APPLIED MICROBIOLOGY, Apr. 1970, p. 658-662
Copyright © 1970 American Society for Microbiology
Vol. 19, No. 4
Printed in U.S.A.

Large-Quantity Production of Chicken Embryo Tracheal Organ Cultures and Use in Virus and Mycoplasma Studies

J. D. CHERRY[1] AND D. TAYLOR-ROBINSON
Clinical Research Centre, Harvard Hospital, Salisbury, Wiltshire, England

Received for publication 21 January 1970

Chicken tracheal organ cultures were made from embryos which were 19 to 20 days old. Transversely cut rings of trachea were placed in screw-capped tissue-culture tubes with Eagle's-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) medium and incubated in roller drums. The method had advantages over other organ culture systems in that these cultures were prepared in numbers similar to conventional tissue cultures, ciliary activity was quickly and accurately evaluated, and contamination occurred less frequently than with organ cultures in petri dishes. Ciliary activity persisted for at least 1 month when the medium was changed at 5- to 7-day intervals and for 10 to 15 days without a change. Infectious bronchitis virus stopped ciliary movement, and this effect was used as a basis for titrating the virus and for determining the neutralizing capacity of immune mouse ascitic fluid. Twenty-four *Mycoplasma* strains were tested. Organisms of 17 strains, both avian and mammalian, multiplied in the organ cultures, and 7 strains, belonging to the species *M. gallisepticum* and *M. mycoides* var. *capri*, inhibited ciliary activity.

The use of organ cultures has enabled several "new" respiratory viruses to be isolated, and such cultures have provided a model system for the study of the effects of various viruses on ciliated epithelium of the respiratory tract (8). In addition, the organ culture technique has been used recently for the study of mycoplasmas (1, 2; S. Read, *unpublished data*). The conventional technique is to place carefully cut pieces of trachea in plastic dishes, to bathe the pieces with medium, and to observe ciliary activity with low power microscopy and reflected light. The disadvantages of this form of culture are the relative difficulty experienced in quantitating ciliary activity, the frequent occurrence of bacterial and fungal contamination, and the small number of culture dishes that can be prepared in a reasonable time period.

Recently, organ cultures of ciliated epithelium contained in glass test tubes have been described (7). This technique, in addition to its simplicity, decreases the chance of contamination. Furthermore, the ciliary activity of tracheal sections cut transversely in the form of rings can be examined microscopically in a conventional manner with direct transmitted light (1, 9). This procedure allows a more accurate assessment of ciliary activity and it is less time consuming than the reflected-light method.

This report describes the preparation of chicken embryo tracheal organ cultures in tubes in quantities similar to conventional tissue cultures. This organ culture system is simple and inexpensive and its potential in virus and *Mycoplasma* studies is outlined.

MATERIALS AND METHODS

Organ cultures. Fertile eggs from an *M. gallisepticum*-free flock were supplied by D. M. Berry (Glaxo Laboratories, Greenford, Middlesex, England). Chicken embryos (19 to 20 days old) were removed aseptically from their shells. Feathers over the neck were removed, and the skin overlying the trachea was cut longitudinally with scissors. The trachea was freed from underlying structures and cut at the levels of the carina and larynx. Excess tissue was removed and 0.5- to 1-mm transverse ring sections were cut with a scalpel; each embryo yielded about 17 tracheal rings. Each ring was placed in a standard screw-capped tissue-culture tube (16 by 125 mm) to which was added 1 ml of the medium described below. Unless otherwise mentioned, tracheal rings were allowed to adhere by capillary action to the sides of the tubes about 20 mm from the bottom. The tubes were placed in a roller drum (15 rev/hr) and incubated at 37 C. The rings remained adherent to the glass during

[1] Visiting worker from the Department of Pediatrics, St. Louis University School of Medicine, St. Louis, Mo.

rolling and were detached every 24 to 48 hr by shaking the tubes and then allowed to readhere.

Media. Medium for organ cultures was Eagle's basal medium with 200 units of penicillin per ml and 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (6, 17). The *p*H of the medium was adjusted to 7.0 with 0.2 N NaOH.

