ENHANCEMENT OF PROPAGATION OF *MYCOPLASMA HYOPNEUMONIAE* BY CULTURE IN A BIPHASIC MEDIUM

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Abstract

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Mycoplasma hyopneumoniae (strain J) was propagated in a liquid and a biphasic media. The solid phase of the biphasic medium was obtained by the addition of 1.9% or 0.19% of agar to the liquid medium. Compared with the liquid medium, a more rapid propagation of mycoplasmas was observed in the biphasic medium after two weeks of incubation. After three weeks of incubation in the liquid and the biphasic medium containing 0.19% of agar in the solid phase, the numbers of mycoplasmas rose 2.7fold and 4.7fold, respectively. The biphasic medium, simulating partly the environment of the porcine respiratory tract, supported better the propagation of *M. hyopneumoniae* than the liquid medium. The biphasic medium containing 0.19% of agar in the solid phase should be preferred when a mass of *Mycoplasma* cells is to be obtained.

Porcine enzootic pneumonia, culture media, biomass

The causal agent of porcine enzootic pneumonia *Mycoplasma hyopneumoniae* ranks with *Mycoplasma* species which are difficult to propagate (Friis 1975, 1979). On solid media, this species does not form the "fried eggs" colonies typical of a majority of mycoplasmas, but rather small, dispersed bodies resembling poppy-seeds. The propagation in liquid media is very slow, taking several weeks.

Larger amounts of *Mycoplasma* cells can be obtained only by gradually increasing the volume of the culture medium (Kuksa et al. 1987).

M. hyopneumoniae lives on the surface of porcine respiratory mucosae (Meyling 1971; Kobisch et al. 1993). The mucus of the respiratory tract protects *M. hyopneumoniae* cells from drying in the environment thus supporting the spread of porcine enzootic pneumonia by droplet infection (Goodwin 1972; Friis 1973). As a typical mucosal pathogenic agent, *M. hyopneumoniae* is adapted to an environment which is more viscous than are the conventional liquid media used for its culture *in vitro*.

A biphasic culture medium, simulating partly the conditions prevailing in the porcine respiratory tract, was used to provide more favourable conditions for the culture of *M. hyopneumoniae in vitro*.

Materials and Methods

The strain J of *Mycoplasma hyopneumoniae* (type strain NCTC 10110, ATCC 25934) was obtained from the Collection of Animal Pathogenic Microorganisms, Brno (CAPM M-38).The biphasic culture medium consisted of solid and liquid phases.

Liquid phase

The medium FF (Friis, 1971) was modified as follows:

Saline A: 160 g NaCl, 8 g KCl, 2 g $MgSO_4.7H_2O$, 2 g $MgCl_2.6H_20$, and 2.8 g anhydrous $CaCl_2$ were dissolved in 1 L bidistilled water.

Saline B: 3 g $Na_2HPO_4.12H_2O$ and 1.2 g KH_2PO_4 were dissolved in 1 L bidistilled water.

The stock salines A and B were diluted by adding to 7.5 ml 125 ml and 17.5 ml bidistilled water, respectively. Then the salines were mixed and 2.6 g *Mycoplasma* Broth Base (Oxoid) and 2.5 g Brain Heart Infusion (Oxoid) were added. The mixture was completed with 225 ml bidistilled water and autoclaved at 121 °C and 110 kPa for 30 min. After cooling, 78 ml horse blood serum, 18 ml yeast extract, 5 ml 10% (w/v) glucose and 1 ml 5% (w/v) thalium acetate solutions and 200 mg ampicillin were added. pH was adjusted to 7.6.

Solid phase

The liquid medium FF was completed with 4.5 or 0.45 g Noble agar (Difco) before autoclaving, supplemented with the same components as the liquid medium FF after autoclaving and cooling to 50 °C and poured into culture vessels. The agar concentrations in the solid media were 1.9% and 0.19%, respectively.

The solid medium for counting *M. hyopneumoniae* bodies was prepared by dissolving 4 g PPLO agar (Difco) in 105 ml bidistilled water. The medium was completed with 30 ml horse blood serum, 15 ml yeast extract, 100 mg ampicillin and 0.75 ml 5% thalium acetate after autoclaving and cooling to 50 °C. The hot medium was poured into plastic 60mm Petri dishes to form a 2-mm layer.

Comparison of *M. hyopneumoniae* propagation in liquid and biphasic media

Experiment 1

Forty ml of the liquid medium or forty ml of the hot solid phase containing 1.9% agar were poured into 100-ml glass bottles. The agar was left to solidify in slant position (Fig. 1). After warming to 37 °C, the media were inoculated with 5 ml of a *M. hyopneumoniae* suspension containing 2.9×10^5 bodies per 1 ml and the cultures were incubated at 37 °C. Forty ml of fresh liquid medium were added after 3 days incubation and 0.2 ml of the suspension were collected and inoculated onto the solid medium for the counting of *M. hyopneumoniae* bodies after another 14 days.

