

Application of Mynox[®] for Mycoplasma Inactivation in Virus Stocks

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A novel procedure for the purification of virus stocks from mycoplasma contaminations was established using the biologically active antimycoplasma agent Mynox[®]. The enveloped viruses SHV-1 and BVDV and the nonenveloped viruses EMCV and PPV were investigated. Two consecutive treatments led to the complete and permanent elimination of mycoplasmas from all of these stocks as monitored by PCR. A negligible titer reduction was found for the nonenveloped viruses. For the enveloped viruses a pronounced infectivity decrease was measured but enough infectious virus particles could be salvaged for propagation. Due to its reliability and simplicity Mynox[®] was found to be a valuable improvement in removing mycoplasmas from virus stocks.

Mycoplasmas are prokaryotes living parasitously on eukaryotic cells and induce severe diseases in man and in animals. It has been shown that a variety of cell lines are contaminated with different mycoplasma strains. They are a major problem of tissue culture cells affecting metabolism, morphology and virus replication, for example. Their elimination from cells and virus stocks is important under the aspect of good laboratory practice (GLP) in basic research and biotechnological production. For the inactivation of mycoplasmas in cell cultures different more or less efficient techniques are available, but the most effective procedure published is the treatment with antibiotics (1, 7, 8). In virological research one of the common sources for contaminations are virus stocks harvested from mycoplasma contaminated cell cultures, collected in the field, or passaged through animals. Therefore, in virology not only the cell cultures but also the virus stocks must be freed from mycoplasmas (2, 4). Only a few techniques are published for the treatment of virus stocks. The only practical procedure in current usage is differential filtration, however impractical when large viruses with a particle size close to that of mycoplasmas (100-300 nm) are contaminated (3). Linn and coworkers published a procedure for the complete removal of mycoplasmas from viral preparations using solvent extraction (4). Recently, the mycoplasma elimination reagent Mynox[®] became commercially available providing an efficient procedure for the elimination of mycoplasmas from adherent and suspension cell lines. This publication describes the application of Mynox[®] for efficient mycoplasma elimination in virus suspensions. The advantages of the Mynox[®] method are compared with the procedure described by Linn *et al.* (4).

MATERIALS AND METHODS

Source of Mynox[®]. Mynox[®] was provided by Minerva Biolabs GmbH (Berlin, Germany) as a directly applicable solution in phosphate-buffered saline (PBS).

Viruses, cells, and culture conditions. Following cell virus systems were used: nonenveloped viruses: murine encephalomyocarditis virus (EMCV) / Hep2 cells (ATCC CCL 231), porcine parvovirus (PPV strain NADL) / ST cells (ATCC CRL 1746); enveloped viruses: bovine diarrhea virus (BVDV) / KL cells (embryonal calf lung), swine herpesvirus type 1 (SHV-1, pseudorabies) / ML cells (mink lung). Virus stocks were prepared by infecting subconfluent cell monolayers with a multiplicity of infection (m.o.i.) of 10³. The culture supernatants were harvested when a pronounced cytopathic effect (CPE) approximately 2 to 6 days post infection (d.p.i.) was visible. Aliquots of the cell-free supernatants were stored at -70°C. Cell lines were propagated as monolayers in Dulbecco's modified Eagle's medium (DMEM; ICN, USA) supplemented with 8% heat inactivated fetal calf serum (FCS; Gibco, USA) in 25 cm² tissue culture flasks (Greiner, Germany).

Virus titration. The virus titers were determined by a standard microtitration assay. Approx. 1 to 2 x 10⁴ cells in 100 µl medium were plated into each well of a 96-well microtiter plate (Nunc). The virus solution was serially diluted 1:5 or 1:10 in culture medium and 100 µl of each dilution were added to each of 8 wells of a 96 well microtiter plate. The microtiter plates were incubated at 37°C and evaluated microscopically for cytopathic effects (CPE). When a pronounced CPE was visible the 50 % tissue culture infectivity doses (TCID₅₀) were calculated according to Reed and Muench (5).

Mycoplasma detection. Mycoplasma contamination was detected by the highly sensitive PCR technique with the mycoplasma group-specific VenorGeM[®] Mycoplasma PCR Detection Kit (Minerva Biolabs).