Occasionally, when mycoplasmas were not being studied, 100 μg of streptomycin and 100 μg of neomycin were added per ml of medium.

Medium for the growth and titration of mycoplasmas has been described in detail previously (11, 15), except that thallium acetate was omitted from medium used to produce organisms for organ-culture inoculation.

Virus and mycoplasmas. The Beaudette strain of infectious bronchitis virus (IBV) was obtained from A. F. Bradburne. It had a titer of 3×10^4 plaque-forming units (PFU) in primary cultures of chicken embryo kidney and a titer of $10^{4.5}$ by the chicken embryo stunting test (A. F. Bradburne, *unpublished data*). Mouse ascitic fluid with a complement-fixing (CF) antibody titer of 1:40 to IBV and control ascitic fluid were obtained from A. F. Bradburne.

Mycoplasmas studied are recorded in Table 1. The source of *M. gallisepticum* strains has been described before (14). The source of strains B3, WR1, and the PG16 strain of *M. gallinarum* has been described also (11). Other strains of *M. gallinarum* (13) were obtained from D. H. Roberts (Central Veterinary Laboratory, Weybridge, England). The strain of *M. mycoides* var. *capri* was obtained from G. R. Smith (Nuffield Institute, Regent's Park, London), and the SP2067A and SP2067E strains of *M. pneumoniae* were obtained from W. Hirsch (Central Laboratory, Kupat Holim, Haifa, Israel). Other strains of *M. pneumoniae*, a strain of *M. hominis* from the human oropharynx, and a T-strain of human genital origin were isolated in this laboratory.

Titration of mycoplasmas. Specimens were diluted in serial 10-fold steps in mycoplasma liquid medium containing phenol red and 0.1% glucose or arginine or urea, depending on which substrate was metabolized. The highest dilution which produced a color change [1 color-changing unit (CCU)] on continued incubation at 37 C was the end point of the titration.

Ciliary activity. Tubes were held in a conventional roller-tube observation platform on a microscope stage with the tracheal rings adherent to the glass surface closest to the objective. The entire inner ciliated aspect of a ring was made visible in one field with magnifications of 60 to 100 ×. Ciliary activity was graded as to its extent, that observed in the entire ring being recorded as 100%, and also as to its vigor of movement. The vigor was arbitrarily graded from 0 to 6+. Tubes were tilted from one side to the other, particularly if the rings were cut unevenly, to observe the maximum amount of ciliary activity.

RESULTS

General. After the organ culture method was developed, one worker could make 200 tube preparations of tracheal rings from 12 eggs in 4 hr. The tube cultures were randomized as they were placed in the roller drum. Although from week to week ciliary activity varied between batches of cultures, less than 3% of the rings were found to be unsatisfactory for use, these having less than 80% extent of ciliary activity and 4+ degree of vigor. Twenty-four hours after preparation, the lumen of the tracheal rings frequently contained considerable cellular debris. This was removed by shaking the tubes vigorously, which resulted in increased longevity of the cultures. Of 148 ring cultures examined 4 to 6 days after incubation, only 9 had less than 50% ciliary activity and only 6 had less than 3+ vigor.

Maintenance of cultures. The ciliary activity of rings in tubes incubated in a stationary vertical position (with the ring submerged in the fluid) was compared with that of rings incubated in a stationary horizontal position (fluid to the top of the ring) and in a conventional tissue-culture roller drum. The medium was changed every 5 to

