PURIFICATION OF RICKETTSIAL CULTURES CONTAMINATED BY MYCOPLASMAS

M.E. EREMEEVA, N.M. BALAYEVA, D. RAOULT*

Unité des Rickettsies, CNRS: EP J 0054, Faculté de Medecine, 27 bvd J. Moulin, 13385 Marseille Cédex 5, France

Received April 25, 1994

Summary. – An experimental biological model is proposed for purification of rickettsiae-infected cell cultures from mycoplasma contamination using intravenous mouse infection and subsequent passages on Vero cell monolayers. Mycoplasma-free rickettsial cultures were obtained in mouse brain or spleen suspension within 3 hrs or 3 days, respectively, after mouse inoculation. Ten strains of spotted fever group rickettsiae were purified from mycoplasmas by this procedure.

Key words: rickettsiae; mycoplasma contamination; cell culture

Introduction

Shell-vial technique is a widely used procedure for an isolation of rickettsiae from patient blood, infected animals or ticks (Peter et al., 1990). In comparison with classical animal models (guinea pigs, mice, hamsters), which are sensitive mainly to virulent strains of rickettsiae (Zdrodowskyi and Golinevitch, 1972), the shell-vial procedure is cheaper, easier and allows isolation of both pathogenic and nonpathogenic rickettsial strains (Peter *et al.*, 1990). However, cell cultures can be often infected with mycoplasmas, originating from cell medium (Hopps *et al.*, 1973), clinical, or tick samples. In these cases a new rickettsial strain can be contamined from the first step of its isolation. In our practice, inspite of periodic testing of the cell cultures for mycoplasma infection, we had an unfortunate accident when cultures of some newly isolated rickettsiae strains were showed to contain *Mycoplasma arginini* and *Acholeplasma laidlawii.*

Mycoplasma contamination represents a serious threat to any cell culture, and its elimination from infected cultures is nearly impossible, especially when they contain another procaryote. Mycoplasma cells have no peptidoglycan and its precursors, and are resistant to antibiotics which inhibit cell-wall synthesis (Razin and Freundt, 1984). Their small size allow them to pass through any bacteriological filter (Razin and Freundt, 1984).

Rickettsiae are obligatory intracellular bacteria which colonized endothelial cells of small and middle vessels (Weiss and Moulder, 1984; Zdrodowskyi and Golinevitch, 1972). Within 2 – 3 hrs after inoculation of mice rickettsiae accumulate in the brain (Balayeva, 1969). A developed asymptomatic infection allows the multiplication of rickettsiae in brain, kidney, lung, liver, and spleen (Balayeva, 1969; Zdrodowskyi and Golinevitch, 1972). Mycoplasma, in contrast, are characterized by an epicellular localization and are associated with epithelium covered organs (Razin and Freundt, 1984).

These differences in cell targets between these two bacteria were used for purification of rickettsial cell culture from mycoplasma contamination. Previously, one of us used the similar approach to purify from mycoplasma yolk sac cultures of *R. canada* (Balayeva and Nikolskaya, 1976). Here, we describe an experimental biological model which was useful for purification of rickettsial cultures from mycoplasma contamination.

Materials and Methods

The procedure was performed as follows. Four 18 – 20 g female Swiss Webster mice were inoculated intravenously via the tail vein with 0.25 ml of Vero cell suspension, containing about 10^4 PFU/ml of rickettsiae (Walker and Cain, 1980) and no less

*Corresponding author

than 10^6 CFU/ml of mycoplasma cells. Two mice were euthanised with ether within 3 hrs, and two on day 3 after inoculation. Brain and spleen of each animal were sampled and separately homogenized with glass beads. A 20% suspension (w/v) was prepared in Minimal Essential Medium (MEM, Biological Industries, Israel) supplemented with 4% foetal calf serum (Eurobio, Les Ulis, France) and 1% glutamine (Seromed, Berlin, Germany). 1 ml of obtained suspensions was inoculated into 25 cm^2 culture flasks containing Vero cell monolayers which were then kept for 1 hr at room temperature. Then inoculum was removed, monolayers were washed once with a 0.2 mol/l potassium phosphate buffer pH 7.2 and 5 ml of fresh medium was added. Infected cultures were incubated at 32 °C in 5% CO_2 atmosphere (Peter *et al.*, 1990). From 1 to 3 serial passages on Vero cells were performed to establish rickettsial culture. Rickettsia propagation was controlled at each passage by Gimenez staining (Gimenez, 1964). Presence of mycoplasma contamination was tested at the same time by an enzyme immunoassay with Mycoplasma Detection Kit, containing polyclonal antibodies to *M. arginini, M. hyorhinis, M. orale* and *A. laidlawii* (Boehringer Mannheim, Germany).