Mycoplasma inactivation procedure.

i) Ether extraction: According to the publication of Linn *et al.* (4) 250 μ l of ether was added to 750 μ l of virus suspension in an Eppendorf tube at 4°C. The mixture was vigorously vortexed for 1 min and centrifugated (10,000xg; 5 min). 100 μ l of the water phase was quickly diluted into 2 ml of fresh medium and used for the infection of mycoplasma-free subconfluent cell cultures.

ii) Mynox[®] treatment: For nonenveloped viruses, 125 μ l of the virus stocks, containing up to 8% FCS, were diluted in 1 ml of cell culture medium without FCS supplemented with 100 μ l Mynox[®] in a sterile 1.5 ml reaction tube with a safety lock top. For enveloped viruses, 0.5 ml of the virus stocks, containing up to 8% FCS, were diluted in 4.4 ml of cell culture medium without FCS supplemented with 100 μ l Mynox[®] in a sterile 15 ml screw cap reaction tube. The elimination mixtures were incubated at room temperature by gentle shaking for 2 hours.

These reactions were stopped by diluting Mynox[®] 1:10 in culture medium. This step could beneficially be done by using the elimination mixture to infect a subconfluent, host cell culture for simultaneous propagation of the mycoplasma free virus culture. The final volume was 10x that of the elimination mixture. The host cell line was tested for mycoplasma contamination prior to infection. The procedure was repeated with viruses harvested from these cultures to ensure that all mycoplasmas had been removed.

RESULTS AND DISCUSSION

Mynox[®] is active against mycoplasmas due to a physico-chemical interaction of the reagent with the lipid membrane bilayer causing permeability changes and leading finally to the burst of the mycoplasma membrane. The Mynox[®] procedure for the removal of mycoplasmas from virus stocks takes advantage of this feature.

The nonenveloped viruses EMCV and PPV and the enveloped viruses BVDV and SHV-1 collected from different sources were PCR positive for mycoplasmas. In order to determine the efficiency of the Mynox[®] exposure, the virus stocks were treated with various concentrations of Mynox[®] for different incubation times. After treatment, viruses were propagated on mycoplasma-free cells cultivated without any antimycoplasm antibiotics, so that any resident mycoplasma would have grown to detectable levels after 1 to 2 subcultivation cycles. At Mynox[®] concentrations higher than recommended the infectivity of the enveloped viruses were either completely destroyed or below acceptable levels. After a single treatment with the recommended procedure for enveloped viruses in SHV-1 and BVDV stock preparation no mycoplasma were detectable. A treatment with low Mynox[®] concentrations did not lead to a long lasting mycoplasma elimination. Nonenveloped viruses are stable against much higher Mynox[®] concentrations which led to a removal of all mycoplasmas from the EMCV and the PPV stocks without a serious titer reduction (Tab. 1). After performing the standard elimination procedure we harvested virus from culture supernatants when a pronounced cytopathic effect approximately 2 to 6 days post infection was visible and treated them a second time to ensure that mycoplasma elimination is successful. All virus strains tested could be freed from mycoplasma by this procedure. We tested the possibility of a re-emergence of residual mycoplasmas cultivating all viruses on cells without any antibiotics for 3 passages (Tab. 1). No re-emergence of the contaminants could be detected.

We determined the loss of virus infectivity during treatment. None or a negligible loss of infectivity of the nonenveloped viruses EMCV and PPV was reserved during treatment with the drug. On the other hand, Mynox[®] reduced the virus titer of the enveloped viruses BVDV and SHV-1 by approximately 6 log₁₀ TCID₅₀ (Tab. 1). Propagation of virus under standard conditions (m.o.i. of 10⁻³) resulted in high titer virus suspensions. After the third passage without Mynox[®] the virus titers were comparable to that of the initial virus stocks. The antimycoplasmicidal activity of Mynox[®] appears to be dependent on the environment, decreasing with an increasing FCS content in the reaction mixture probably as a result of the competitive binding of Mynox[®] to proteins or lipids in the medium. In medium containing 10% FCS we observed none or a negligible inactivation of mycoplasmas by Mynox[®] at low concentrations, but at the recommended concentration all mycoplasmas in medium containing 5% FCS had been eliminated. Therefore, to exclude the mitigating effect of serum on Mynox[®] mycoplasma inactivation should be performed under reproducible conditions in standard cell culture medium without FCS.

