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Short communication

Mycoplasma removal: Simple curative methods for viral supernatants

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A B S T R A C T

As a partner of the European Virus Archive (EVA) FP7 infrastructure, our research group is maintaining and developing a large virus collection. To meet the standards of the quality management system adopted by all European Virus Archive partners, the detection and eradication of mycoplasma in cell culture supernatants (stored at −80 °C or freeze-dried) has to be improved. Although the methods for mycoplasma elimination from infected cell lines were largely described, the decontamination procedures of precious cell culture supernatants was poorly documented. In this study, a large panel of mycoplasma-contaminated virus stocks (enveloped and non enveloped, RNA and DNA viruses) was tested successfully for mycoplasma removal using two simple optimized methods. These easy-to-perform protocols, using respectively Plasmocin™ (InvivoGen, Cayla, France) and chloroform, were shown to remove mycoplasma completely from cell supernatant without incidence in viral infectivity.

1. Introduction

Mycoplasmas, which belong to the class Mollicutes (*Mycoplasma* and *Acholeplasma*), are a frequent contaminant of cell cultures and therefore of cell-culture derived virus stocks (Mariotti et al., 2012; Rawadi and Dussurget, 1995; Volokhov et al., 2011). The sources of cell culture contamination by the genus *Mycoplasma* are any materials derived from plants or animals or, in theory, almost anything living, including the manipulator (Rottem and Barile, 1993). The difficulties for prevention and detection of mycoplasma contamination are coupled to the fact that contamination can be due to the virus isolate itself and it may remain undiscovered without the establishment of a systematic and efficient detection. Of the 100 mycoplasma species recognized currently, at least 20 have been found as cell culture and virus stock contaminants. Five species, *M. arginini*, *M. orale*, *M. hyorhinis*, *M. fermentans*, *Acholeplasma laidlawii*, are responsible for about 95% of contamination episodes (Hu et al., 1995; Uphoff and Drexler, 2005a). *Mycoplasmas*, generally, grow in culture supernatant and cannot be conveniently visualized under inverted microscope. Moreover, these contaminations are most often not associated with a culture fluid turbidity and are resistant to the commonly used antibiotics. As mycoplasma infection induce cell physiology perturbation, the use of contaminated cells and culture supernatants may compromise the results and conclusions from any experiments. In addition, mycoplasma was recognized as a significant and costly problem for the biopharmaceutical industry (Armstrong et al., 2010).

Over the last years, several methods have been employed to eradicate mycoplasma from infected cells; as a result antibiotic treatment seemed to be the simplest, the cheapest and the most effective (Singh et al., 2008; Uphoff and Drexler, 2005b).

Contaminated cell lines can generally be replaced easily with mycoplasma-free lines. However, for virus collections in which there are many unique strains, few techniques for mycoplasma elimination have been described and all the methods were difficult to perform, cytotoxic or induced a decrease of virus infectivity (Ikoev et al., 1973; La Linn et al., 1995; Nissen et al., 1997; Simpson et al., 1983; Wolford and Hetrick, 1972). The challenge is to eliminate mycoplasma contamination without losing the infectiousness of the viral material by using a simple and cost-effective method versatile enough to be efficient for a large variety of viruses (DNA/RNA genome, enveloped/non enveloped). Indeed, in recent years, several kits have been commercialized for mycoplasma removal from cell lines; most of them were also proposed for treating viral supernatants. However, few studies have tested simple and efficient methods to eliminate mycoplasma from a large panel of contaminated virus samples (Ikoev et al., 1973; La Linn et al., 1995; Nissen et al., 1997; Simpson et al., 1983; Wolford and Hetrick, 1972).

In this study, the efficiency of two optimized procedures, using respectively Plasmocin™ (InvivoGen, Cayla, France) and chloroform, was demonstrated for the elimination of mycoplasma from a panel of 20 representative virus strains (enveloped and non enveloped, RNA and DNA viruses) passaged onto four different cell lines (Vero, SW13, MDCK, BGM) (Table 1).

Virus strains were produced using quality controls and safety standards recommended by the European Virus Archive internal

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Table 1
List of contaminated virus strains and cell lines used for their propagation.