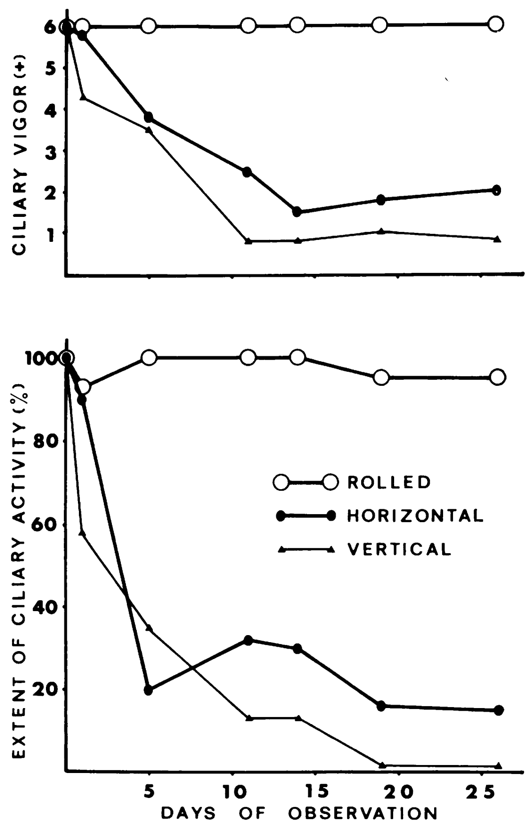

FIG. 1. *Ciliary activity (vigor and extent) of chicken tracheal organ cultures maintained at 37 C under different conditions. Four cultures were used for each experiment and medium was changed every 5 to 7 days.*

7 days. The results are presented in Fig. 1. Rolled cultures maintained nearly complete ciliary activity for 26 days, whereas the stationary cultures had lost more than half their activity by the 9th day of incubation. Chicken tracheal rings placed conventionally in plastic dishes occasionally maintained ciliary activity for long periods of time. However, debris in the middle of the rings made observation difficult, and contamination occurred more frequently.

Tracheal ring cultures were maintained without a medium change at *p*H 7 ± 0.05 and at *p*H 7.27 ± 0.07, and no difference in ciliary activity was noted (Fig. 2). Without medium change, the cultures maintained over 50% activity and 5+ vigor for 10 days.

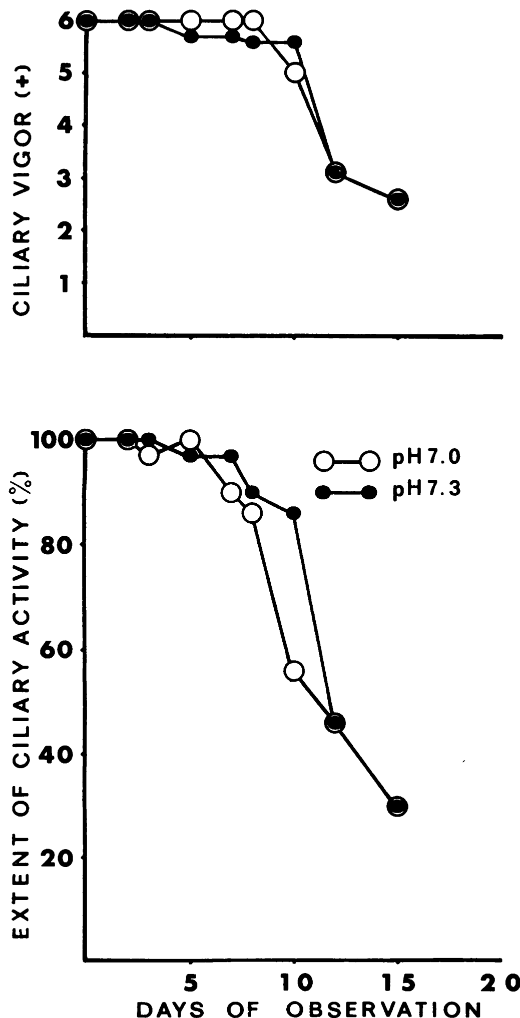

FIG. 2. *Ciliary activity (vigor and extent) of rolled chicken tracheal organ cultures kept at pH 7 ± 0.05 and 7.27 ± 0.07 without a change of medium. Seven cultures were used at each pH value.*

IBV studies. The effect of IBV on ciliary activity is shown in Fig. 3. The cilia-stopping effect (CSE) was virtually complete 72 hr after the inoculation of 100 cilia-stopping doses (CSD_{50}). The virus was easily titrated in the organ culture system by using five tube cultures for each 10-fold dilution of virus, a clear-cut end point being noted within 5 days. The titer was $10^{4.5}$ CSD_{50}, which was similar to the titer obtained by other methods of titration (*see above*). A conventional virus neutralization test was done with five tube cultures for each dilution of mouse ascitic fluid and 100 CSD_{50} of virus. The CSE was clearly neutralized by a 1:10 dilution of the mouse ascitic fluid (CF titer 1:40) and protection persisted for 48 hr.