We compared the Mynox[®] procedure with the protocol based on solvent extraction as recently described by Linn *et al.* (4). We applied this technique to the mycoplasma infected virus stocks and determined the elimination effectivity. The nonenveloped viruses EMCV and PPV showed none or a negligible reduction of virus infectivity. The enveloped virus BVDV was not effected by solvent extraction, whereas SHV-1 lost over 3 log₁₀ TCID₅₀. Linn *et al.* (4) reported for the enveloped Ross River virus a titer reduction of 2 log₁₀ TCID₅₀. Obviously, both the solvent extraction and the Mynox[®] treatment caused the inactivation especially of enveloped viruses. In contrast to the Mynox[®] treatment and the results reported by Linn *et al.* (4) we were not able to eliminate the mycoplasma contamination from the virus stocks by the ether extraction procedure (Tab. 1). Only after supplementing the reaction mixture with Mynox[®] all mycoplasmas were eliminated from the virus stocks. The apparently disparant effectivity of the solvent extraction method may be due to the infection with different mycoplasma species. The composition of membrane lipids differ in mycoplasma species (6), a characteristic which

is likely to be responsible for their different to organic solvents or Mynox®.

Compared with other mycoplasma elimination protocols using organic solvents or antibiotics (1, 4, 7, 8) the Mynox® treatment described has the advantage of effectiveness. Furthermore it is easy to handle. The novel physico-chemical way of action of this drug minimizes the potential of the development of resistant mycoplasma strains. Therefore, Mynox® is a potent antimycoplasma agent for the development of mycoplasma-free virus stocks.

LEGEND

Table 1: Treatment of virus stocks with Mynox® or ether.

Virus ^a	Initial Virus Titer	Cell Line ^b	Treatment ^c	Passages ^f after Treatment					
				1st.		2nd.		3rd.	
				Titer ^d	PCR ^e	Titer ^d	PCR ^e	Titer ^d	PCR ^e
non enveloped viruses:									
EMCV	11.02	Hep2	M -	10.27	+				
			M +	10.02	-	9.75	-	10.25	-
			E / M -	10.52	+				
			E / M +	11.02	-				
PPV	8.25	ST	M -	7.75	+				
			M +	7.50	-	7.75	-	9.17	-
			E / M -	8.50	+				
			E / M +	7.75	-				
enveloped viruses:									
BVDV	8.66	KL	M -	7.75	+				
			M +	2.25	-	6.66	-	7.83	-
			E / M -	8.75	+				
			E / M +	2.50	-				
SHV-1	7.73	ML	M -	7.17	+				
			M +	< 1.14	-	1.39	-	8.67	-
			E / M -	3.99	+				
			E / M +	< 1.14	-				

^a All virus stocks were PCR positive for mycoplasma.

^b The cell lines used for virus propagation were PCR negative for mycoplasma.

^c Treatment: M - control without Mynox
M + Mynox treatment
E ether extraction
E / M+ reaction mixture for ether extraction was supplemented with Mynox.

^d The virus titers are given as log TCID₅₀.

^e PCR for mycoplasma detection.

^f Passages of viral stocks without mycoplasma inhibitors, following a single treatment with Mynox, on cell lines cultivated free of antibiotics.

REFERENCES

- Drexler, H. G., Gignac, S. M., Hu, Z. B., et al. Treatment of mycoplasma contamination in a large panel of cell cultures. *In Vitro* **30A**:344-347; 1994.
- Hu, M., Buck, C., Jacobs, D., Paulino, G., Khouri, H. Application of PCR for detection and identification of mycoplasma contamination in virus stocks. *In Vitro Cell. Dev. Biol.* **31A**:710-716; 1995.
- Ikoiev, V. N., Gonski, G. M., Dzagurov, S. G. A method of ultracentrifugation used for control of live viral vaccines with regard to removal of mycoplasma. *Vopr. Virusol.* **18**:625-628; 1973.
- Linn, M. L., Bellett, A. J. D., Parsons, P. G., Suhrbier, A. Complete removal of mycoplasma from viral preparations using solvent extraction. *J. Virol. Methods* **52**:51-54; 1995.
- Reed, L. J., Muench, H. A simple method for estimating fifty percent endpoints. *Am. J. Hyg.* **27**:493-497; 1938.
- Rottem, S. Membrane lipids of mycoplasmas. *Biochim. Biophys. Acta* **604**:65-90; 1980.
- Schmidt, J., Erfle, V. Elimination of mycoplasmas from cell cultures and establishment of mycoplasma-free cell lines. *Exp. Cell. Res.* **152**:565-570; 1984.
- Uphoff, C. C., Gignac, S. M., Drexler H. G. Mycoplasma contamination in human leukemia cell lines. II. Elimination with various antibiotics. *J. Immunol. Meth.* **149**:55-62; 1992.