COMPARISON OF DIFFERENT MEDIA FOR PRESERVATION AND TRANSPORT OF VIABLE RICKETTSIAE

Hagen Frickmann^{1,2,*} and Gerhard Dobler³

¹Department of Tropical Medicine at the Bernhard Nocht Institute, German Armed Forces Hospital of Hamburg, Germany

²Institute for Microbiology, Virology and Hygiene, University Hospital Rostock, Germany

³Institute for Microbiology of the German Armed Forces, Munich, Germany

Received: May 24, 2013; Accepted: May 29, 2013

Rickettsiae tend to have a rapid decrease of viability outside living cells. Therefore, the transport of samples containing viable rickettsiae for culturing in cell culture for diagnostic purposes is challenging.

The viability of rickettsiae in different transport media (commercially available transport medium COPAN “UTM-RT transport medium for viruses, chlamydia, mycoplasma, and ureaplasma,” minimal essential medium (MEM) with and without 10% foetal calf serum) at various time points at 4 °C and at ambient temperature (22 °C) was compared. *Rickettsia honei* was used as model organism.

After 2 weeks of storage at room temperature, no viable rickettsiae were detectable any more while storage at 4 °C kept rickettsiae viable for up to 4 weeks. The commercially available COPAN medium showed similarly good or slightly better stabilizing effects on rickettsiae compared with MEM + 10% foetal calf serum, pure MEM demonstrated the poorest results.

It is important to transport and store media with potentially rickettsiae-containing samples at 4 °C to prevent inactivation. MEM + 10% foetal calf serum can be used if no commercial medium is available with similarly good results.

Keywords: transport medium, rickettsia, preservation, cell culture, diagnostic

Introduction

Rickettsiae are gram-negative, pleomorphic, intracellular, rod-like organisms that are sensitive to environmental influences. Transport of clinical samples containing rickettsiae is difficult due to rapid decrease of viability. For this reason, isolation of rickettsiae from clinical samples in cell culture is difficult and rarely successful [1].

Materials and methods

Tick-transmitted *Rickettsia (R.) honei* (ATC VR-1472, passage numbers 30–40), the causative agent of Flinders-Island-spotted fever in Australia [2], was chosen for the experiments as model organism [3, 4]. *R. honei* is especially suitable for infection trials because it can be grown to high titres under bio-safety level (BSL) 2 conditions in Vero E6 cell mono-layers where it induces a cytopathic effect [2–4]. The epithelial Vero E6 clone (Friedrich Loeffler Institute, Riems Island, Germany) was used for the experiments. The cytopathic effect in the cellular mono-layers usually appeared between the 3rd and the 5th day of incubation.

The rickettsial growth was additionally confirmed using a semi-quantitative real-time light-cycler PCR targeting the *gltA* gene [5].

The commercially available transport medium COPAN “UTM-RT transport medium for viruses, chlamydia, mycoplasma, and ureaplasma” (Copan Diagnostics Inc., Murrieta, CA) (containing about 3 ml of the medium) was analyzed for its preserving effects on rickettsiae. According to the manufacturer, the medium contains a not-further-specified combination of antibiotics to inhibit interfering bacterial or fungal growth. The results were compared with minimal essential medium (MEM; GIBCO, Invitrogen Ltd., Karlsruhe, Germany) with and without 10% foetal calf serum (FCS, GIBCO, Invitrogen, Karlsruhe, Germany). Experiments were performed at two different temperature conditions, i.e., at room temperature (22 °C) and at 4 °C.

Cell culture supernatants containing *R. honei* were mixed with the transport medium in a ratio of 1 : 3 ensuring a rickettsial density of at least 10³ tissue culture infecting doses (TCID). The initial TCIDs and the viability of rickettsiae after storage for various time spans at 4 °C and 22 °C were determined in Vero E6 cell mono-layers

*Corresponding author: Hagen Frickmann; Department of Tropical Medicine at the Bernhard Nocht Institute, German Armed Forces Hospital of Hamburg, Bernhard Nocht street 74, D-20359 Germany; Phone: 0049-40-6947-28743; Fax: 0049-40-6947-28709; E-mail: Frickmann@bni-hamburg.de

after diluting rickettsiae-containing suspensions in decadic logarithmic steps. Rickettsial growth after inoculation in Vero E6 cell mono-layers was confirmed by detection of characteristic cytopathic effects.

