A SOLID MEDIUM FOR CULTURE AND IDENTIFICATION OF HUMAN T-MYCOPLASMAS

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PLATE X

T-MYCOPLASMAS produce very small colonies on solid media. This may be due to their urease activity, leading to local accumulation of ammonia (Manchee and Taylor-Robinson, 1969); these authors increased the size of colonies by adding to the medium a new hydrogen-ion buffer, N-2-hydroxy-ethylpiperazine-N-2-ethanesulphonic acid (HEPES). This observation suggested to us that other buffers might have a similar effect, and the present paper describes the development of a solid medium containing phosphates for the growth and identification of T-mycoplasmas. The latter have recently been placed in the new genus *Ureaplasma*, the specific epithet for human isolates being *urealyticum* (Shepard *et al.*, 1974).

MATERIALS AND METHODS

Organisms. All the mycoplasmas examined were human genital strains. T-mycoplasma, strain T₀, had been subcultured 11 times, and strains T₁, T₂, T₇ and T₁₀ only five times after isolation. Stock suspensions were stored, as broth cultures, at -70°C , in small amounts. *Mycoplasma hominis*, strain 164, was isolated at Dulwich Hospital and passaged three times on conventional mycoplasma medium before storage at -70°C .

Media. The basic medium used consisted of 1.2% Oxoid Agar, no. 3, in distilled water, 60 ml; horse serum (Wellcome no. 3), 20 ml; 25% extract of baker's yeast containing 18% Tryptone Soya Broth (Oxoid), 10 ml; 10% solution of urea (BDH Analar), 1 ml; 0.4% phenol red, 0.5 ml; 1% thallium acetate, 1 ml; and penicillin (10,000 units per ml), 2 ml. The pH was suitably adjusted, as described in the text, with 1.5M-solutions of KH_2PO_4 , Na_2HPO_4 , $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (all BDH Analar) or K_2HPO_4 (BDH Lab. reagent), or, in the case of non-buffered batches of media, with 1N- or 0.1N-HCl (BDH Analar) or 0.1N-NaOH (BDH Analar). The final volume of medium was made up to 100 ml with distilled water. After inoculation, the plates were incubated at $36-37^{\circ}\text{C}$ in an atmosphere of 95% nitrogen and 5% CO_2 .

The fluid medium used for the original isolation of the T-mycoplasmas had the same composition as the solid medium but without agar and phosphate buffers, and the pH was adjusted to 6.5 with N-HCl. Lincomycin, 12.5 μg per ml, was added to suppress the growth of *M. hominis* (Braun *et al.*, 1970).

Evaluation of colonial growth. Stock suspensions of T-mycoplasmas, suitably diluted to produce about 100 colonies per plate, were inoculated, in 0.05 ml volumes, across the agar surface. Counts were done after 3-days' incubation, at 25-fold magnification for colonies on phosphate-buffered medium and 60-fold magnification for colonies on medium without buffer. Colony diameters were measured with a graticule and expressed as the average of ten colonies per plate.

Removal of soluble constituents from agar plates. The properties of the precipitate formed in T-mycoplasma colonies were examined after soluble medium components had been removed by washing. The agar gel was taken out of the petri dish, placed in a nylon net

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and immersed in approximately 1 litre of distilled water kept at 4°C and stirred magnetically. The water was changed after 2 h, and the washing continued for a further 18h.

RESULTS

Preliminary experiments had shown that the addition of a mixture of 7 parts of 1.5M KH_2PO_4 and 3 parts of 1.5M Na_2HPO_4 to solid medium, producing a pH of 6.5, increased the size of T-mycoplasma colonies. In the presence of urea, a white precipitate also appeared within the enlarged colonies and there was an alkaline shift in the pH of the medium, recognised by change in colour of the phenol red. Use of filter-sterilised instead of autoclaved tryptone soya broth and the addition of yeast extract caused a further slight increase in the size of colonies.

To investigate more fully the effect of phosphate buffer on the size of T-mycoplasma colonies, 7 : 3 part mixtures of 1.5M-solutions of (1) KH_2PO_4 and Na_2HPO_4 , (2) KH_2PO_4 and K_2HPO_4 , (3) $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and Na_2HPO_4 or (4) $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and K_2HPO_4 were added to medium, in a range of concentrations from 0.25% to 7.5%, and tested with strain T_c. With all four mixtures, maximum colony size was reached at 2.5% concentration, with no further increase at 5% and 7.5%. Similar numbers of colonies were present throughout the concentration range but at 7.5% the colonies were largely obscured by precipitation throughout the medium. At 0.25% and 7.5% the colonies appeared translucent under the microscope but between 0.5% and 5.0% they were opaque because of a white precipitate within the colony. The colonies could be moved with a loop bodily across the surface of the agar.

Since significant differences were not found among the various phosphate mixtures the KH_2PO_4 - Na_2HPO_4 combination, at a concentration of 2.5% in the medium, was selected for further study of pH effect. Batches of solid media were prepared containing 2.5% of various mixtures of 1.5M KH_2PO_4 and 1.5M Na_2HPO_4 , to give a range of pH, namely, 6.5 (7 parts KH_2PO_4 to 3 parts Na_2HPO_4), 7.0 (4 : 6 parts), 7.5 (2 : 8 parts) and 8.0 (0.5 : 9.5 parts). Similar batches of media without buffer were adjusted to the same range of pH with 0.1N-HCl or 0.1N-NaOH. Plates with and without phosphate buffer were then inoculated, in parallel, with strain T₁ or T₁₀. Neither the presence or absence of buffer nor the pH itself significantly affected the number of T₁ colonies that appeared but colony size decreased with increasing pH (table I). In the case of T₁₀, both number and size of colonies decreased with rise of pH. However, with both T₁ and T₁₀, medium containing buffer at pH 6.5 and pH 7.0 produced larger colonies than did medium at the same pH but without buffer, and the colonies showed precipitate formation; neither enhancement of colony size nor precipitate was seen at pH 7.5 and pH 8.0. Accordingly, medium containing 2.5% of the 7 : 3-part mixture of KH_2PO_4 and Na_2HPO_4 , pH 6.5, was chosen for further work.

