A simple method for detecting mycoplasma infection of cell cultures

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SYNOPSIS This paper describes the overnight Giemsa staining method for detecting mycoplasma infection of cell cultures. This simple method requires no special equipment and has practical application in most cell culture laboratories. This technique has been used conveniently to monitor stock cell cultures for mycoplasma infection over a 12-month period avoiding any contamination.

Mycoplasma contamination of cell cultures is a widespread and variable factor which can give rise to fallacious results in many experiments. Mycoplasma infection can produce acidity and a cytopathic effect in some cells (Butler and Leach, 1964; Girardi, Hamparian, Somerson, and Hayflick, 1965), may cause chromosome changes (Paton, Jacobs, and Perkins, 1965; Fogh and Fogh, 1965), and may also affect virus growth (Singer, Ford, Barile, and Kirschstein, 1972). On occasion, mycoplasmas present in cell cultures have been mistaken for viruses (Cross, Goodman, Chatterji, Beswick, and Chapman, 1970), and as contaminating antigens in virus preparations used to immunize animals they may give rise to troublesome cross-reacting antibodies particularly in fluorescent antibody studies (Harnett, unpublished observations, 1972). We describe a simple and sensitive method for detecting mycoplasma infection of cell cultures suitable for routine testing of cultures in diagnostic and research laboratories.

Materials and Methods

CULTURE MEDIA

Powdered Eagle's basal medium (Grand Island Biological Co, NY, USA) was made up according to the manufacturer's directions. Routine growth medium for cell culture was made by supplementing this with 10% foetal calf serum, 0.07% sodium bicarbonate, 100 units of penicillin, and 100 micrograms of streptomycin. For maintenance of tube cultures in the titration experiment, foetal calf serum was reduced to 2% and sodium bicarbonate increased to 0.2%. For coverslip culture, growth medium was

modified by increasing the sodium bicarbonate concentration to 0.14% and adding 28 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) (Good, Winget, Winter, Connolly, Izawa, and Singh, 1966). With the foetal calf serum reduced to 2% this medium was also used for maintenance of tubes in the titration experiment.

COVERSLIP CULTURES

Cells to be tested for mycoplasma infection were harvested with trypsin or versene and seeded in 1 ml of growth medium into sterile, disposable, flat-bottomed plastic bottles each of which contained a sterile, circular 10 mm diameter glass coverslip. The cell dose used was that which formed an incomplete monolayer on the coverslip after 24 hours' incubation at 36°C.

FIXATION AND STAINING

These manoeuvres were carried out in the original vessels. Cells on coverslips were washed once with phosphate buffer pH 6.8 obtained from G T Gurr, after discarding the culture medium, and then fixed for five minutes in methanol. The coverslips were then stained overnight with a 1/200 dilution of Giemsa stain (Merck) in phosphate buffer. Staining was followed by rinsing the coverslips for two to five minutes in phosphate buffer, after which they were removed from the bottles, air-dried, and mounted on microscope slides in DPX (G T Gurr) and examined under an oil-immersion objective at 1000 × magnification.

EXPERIMENTS USING MEDIA BUFFERED BY HEPES

As HEPES buffer is widely used in cell culture

media and there is evidence that its use in artificial media enhances growth of mycoplasma (Manchee and Taylor-Robinson, 1969; Spendlove, Crosbie, Hayes, and Keeler, 1971), it was decided to test bicarbonate and HEPES/bicarbonate buffers in a titration of mycoplasma infectivity.

A cell-free pool of *Mycoplasma hyorhinis*, stored as aliquots in liquid nitrogen, was prepared as tenfold dilutions in Hanks' balanced salt solution and inoculated into tube cultures of FL amnion cells (Fogh and Lund, 1957). The tubes were divided into two replicate sets and maintained respectively on bicarbonate-buffered medium and HEPES/bicarbonate-buffered medium. After seven days' incubation the cells were harvested and seeded into coverslip culture bottles, incubated, fixed, and stained as above.