Experiment 2

Forty ml of the liquid medium or forty ml of the hot solid phase containing 0.19% agar were poured into 100-ml glass bottles. The agar was left to solidify in slant position (Fig. 1). After warming to 37 °C, the media were inoculated with 5 ml of a *M. hyopneumoniae* suspension containing 1.2×10^5 bodies per 1 ml and the cultures were incubated at 37 °C. Forty-five ml of fresh liquid medium were added after 3 days of incubation and 0.2 ml to 0.4 ml of the suspension were collected and inoculated onto the solid medium for the counting of *M. hyopneumoniae* bodies at weekly intervals during the three-week incubation period. The experiment was repeated twice.

Counting of *M. hyopneumoniae* bodies

A known volume (0.2 ml to 0.4 ml) of a well shaken *Mycoplasma* suspension was applied onto the solid medium in a 60-mm Petri dish to cover the whole surface of the medium. The dishes were left to stand for 30 min and then incubated at 37 °C for 3 days.

M. hyopneumoniae bodies were counted at 10 randomly selected sites at the magnification 630x using an eye-piece screen.

Sterility checks

A sample of the suspension was collected at each opening of a culture vessel, inoculated onto blood agar and incubated at 37 °C. Attention was paid to *Mycoplasma* colonies suggestive of contamination at the counting of the *M. hyopneumoniae* bodies.

Results

Mycoplasma hyopneumoniae propagated in both the liquid and the biphasic media and *M. hyopneumoniae* bodies resembling dispersed poppy-seeds were observed with a light microscope after reinoculation onto the solid medium.

Experiment 1

The number of *M. hyopneumoniae* bodies increased 1.5fold (from 2.9×10^5 bodies per 1 ml of inoculum to 4.5×10^5 bodies per 1 ml, $P < 0.01$) and 4.8fold (to 1.4×10^6 bodies per 1 ml, $P < 0.01$) during the 14-day incubation in the liquid and the biphasic media, respectively. The medium containing 1.9% agar disintegrated when the culture vessels were shaken, but large fragments of solid agar remained in the liquid phase.

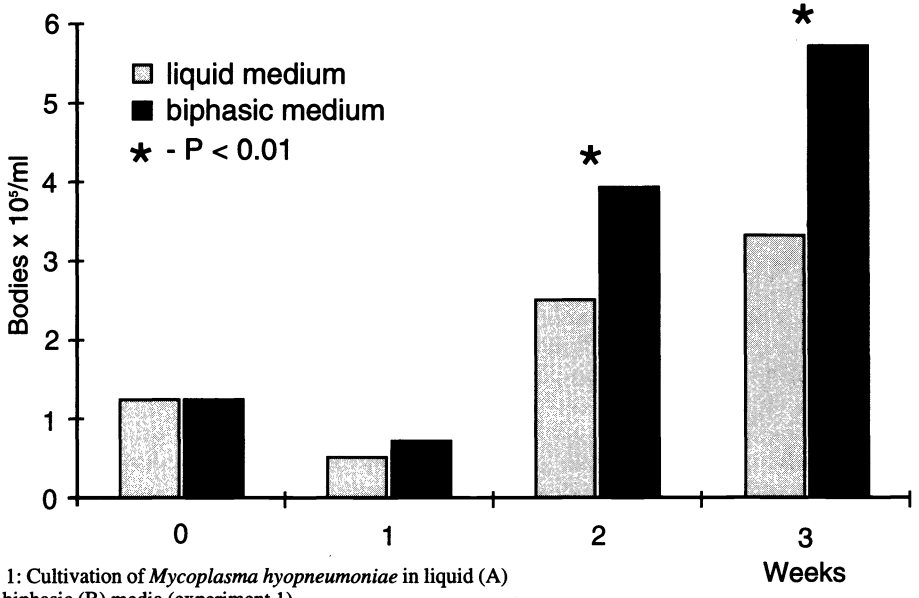

Fig. 1: Cultivation of *Mycoplasma hyopneumoniae* in liquid (A) and biphasic (B) media (experiment 1).

Experiment 2

Mean values obtained in three experiments made with various batches of the media are shown in Fig. 2. The numbers of *M. hyopneumoniae* bodies decreased both in the liquid and the biphasic media and the difference in counts of *M. hyopneumoniae* bodies between the two media was non-significant ($P > 0.05$) by the end of the first week of incubation.

During the second week, the numbers of mycoplasmas rose twofold (from 1.2×10^5 to 2.5×10^5 bodies per 1 ml, $P < 0.01$) and threefold (to 3.9×10^5 bodies per 1 ml, $P < 0.01$) in the liquid and the biphasic media, respectively.

Further 2.7fold (3.3×10^5 bodies per 1 ml, $P < 0.01$) and 4.7fold (5.7×10^5 bodies per 1 ml, $P < 0.01$) increases were recorded after three weeks of incubation in the liquid and the biphasic media, respectively. The 0.19% agar dissolved almost completely in the liquid medium. No contamination by bacteria or colony-forming *Mycoplasma* species was demonstrable.

Discussion

Bruggmann et al. (1977) demonstrated *M. hyopneumoniae* in frozen lung sections collected from swine suffering from enzootic porcine pneumonia by the immunoperoxidase reaction. *M. hyopneumoniae* cells were visible as reddish brown pleiomorphic spots on the mucosa of the respiratory tract and on the surface of scraped-off epithelial cells.