Twenty-four well-plates were inoculated with the stored rickettsiae-containing solutions. A 10-fold dilution series was created including the four dilution steps $1:10^0$ to $1:10^3$. One milliliter was inoculated per well at 37 °C for 1 h to infect Vero E6 cells. Afterward, the inoculum was removed and replaced by MEM enriched with non-essential amino acids (NEA) and 5% foetal calf serum (FCS) (GIBCO, Invitrogen Ltd., Karlsruhe, Germany). The inoculation procedures were performed in duplicate for MEM with and without FCS at 4 °C and 22 °C as well as for COPAN medium at 22 °C and in quadruplicate for COPAN medium at 4 °C. The cytopathic effects were determined by microscopic examination after 7 days of growth after inoculation.

The time points chosen for storage ranged from 5 min up to 28 days. The mean values of the numbers of 10-fold dilution steps expressing cytopathic effects were determined.

Results

Rickettsial viability decreased in the course of time in all three tested transport media

Storage at room temperature led to a complete loss of rickettsial viability within 2 weeks under all analyzed conditions. After 15 days at 22 °C, no viable rickettsiae were detectable any more in the COPAN medium. In MEM enriched with 10% FCS, infections were observed due to samples that were stored for up to 12 days, in pure MEM due to samples after up to 5 days of storage. Results for growth in duplicate were even worse; the duration of infectivity depended on the initial rickettsial dose (Table 1). However, the loss of infectivity for the different dilutions was no linear function of storage time. Already after a few days, infectivity decreased rapidly due to storage at room temperature (Fig. 1).

However, by storage at 4 °C, rickettsial viability decreased slowly and viable rickettsiae were detectable for up to 4 weeks. The commercial COPAN medium showed the best stabilizing effect on rickettsiae. Viable rickettsiae were even detectable at the 28th day at the end of the study in COPAN medium. However, MEM enriched with 10% foetal calf serum showed a good stabilizing effect as well with viable rickettsiae at the 27th day of storage. Pure MEM at 4 °C demonstrated the poorest, but still acceptable, results concerning the preservation of the viability with infectious rickettsiae being still detectable after 3 weeks. Similar to the storage at room temperature, results for growth in duplicate were a little bit worse, and the duration of infectivity depended on the initial rickettsial dose (Table 1). However, different from storage at room temperature, the observed decrease of rickettsial infectivity was very slow for both the COPAN medium and MEM enriched with 10% FCS at 4 °C. Only pure MEM led to a rapid decrease of rickettsial infectivity after the first week of storage even at 4 °C (Fig. 2). All tested media provided better results at 4 °C than any analyzed medium at room temperature (Table 1).

The direct comparison of different temperature conditions showed a generalized stabilizing effect of cooling to 4 °C. By storage at 4 °C, the storage duration could be doubled in COPAN medium, even a four-fold increase was observed for pure MEM. If the preserving effect of the medium itself at room temperature was poor, the additional effect of temperature was particularly pronounced (Table 1). All inactivation curves were much steeper at room temperature than at 4 °C (Figs. 1 and 2).

Discussion

The stability of intra-cellular bacteria during the transport process is species-dependent. *Orientia tsutsugamushi* can be isolated in vitro from blood of scrub typhus patients even when the samples are transported at tropical temperatures for 2–5 weeks [6]. On the other hand, chlamydiae tend to destabilize outside living cells and are very sensitive to

Table 1. Rickettsial growth in quadruplicate (COPAN medium at 4 °C) or duplicate (all other storage conditions) of the dilution steps $1:10^0$ to $1:10^3$ of the rickettsiae-containing suspension after storage in various media at 4 °C and 22 °C

	Any rickettsial growth	Growth in dilution step $1:10^0$	Growth in dilution step $1:10^1$	Growth in dilution step $1:10^2$	Growth in dilution step $1:10^3$
COPAN 4 °C	28 days	20 days	19 days	12 days	1 day
COPAN 22 °C	14 days	10 days	7 days	2 days	1 day
MEM + FCS 4 °C	27 days	25 days	21 days	9 days	5 days
MEM + FCS 22 °C	12 days	12 days	9 days	6 days	–
MEM 4 °C	21 days	21 days	9 days	9 days	–
MEM 22 °C	5 days	5 days	8 hours	8 hours	1 hour

Any rickettsial growth = rickettsial growth in at least one inoculated well, MEM + FCS = MEM enriched with 10% FCS, MEM = MEM without FCS