Mycoplasma studies. The growth of 24 selected mycoplasma strains and their effect on ciliary activity are shown in Table 1. Of the strains, 17 multiplied in the organ cultures, as indicated by an increase in the number of viable organisms in the culture media. Seven of the strains stopped ciliary movement. It was noteworthy that of the

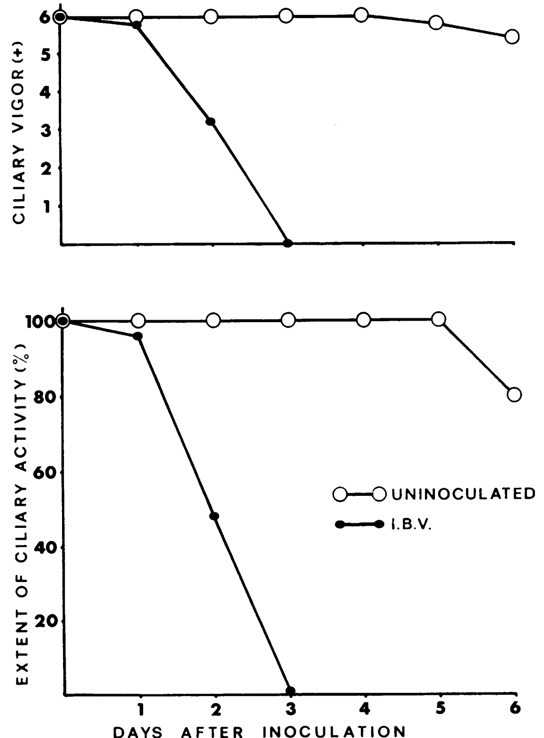

FIG. 3. *Ciliary activity (vigor and extent) of chicken tracheal organ cultures inoculated with infectious bronchitis virus. Five cultures were inoculated and five were uninoculated controls.*

TABLE 1. *Growth of selected Mycoplasma strains in chicken tracheal organ cultures and their effect on ciliary activity*

Strains	No. of viable organisms in culture fluid[a]		Cilia-stopping effect
	Initially	At 5-12 days after inoculation	
M. gallisepticum (J1)	10^4	10^6	+
M. gallisepticum (MG2)	10^4	10^6	+
M. gallisepticum (A5969)	10^4	10^6	±
M. gallisepticum (C54)	10^4	10^8	+
M. gallisepticum (T34)	10^4	10^6	+
M. gallisepticum (X95)	10^3	10^6	±
M. gallisepticum (A514)	10^4	10^5	+
M. gallinarum (CD497)	10^1	10^4	−
M. gallinarum (PG16)	10^4	10^6	−
M. gallinarum (K2, W62, W57, 271)	10^4	10^5	−
M. gallinarum (W115)	10^3	10^6	−
WR1	10^4	10^8	−
M. mycoides var. *capri*	10^4	10^7	+
B3	10^4	10^6	+
M. pneumoniae (SP2067A)	10^2	10^2	−
M. pneumoniae (SP2067E)	10^4	10^8	−
M. pneumoniae (SP1749)	10^3	10^3	−
M. pneumoniae (SP1427)	10^1	10^5	−
M. pneumoniae (SP1612)	10^3	10^2	−
M. hominis (73T)	10^5	10^5	−
T-strain (SP1467)	10^3	0	−

[a] Expressed as color-changing units per milliliter.

mycoplasmas of avian origin, *M. gallisepticum* strains stopped ciliary activity, whereas *M. gallinarum* strains and the WR1 strain did not. This failure was not due to poorer mycoplasma growth; indeed the viable titer of the WR1 strain attained 10^8 CCU and there was no loss of ciliary activity. Several mycoplasma strains of nonavian origin also grew. There was clear evidence for this in the case of the caprine species *M. mycoides* var. *capri*, the related strain B3, and for at least one strain of *M. pneumoniae*. The strain of *M. hominis* probably grew, since the titer of viable organisms 12 days after inoculation was the same as the titer at the time of inoculation, and organisms did not survive in medium without tissue for more than 3 days. However, of these mammalian species, only the caprine one caused cessation of ciliary activity.