Zielinski et al. (1990) observed adhesion of *M. hyopneumoniae* to porcine and human cell cultures. The adhesion to the porcine cells was more intensive. De Bey and Ross (1994) described *M. hyopneumoniae*-induced ciliostasis and loss of ciliae in cells of organ cultures prepared from porcine tracheal rings.

For solid medium either Ionagar No. 2 or Agar-Agar (both from Oxoid Ltd., London) can be used at a concentration 0.8 %. Agarose 0.9 % without DEAE-Dextran may be used instead of agar (Friis 1975).

The culture in a biphasic medium combines the advantages of the liquid and the solid culture media and partly simulates the conditions prevailing in the porcine respiratory tract. While mycoplasmas grown in a liquid medium can adhere only to the glass surface of culture vessels, in a biphasic medium they can first propagate in the nutrient-rich agar and the continuing growth is supported by the addition of fresh liquid medium providing further nutrients and diluting metabolic products of mycoplasmas. The semisolid agar swells in the liquid medium and eventually dissolves almost completely. *Mycoplasma* bodies are thus released into the liquid phase of the medium and can be harvested by washing and the centrifugation. The remains of the undissolved agar area very suitable substrate for the propagation of mycoplasmas.

M. hyopneumoniae propagates slowly in liquid media, fermenting glucose and trehalose, but not saccharose, lactose and mannitol (Bannerman and Nicolet 1971), and can be distinguished from the non-pathogenic, slowly growing and glucose fermenting *M. flocculare* by serological tests (Bölske et al. 1987) or biological assay (Abiven et al. 1990).

Comparative morphologic studies of *M. hyopneumoniae* and *M. flocculare* were made by Hovind-Hougen and Friis (1991). Both the species carried on their surfaces fine, 30 to 100 nm long hairs, were surrounded by a simple membrane consisting of three layers and propagated by binary fission. The size of the spherical or oval bodies of *M. hyopneumoniae* varied between 0.8 and 2.5 μm, being within the considerably broader range 0.4 to 4.0 μm of *M. flocculare*. Unlike *M. hyopneumoniae*, *M. flocculare* formed filamentous cords between cells measuring 140 x 30 to 90 nm. However, special microscopic techniques are necessary for the recognition of this difference. Tajima and Yagihashi (1982), Blanchard et al. (1992), Jacques et al. (1992) in their electron microscopic studies of mycoplasmas found *M. hyopneumoniae* apparently lying free in the bronchiolar lumen of pigs, but in close contact with tips of several microvilli. Very fine fibrils, measuring 0.005 μm in diameter and 0.25 μm in length, linked mycoplasmas with microvilli or cilia of host cells and interconnected also individual *Mycoplasma* bodies. Mycoplasmas propagated in a liquid medium lacked the fibrils. The size of the bodies grown in the liquid medium and found in the porcine respiratory tract ranged from 0.4 to 1.2 μm and from 0.5 to 1.0 μm, respectively. While spherical and oval forms prevailed among mycoplasmas propagated *in vitro*, those observed *in vivo* among host cell cilia were elongated.

All the media were warmed to 37 °C prior to inoculation to avoid cold shock.

The modified medium FF has proven effective for the propagation of *M. hyopneumoniae*. Although the counts decreased in the initial phase of incubation due to dilution and death of a part of the inoculum, a marked increase could be recorded after the adaptation of mycoplasmas to the culture conditions in the 2nd and 3rd weeks of culture. The experiments were discontinued after two or three weeks of incubation, because the quality of the media decreased owing to the accumulation of toxic metabolic products of mycoplasmas and dead, disintegrating cells after prolonged incubation.

Although a part of the mycoplasma culture adhered to undissolved remains of agar gel upon the harvest, the yield from the propagation in the biphasic medium was higher than that from cultures in liquid media.

Zvýšení schopnosti dělení *Mycoplasma hyopneumoniae* kultivací ve dvoufázovém mediu

Mycoplasma hyopneumoniae (kmen J) bylo kultivováno ve dvou typech medií, tekutém a dvoufázovém. Tuhá fáze dvoufázového media byla vytvořena zahuštěním tekutého media

1,9 nebo 0,19 % agaru. Po dvou týdnech kultivace bylo zaznamenáno vyšší množství mykoplazmat v mediu dvoufázovém než v mediu tekutém.

Po třech týdnech kultivace s v tekutém mediu zvýšil počet mykoplazmat 2,7×, zatímco ve dvoufázovém mediu, jehož pevná fáze obsahovala 0,19 % agaru, 4,7×.

Dvoufázové medium, které částečně napodobuje poměry v dýchacích cestách prasete, je pro množení *M. hyopneumoniae* vhodnější než medium tekuté.

Pro snadnější získávání mykoplazmové biomasy je vhodnější používat dvoufázové medium, jehož tuhá fáze obsahuje 0,19 % agaru.

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Fig. 1: Cultivation of *Mycoplasma hyopneumoniae* in liquid (A) and biphasic (B) media (experiment 1).