Fig. 1. Preservation of rickettsial viability at 22 °C in COPAN medium, MEM and MEM enriched with 10% FCS; p. c. = positive control, min = minutes, h = hour, d = days, dilution step 1 = non-diluted suspension, dilution step 2 = 1:10¹ dilution, dilution step 3 = 1:10² dilution, dilution step 4 = 1:10³ dilution of the rickettsiae-containing suspension

Fig. 2. Preservation of rickettsial viability at 4 °C in COPAN medium, MEM and MEM enriched with 10% FCS; p. c. = positive control, min = minutes, h = hour, d = days, dilution step 1 = non-diluted suspension, dilution step 2 = 1:10¹ dilution, dilution step 3 = 1:10² dilution, dilution step 4 = 1:10³ dilution of the rickettsiae-containing suspension

environmental effects. The viability of *Chlamydia pneumoniae* decreases rapidly outside of host cells [7]. Only about 1% of chlamydiae remain viable if they are stored for 24 h at 22 °C, while this percentage can be increased to 70% by a storage temperature of 4 °C. About 77% of

chlamydiae remain viable if they are frozen at −75 °C 4 h after being collected and stored at 4 °C prior to the freezing procedure [8]. Both refrigeration during transport and presence of foetal calf serum in transport media are factors that support the viability of chlamydiae. Wild-type strains

demonstrate good stability about 4 h at room temperature and 24 h in Eagle's minimal essential medium or sucrose-phosphate-glutamine medium while further extensions of the storage time require freezing at -75°C to keep the microbes infectious [7]. In this study, we analyzed the transport stability of rickettsiae.

We demonstrated that temperature is a more important factor for the viability of rickettsiae than the ingredients and composition of the medium. Storage at a temperature of 4°C can prolong the infectivity of rickettsiae in a suspension compared with room temperature by more than factor 2.

We demonstrated that the transport medium COPAN "UTM-RT transport medium for viruses, chlamydia, mycoplasma, and ureaplasma" provides good results regarding storage and transport of rickettsiae. The sample-containing medium should be transported at 4°C to achieve optimum results concerning the preservation of rickettsial viability. The antibiotics in the medium obviously do not harm the viability of the stored rickettsiae.

MEM enriched with 10% foetal calf serum can be used if no COPAN medium is available with similarly good results. The COPAN medium is more suitable for the routine sample transport because it does not require special preparation steps. MEM with 10% foetal calf serum must be mixed under sterile conditions; so it may be the medium of choice for specialists working in laboratories. If no other alternative is available, a transport in pure MEM can be considered as well as long as it is performed in a fridge or a comparable cooling device.

References

1. La Scola B, Raoult D: Laboratory diagnosis of rickettsioses: current approaches to diagnosis of old and new rickettsial diseases. *J Clin Microbiol* 35, 2715–2727 (1997)
2. Parola P, Paddock CD, Raoult D: Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. *Clin Microbiol Rev* 18, 719–756 (2005)
3. Stenos J, Ross B, Feng HM, Crocquet-Valdes P, Walker D: Protein characterization of Australian spotted fever group rickettsiae and monoclonal antibody typing of *Rickettsia honei*. *J Clin Microbiol* 35, 261–263 (1997)
4. Stenos J, Roux V, Walker D, Raoult D: *Rickettsia honei* sp. nov., the aetiological agent of Flinders Island spotted fever in Australia. *Int J Syst Bacteriol* 48 Pt 4, 1399–1404 (1998)
5. Wölfel R, Terzioglu R, Kiessling J, Wilhelm S, Essbauer S, Pfeffer M, Dobler G: *Rickettsia* spp. in *Ixodes ricinus* ticks in Bavaria, Germany. *Ann N Y Acad Sci* 1078, 509–511 (2006)
6. Luksameetanasan R, Blacksell SD, Kalambaheti T, Wuthiekanun V, Chierakul W, Chueasuwanchai S, Apiwattanaporn A, Stenos J, Graves S, Peacock SJ, Day NP: Patient and sample-related factors that effect the success of in vitro isolation of *Orientia tsutsugamushi*. *Southeast Asian J Trop Med Pub Health* 38, 91–96 (2007)
7. Maass M, Dalhoff K: Transport and storage conditions for cultural recovery of *Chlamydia pneumoniae*. *J Clin Microbiol* 33, 1793–1796 (1995)
8. Kuo CC, Grayston JT: Factors affecting viability and growth in HeLa 229 cells of *Chlamydia* sp. strain TWAR. *J Clin Microbiol* 26, 812–815 (1988)