DISCUSSION

As indicated above, for our purposes tracheal organ cultures in tubes are preferable to those in dishes. Furthermore, we found that rolling cultures was decidedly better than keeping them in either the vertical or horizontal positions. Since some chicken tracheal rings in dishes had ciliary activity for as long as rings in rolled tubes, it is surprising that our horizontal stationary cultures were not more successful. Fisk and Pathak (5) recently reported that HEPES buffer is valuable for maintaining the structure of rabbit pituitary organ cultures, and we hoped that medium kept at a constant *p*H by this buffer would maintain ciliary activity for long periods of time. We found that the *p*H of the medium did not vary in cultures kept for 15 days without being changed and that ciliary activity was often adequately maintained for this period of time. Even growth of strong acid-producing mycoplasmas caused little change in the *p*H of the medium. We also considered that the ability to maintain cultures without changing the medium might be valuable in demonstrating effects caused by mycoplasmas since their metabolic products would not be removed. For this reason, we made few attempts to maintain cultures for prolonged periods by frequently replenishing the medium. It seems, however, that by doing this the ciliary activity of tracheal rings in HEPES-containing medium could be maintained for more than 1 month.

Chicken embryo tracheal organ cultures are particularly easy to produce in quantity. Aside from the ready availability of fertile eggs, the trachea is very easy to remove from the surrounding tissues and it is long for the size of the animal, so that 17 rings can be made from each one. In contrast, we have used a similar organ culture technique with weanling mice and have found the yield of rings poorer, about four to eight per animal. Although infectious agents are occasionally transmitted from hens to their eggs, careful selection and monitoring should ensure an organ culture system as free from adventitious agents as conventional chicken embryo tissue cultures. Furthermore, it seems that this system is valuable for the study of viruses and mycoplasmas. Titration and neutralization of IBV are easier than in the embryo stunting test or in available tissue-culture systems. Johnson et al. (9) have used a system of tracheal rings in dishes for the study of IBV infection in immune and nonimmune birds and have also noted the ease of titrating the virus. Our method would appear to be potentially useful for large-scale screening of populations for IBV antibody. In addition, organ cultures of human embryo trachea have been used for the primary isolation of respiratory viruses of man (16; M. Votava and D. A. J. Tyrrell, Arch. Virusforsch., *in press*), and the use

of chicken embryo tracheal organ cultures for the isolation of wild IBV deserves consideration.

It was logical for us to test several *Mycoplasma* species of avian origin in the chicken organ cultures. *M. gallisepticum* produces a mild respiratory infection in chickens which may be clinically inapparent (4), and a more severe disease in conjunction with *Escherichia coli*, IBV, and Newcastle disease virus. *M. gallinarum* is considered to be nonpathogenic. Our finding that strains of *M. gallisepticum* inhibit ciliary activity is a further indication of the pathogenicity of this mycoplasma in its own right, in the absence of the potentiating effect of bacteria and viruses. This damaging effect and the failure to demonstrate it with strains of *M. gallinarum* are in accord with the clinical observations and indicate the value of the organ culture system as a model for the study of these organisms. Collier and Clyde (2) used a similar organ culture technique for *M. pneumoniae*, except that they employed mycoplasma medium to bathe the human embryo tracheal tissue, whereas Butler (1) and ourselves have used Eagle's medium, which in itself does not support the growth of mycoplasmas. We feel that this provides conditions which are more akin to those found in the intact animal.