A single batch of horse serum had been used for all the previous experiments. Table II shows the effect of four different batches of horse serum, which had been stored at -20°C, on colony size. Each serum was tested in solid medium with and without phosphate buffer (media without buffer were adjusted to pH 6.5 with 0.1N-HCl). In general, with all four sera, colonies of strains T_c, T₁, T₂, T₇ and T₁₀ were larger in the buffered medium; the only exceptions were the colonies of strains T₇ and T₁₀ on media containing horse serum batch no. 3. All colonies on buffered medium contained white precipitate and the colour of the medium changed from yellow to red.

Most of the precipitate that developed with T-mycoplasma colonies grown on buffered medium was removed by prolonged washing in distilled water. It was soluble in 33% HNO_3 (BDH Analar, sp. gr. 1.42), in 10% trichloroacetic acid (BDH Lab. Reagent) and in 0.01M-ethylenediamine tetra-acetate (EDTA) (BDH Analar). The presence of inorganic phosphate was detected by a modified vanadate-molybdate test (Michelsen, 1957), a positive yellow coloration being obtained with as few as two washed colonies; washed, uninoculated gel gave a negative test.

The low-passage strain, *M. hominis* no. 164, never produced a precipitate on buffered medium. This greatly facilitated the detection of small numbers of T-mycoplasmas, in freshly cultured specimens from patients, among numerous *M. hominis* colonies (figure).

TABLE I

Effect of pH and phosphate buffer on the colonial growth of T-mycoplasmas

Strain of mycoplasma	Size (μm)* and number† (in parentheses) of colonies produced at							
	pH 6.5		pH 7.0		pH 7.5		pH 8.0	
	with buffer	without buffer	with buffer	without buffer	with buffer	without buffer	with buffer	without buffer
T ₁	168 (212)	67 (210)	115 (216)	56 (274)	58 (226)	53 (213)	53 (225)	43 (208)
T ₁₀	199 (21)	48 (20)	137 (31)	39 (13)	59 (6)	42 (6)	50 (2)	33 (4)

* Expressed as the average measurement of two sets of ten colonies on two plates. On plates with fewer than ten colonies, all colonies were measured.

† Mean plate count of duplicate cultures.

TABLE II

Effect of different batches of horse serum on the enhancing activity of phosphate-buffered medium for colony size of T-mycoplasmas

Strain of T-mycoplasma	Proportionate increase in size of colonies grown on phosphate-buffered medium, compared with unbuffered medium, containing horse-serum batch number			
	1	2	3	4
T _c	3.9	2.3	...	3.5
T ₁	1.7	1.8	1.8	1.3
T ₂	2.9	...	1.6	...
T ₇	2.5	2.2	1.0	2.4
T ₁₀	...	2.7	0.8	2.0

DISCUSSION

A differential agar medium, containing urea and MnSO_4 , was described by Shepard and Lunceford (1970) for the identification of T-mycoplasmas. Specific urease activity produced a white reaction product in the colonies (Shepard, 1973). We obtained colonies with a similar internal precipitate on the phosphate-buffered medium, and treatment of the colonies with 1% urea solution containing 0.8% MnCl_2 (Shepard, 1973) produced no change in their appearance. This suggests that the specific urease activity of T-mycoplasmas can be demonstrated in a urea-containing medium, buffered with phosphate to increase colonial size, without the further addition of a heavy metal salt.

The precipitate was found to contain phosphate, and its solubility in EDTA suggests that the phosphate is combined with an alkaline earth, which is probably calcium because this is the predominant alkaline earth in serum. Its formation can be accounted for by the production of ammonium ions, by the urease activity of the T-mycoplasmas, which then combine with calcium and phosphate ions to give insoluble calcium ammonium phosphate. At high concentrations of phosphate buffer (7.5%), ammonium ions would combine solely with the phosphate ions to form soluble ammonium phosphate. The production of magnesium ammonium phosphate by T-mycoplasmas has recently been reported in rats (Friedlander and Braude, 1974).

Thallium acetate, 0.01%, was incorporated routinely in the medium, although it has been the practice of Shepard (1969) never to use this inhibitor of bacteria. However, Edward (1947) noted that it had some inhibitory activity against classical mycoplasmas, and recommended the incorporation of 0.0125% in solid media. Concentrations as high as 0.02% have been found to have little effect on the isolation rate of T-mycoplasmas (Lee, Bailey and McCormack, 1972).

Although we have found that T-mycoplasmas freshly isolated in liquid media tend to produce smaller colonies on the phosphate-buffered solid medium than do laboratory stock cultures, all of them produce colonies with precipitate, visible usually within 48 h at 25-fold magnification, and colour change in the medium. Although the experimental work has been confined to human isolates, the same medium is probably also suitable for the isolation and identification of animal T-mycoplasmas.

SUMMARY

A solid, urea-containing medium buffered to pH 6.5 with a suitable mixture of KH_2PO_4 and Na_2HPO_4 produced enlarged T-mycoplasma colonies containing a white precipitate. This was absent from *M. hominis* colonies. The medium can be used for the isolation and identification of T-mycoplasmas.

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FIGURE—Subculture on to phosphate-buffered solid medium from a cervical swab culture in liquid medium. After 48 hours' incubation, three T-mycoplasma colonies (dark) are visible among numerous *M. hominis* colonies. $\times 40$.

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