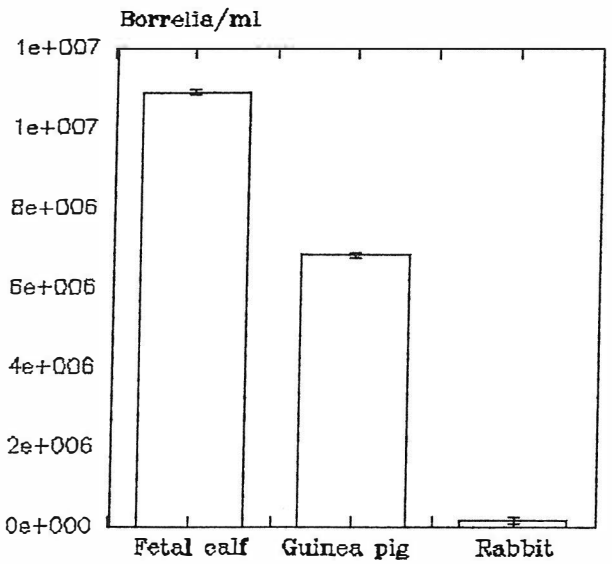

Fig. 3. Effect of different sera in BSK medium on *B. microtti* growth.

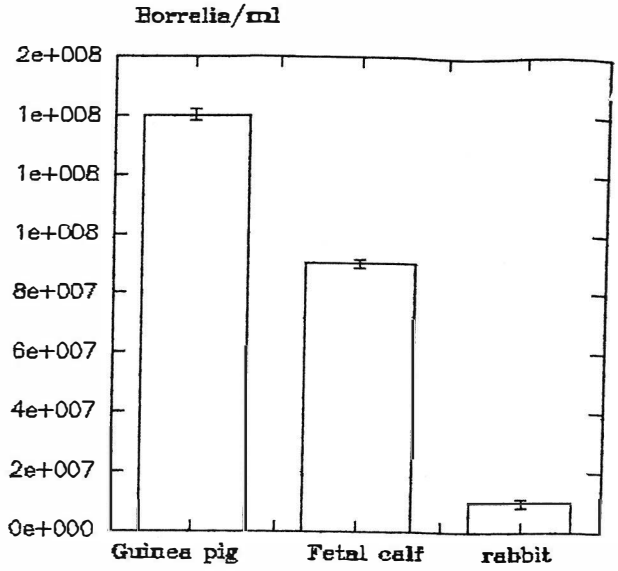

Fig. 4. Effect of different sera in BSK medium on *B. persica* growth.

peptidoglycans involved in cell wall synthesis. Interestingly, chitin of ticks too contains NAGA.[6] Albumin is another essential nutrient as it provides long chain fatty acids which take part in the structure of cells, however, unsaturated fatty acids, when added to the medium, allow the growth of *B. hermsii*, although this was not the case with these two strains of Borrelia. Cholesterol is another essential nutrient. An earlier report had been made of growth of Borrelia on solid medium,[9] but neither *B. microtti* nor *B. persica* were able to grow on solid medium. In fact, it was *B. burgdorferi*, the Borrelia responsible for lyme disease, which prefers solid medium, but continuous culturing can cause a change in protein and LPS content.[21] As mentioned earlier, Philips et al have succeeded in culturing L forms of *B. burgdorferi* in solid MPM medium containing agar at 30°C for 1-3 weeks,[10] In fact, this medium is now under consideration for culturing *Treponema pallidum,* the causative agent of syphilis.[22]

As the density of the medium increases, better growth of Borrelia is observed, hence gelatine, though not essential, causes better growth.[23] In 1971, Kelly showed that *B. hermsii* reaches 3×10^7 after 7 days which causes an 18h[3] generation time, whereas *B. microtti* has 7.43h generation time. It is important to seed the medium with at least 9×10^5-10^6, but with 5×10^5 hardly any growth was seen.

For *B. persica*, it takes 11h for its generation time to reach maximum. Growth tests with fetal calf serum showed slightly lower readings (Fig. 4) than guinea pig serum, but rabbit serum had no effect (Fig. 4) and no growth was seen. Hence, this medium can be used for growth of *B. persica* and *B. microtti*.

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