The chicken tracheal organ culture system is also valuable for the study of some mycoplasmas other than those of avian origin. Just as mycoplasmas may, under experimental conditions, infect animals in which they are not normally found, for example, *M. pneumoniae* of human origin in cotton rats, hamsters, and fertile eggs (3, 10), so also may they infect organ cultures of species they do not naturally infect. Thus, at least one strain of *M. pneumoniae* grew in the chicken organ cultures. In this case, ciliary movement was unaffected. On the other hand, a strain of *M. mycoides* var. *capri*, reported by Butler (1) to have marked cytopathogenicity for human tracheal organ cultures, stopped cilia from moving. Furthermore, the B3 strain, known to be closely related to *M. mycoides* var. *capri* (12; Taylor-Robinson, *unpublished data*) did likewise. Clearly, the system will be useful in attempts to elucidate the mechanism of pathogenicity of such organisms.

ACKNOWLEDGMENTS

We thank Pamela F. Clayton and Kathleen A. Keast for excellent technical assistance.

LITERATURE CITED

1. Butler, M. 1969. Isolation and growth of mycoplasmas in human embryo trachea cultures. Nature (London) 224:605–606.
2. Collier, A. M., W. A. Clyde, Jr., and F. W. Denny. 1969. Biologic effects of *Mycoplasma pneumoniae* and other mycoplasmas from man on hamster tracheal organ culture. Proc. Soc. Exp. Biol. Med. 132:1153–1158.
3. Eaton, M. D., G. Meiklejohn, and W. Van Herick. 1944. Studies on the etiology of primary atypical peumonia. A filterable agent transmissible to cotton rats, hamsters and chick embryos. J. Exp. Med. 79:649–668.
4. Fabricant, J. 1969. Avian mycoplasmas, p. 621–641. *In* L. Hayflick (ed.), the mycoplasmatales and the L-phase of bacteria. Appleton-Century-Crofts, New York.
5. Fisk, A., and S. Pathak. 1969. HEPES-buffered medium for organ culture. Nature (London) 224:1030–1031.
6. Good, N. E., G. D. Winget, W. Winter, T. N. Connolly, S. Izawa, and R. M. M. Singh. 1966. Hydrogen ion buffers for biological research. Biochemistry 5:467–477.
7. Harnett, G. B., and W. L. Hooper. 1968. Test-tube organ cultures of ciliated epithelium for the isolation of respiratory viruses. Lancet 1:339–340.
8. Hoorn, B., and D. A. J. Tyrrell. 1969. Organ cultures in virology. Progr. Med. Virol. 11:408–450.
9. Johnson, R. B., J. A. Newman, and F. K. Wills. 1969. Titration of infectious bronchitis virus in tracheas of susceptible and immune chickens. Avian Dis. 13:632–635.
10. Liu, C. 1957. Studies on primary atypical pneumonia. I. Localization, isolation, and cultivation of a virus in chick embryos. J. Exp. Med. 106:455–467.
11. Manchee, R. J., and D. Taylor-Robinson. 1968. Haemadsorption and haemagglutination by mycoplasmas. J. Gen. Microbiol. 50:467–478.
12. Razin, S. 1968. *Mycoplasma* taxonomy studied by electrophoresis of cell proteins. J. Bacteriol. 96:687–694.
13. Roberts, D. H. 1964. Serotypes of avian mycoplasma. J. Comp. Pathol. 74:447–456.
14. Taylor-Robinson, D., and D. M. Berry. 1969. The evaluation of the metabolic-inhibition technique for the study of *Mycoplasma gallisepticum*. J. Gen. Microbiol. 55:127–137.
15. Taylor-Robinson, D., M. H. Williams, and D. A. Haig. 1968. The isolation and comparative biological and physical characteristics of T-mycoplasmas of cattle. J. Gen. Microbiol. 54:33–46.
16. Tyrrell, D. A. J., M. L. Bynoe, and B. Hoorn. 1968. The cultivation of "difficult" viruses from patients with common colds. Brit. Med. J. 1:606–610.
17. Williamson, J. D., and P. Cox. 1968. Use of a new buffer in the culture of animal cells. J. Gen. Virol. 2:309–312.