Table 1. Purification of spotted fever group rickettsiae from mycoplasmas

Strain	Time after mice infection		
	3 hours	3 days	
	brain	brain	spleen
Bar 29	+	–	+
Bar 31	ND	–	+
A–H	+	–	+
GS	ND	–	+
Mtu 1	ND	–	+
A	+	–	++
A–167	+	ND	+
S	++	+	++
R. japonica	ND	+	++
R. helvetica	+	+	++

Purification resulted in isolation of the rickettsial strain from one mouse (+) or two mice (++) inoculated; or the isolation was negative (–). ND – not done.

Results and Discussion

The described purification procedure was employed for spotted fever group rickettsia strains having different laboratory passages (Table 1). Infection of cell cultures with a brain suspension obtained within 3 hrs after mice inoculation was observed for all strains. Rickettsial growth was established on the second cell culture passage for strains S, *R. helvetica*, A-167 and A, and on the third passage for Bar-31 and A-HZ. On day 3 of the challenge subculture from brain of mice was achieved with *R. japonica, R. helvetica* and strain S only. All rickettsial strains could be recovered by spleen culture on day 3, mycoplasma antigen detection was negative for all subcultured strains.

However, it should be noted that not all experiments were successful starting from the first mouse inoculation. We had to repeat the above procedure from three to four times with *R. japonica* and *R. helvetica* in order to get mycoplasma-free rickettsial cultures. Cell cultures of these two strains with a relative long laboratory passage stories had higher concentration of mycoplasmas and needed several mouse passages. Occasional contamination of rickettsial cultures with Gram-negative and positive bacteria could be observed.

As for purification, no filtration system is effective for mycoplasma elimination. Mycoplasmas are resistant to antibiotics which inhibit cell-wall synthesis. It is impossible to use quinolones, which are effective for mycoplasma infection treatment (Razin and Freundt, 1984), as rickettsiae are sensitive to these antibiotics too (Weiss and Moulder, 1984).

It was reported, that a treatment of contaminated cell lines with Mycoplasma Removing Agent (MRA, ICN) was successfuly used (Spaepen *et al.*, 1992). Unfortunately, there was no description of the mechanism of anti-mycoplasma action, and it is difficult to say that this drug will have some effect on rickettsiae too or not. We previously tried to purify rickettsiae by plaque cloning, renografin gradient centrifugation, and prolonged use of thrimethoprim and sulfamethoxazole without success. Although the proposed method is not characterized by high reproducibility, at present it seems to be the only way for purification of rickettsial cultures from mycoplasma contamination.

References

Balayeva, N.M. (1969): Rise in virulence of vaccinal strain of *Rickettsia prowazekii* in the course of passages in albino mice lungs. *Vestn. Akad. med. Nauk* **10**, 51–56 (in Russian).

Balayeva, N.M., and Nikolskaya, V.N. (1976): Cultural peculiarities and experimental forms of infection of *Rickettsia canada*. In *Questions of Infectious Pathology and Immunology*. Meditsina, Moscow, pp. 192–200, (in Russian).

Gimenez, D.F. (1964): Staining rickettsiae in yolk-sac cultures. *Stain Technol.* **39**, 135–140.

Hopps, H.E., Meyer, B.C., Barile, M.F., and Del Giudice, R.A. (1973): Problems concerning "non-cultivable" mycoplasma contamination in tissue cultures. *Ann. N.Y. Acad. Sci.* **225**, 265–276.

Peter, O., Raoult, D., and Gilot, B. (1990): Isolation by sensitive centrifugation cell culture system of 52 strains of spotted fever group rickettsiae from ticks collected in France. *J. clin. Microbiol.* **28**, 1597–1599.

Razin, S., and Freundt, E.A. (1984): Order I. *Mycoplasmatales* Freundt 1955, 71^{AL}. In N.R. Krieg and J.G. Holt (Eds): *Bergey's Manual of Systematic Bacteriology*. Vol. 1, Wiliams & Wilkins, Baltimore, MD, pp. 741–787.

Spaepen, M., Angulo, A.F., Marynen, P., and Cassiman, J.-J. (1992): Detection of bacterial and *Mycoplasma* contamination in cell cultures by polymerase chain reaction. *FEMS Microbiol. Lett.* **99**, 89–94.

Walker, D.H., and Cain, B.G. (1980): The rickettsial plaque. Evidence for direct cytopathic effect of *Rickettsia rickettsii*. *Lab. Invest.* **43**, 388–396.

Weiss, E., and Moulder, J.W. (1984): Order I. *Rickettsiales* Gieszczkiewicz 1939, $25^{AL^{*}}$. In N.R. Krieg and J.G. Holt (Eds): *Bergey's Manual of Systematic Bacteriology*. Vol 1, Williams & Wilkins, Baltimore, MD, pp. 687–701.

Zdrodowskyi, P.F., and Golinevitch, H.M. (Eds) (1972): *Rickettsiae and Rickettsioses*, Meditsina, Moscow, (in Russian).