Genome	Family	Genus	Virus	Strain	Cell line	Molecular detection
Enveloped viruses						
ss RNA+	*Flaviviridae*	*Flavivirus*	*Alkhurma hemorrhagic fever virus*	8S1	Vero	Moureau et al. (2007)
	Flaviviridae	*Flavivirus*	*Alkhurma hemorrhagic fever virus*	3B1	Vero	
	Flaviviridae	*Flavivirus*	*Louping ill virus*	369T2	SW13	
	Togaviridae	*Alphavirus*	*Mayaro virus*	TC625	Vero	Sanchez-Seco et al. (2001)
	Togaviridae	*Alphavirus*	*Chikungunya virus*	MARS2011 864	Vero	Pastorino et al. (2005)
ss RNA−	*Bunyaviridae*	*Phlebovirus*	*Karimabad virus*	I-58	Vero	Sanchez-seco et al. (2003)
	Bunyaviridae	*Phlebovirus*	*Toscana virus*	AR France	Vero	
	Orthomyxoviridae	*Influenzavirus*	*Influenza A virus (H3N2 saisonnier 2011), MRS 43064111, 2011*	A/Marseille/43064111/2011 (H3N2)	MDCK	Ninove et al. (2010)
	Orthomyxoviridae	*Influenzavirus*	*Influenza A virus, H1N1 pandémique, MRS 50046111, 2011*	A/Marseille/50046111/2011 (H1N1 pandémique)	MDCK	
	Arenaviridae	*Arenavirus old world*	*Mopeia virus*	Mozambique	Vero	Lecompte et al. (2006)
	Arenaviridae	*Arenavirus new world*	*Pinchinde virus*	CoAN 3739	Vero	
DNA	*Poxviridae*	*Orthopoxvirus*	*Cowpox virus*	Compiègne 2009 isolat KP ou LP	Vero	Unpublished
Non enveloped viruses						
ss RNA+	*Picornaviridae*	*Enterovirus*	*Human Coxsackievirus B1*	MRS 053604/2000	BGM	
	Picornaviridae	*Enterovirus*	*Human Coxsackievirus B2*	MRS 2932/1989	BGM	Watkins-Riedel et al. (2002)
	Picornaviridae	*Enterovirus*	*Human Coxsackievirus B4*	MRS 2255041/2001	BGM	
	Picornaviridae	*Enterovirus*	*Human Coxsackievirus B5*	MRS 546504/2000	BGM	
	Picornaviridae	*Enterovirus*	*Human Echovirus 1*	MRS 133/1985	BGM	
	Picornaviridae	*Enterovirus*	*Human Echovirus 30*	MRS 422504/2000	BGM	
ds RNA	*Reoviridae*	*Orthoreovirus*	*Mammalian orthoreovirus 1*	Lang	Vero	Unpublished
	Reoviridae	*Orthoreovirus*	*Mammalian orthoreovirus 4*	Ndelle v. Cameroon	Vero	

experts (Gould et al., 2012). The presence of mycoplasma within the virus batches (frozen or freeze dried vials containing infection culture supernatants) was detected using a modified broad-range mycoplasma quantitative PCR (qPCR) detection system (Stormer et al., 2009). Briefly, after sample nucleic acid extraction (EZ1 virus mini kit) using a EZ1-XL Biorobot (both from Qiagen), qPCR detection of mycoplasma was carried out using 10 μl of extracted sample, 0.5 μM of each primers Tuf414 and Tuf541, 12.5 μl of 2× QuantiTect SYBR Green RT-PCR Master Mix according to the manufacturer's instructions (Qiagen, Hilden, Germany). Cycling program was 95 °C for 15 min, then 45 cycles (95 °C for 15 s, 54 °C for 30 s, 72 °C for 30 s) onto CFX 96 Real-time system (BioRad, Hercules, USA). qPCR products were then analyzed by melting curves for unspecific products or primer dimers formation. Quantification was based on standard curves generated from targeted cloned gene fragments of *M. orale* (127 bp in pCR®II; Invitrogen, Paisley, United Kingdom) with a lower detection limit of approximately 500 DNA copies per reaction. The cell lines and the fetal bovine serum were tested for the presence of mycoplasma through the same PCR assay and revealed negative. The qPCR products were sequenced and compared to existing sequences in the GenBank database using BLAST program which indicated that supernatants were infected exclusively with *M. orale and M. arginini.*

2. Procedure for the elimination of mycoplasma for enveloped and non-enveloped virus stocks by Plasmocin

As described in Fig. 1A, mycoplasma-contaminated cell culture supernatants were clarified first by low speed centrifugation and serial dilutions were used to infect permissive cell lines (Table 1). The highest dilution allowing viral growth (determined by specific molecular detection and/or cytopathic effects) was recovered and filtrated through a 0.22 μm filter. The filtrate was then used for cell infection with media containing 25 μg/ml Plasmocin™. After 3–7 days (depending of the virus strain), viral production was tested for effective virus growth and presence of mycoplasma using qPCR described previously. In case of low-titer contaminated virus stocks where clarified supernatant dilutions induced no viral growth, the same procedure was performed using non-diluted samples obtained after virus re-amplification on permissive cell lines.

3. Procedure for the elimination of mycoplasma for non-enveloped virus stocks by chloroform

As described in Fig. 1B, mycoplasma-contaminated cell culture supernatants were clarified first by low speed centrifugation then supplemented directly with one volume of chloroform. After