Results

The appearance of a normal uninfected FL amnion cell stained with Giemsa is shown in figure 1A. Mycoplasma appeared as dark blue to purple coccoid bodies, tending to be most numerous at the

periphery of infected cells (fig 1B). Detection was for this reason facilitated by an incomplete monolayer of cells.

Growth of the mycoplasma was generally profuse and high levels of infection were attained rapidly after inoculation of susceptible cell cultures. The level of infection was apparently unaffected by subculture of the infected cells 24 hours before staining.

The results of the experiments to test the effect of different buffer systems on the titration of mycoplasma are shown in the table.

Buffer System	TCID ₅₀ (Reed and Muench)
NaHCO ₃	10 ^{-5.80} ¹
HEPES/NaHCO ₃	10 ^{-6.67}

Table Fifty percent endpoint values of titration of *Mycoplasma hyorhinis* in FL amnion cells maintained in two different buffer systems

¹Two separate titrations, total of eight (8) tubes per dilution.

Fig 1A

Fig 1B

Fig 1 Giemsa-stained FL amnion cells X1500: (A) uninfected; (B) infected with *Mycoplasma hyorhinis*.

Discussion

Many methods have been described for detecting mycoplasma infection of cell cultures. They include culture on special cell-free media (Hayflick, 1965) which suffers from being slow to yield results (4-14 days) and unsatisfactory for some strains of mycoplasma; chemical methods of detection (Barile and Schimke, 1963; Levine, 1972) which require relatively large amounts of cell material, special apparatus and reagents, and rely on uniform biochemical behaviour of different mycoplasmas for success; fluorescent antibody techniques (Barile, Malizia, and Riggs, 1962) which are inconvenient to employ since they require many antisera and are unreliable in that new serotypes may be missed; and direct staining methods (Fogh and Fogh, 1964).

In our experience direct staining gives good results and constitutes an important advance on other methods because of its speed, simplicity, and sensitivity. The orcein staining method of Fogh and Fogh (1964) has the disadvantage of requiring phase contrast optics for reading results, and, although the staining procedure is rapid, it is said to work better on some cell lines than others. We selected an overnight Giemsa technique similar to that of Surman (1968) who used it for cytology and demonstration of mycoplasma in the 'pink eye' disease of sheep, because in our hands it yields equally good results as the orcein method in all cells so far tested, and has the advantage of being technically undemanding, thus having application in all cell culture laboratories. The routine 'short' Giemsa method gave only slightly inferior staining results.

We have so far tested the Giemsa staining method on cell cultures infected with *M. hyorhinis*, *M. pharyngis* type 1, and *M. hominis* with similar results; it therefore seems likely to be successful with all types encountered as cell culture contaminants. A study to determine this, and also whether the inoculation of cell cultures can be used as a sensitive method for isolation of mycoplasmas from clinical material, is currently in progress.

The HEPES/bicarbonate buffer was selected for routine use in our laboratories due to its convenience in open atmosphere culture systems and slight increase in sensitivity over the bicarbonate buffer.

It has been shown that cell culture handling procedures rapidly disseminate mycoplasma contamination (O'Connell, Wittler, and Faber, 1964; Harnett, unpublished observations, 1971). Frequent checks are therefore desirable to allow early detection of outbreaks of infection. Contamination of standard cell lines may arise from four main sources: (1) infection from the operator with serotypes

present in the respiratory tract; (2) contaminated cells or products of such cells introduced from outside laboratories (Stewart and Young, 1971); (3) contaminated animal sera used to supplement media (Barile and Kern, 1971); (4) new cell cultures derived within the laboratory from infected tissues.

Since eliminating all mycoplasma-contaminated cell lines from our laboratories, regular weekly checks using the Giemsa staining method have revealed no contamination of stock cultures over a 12-month period although *M. hyorhinis* and *M. pharyngis* type 1 have been detected in virus seeds propagated previously in mycoplasma-infected stocks and in some virus seeds and cell cultures received from external sources.

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