Elimination of mycoplasma contamination of virus stocks

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ABSTRACT: We studied the effectivity of a commercial antibiotic kit Mycokill AB for the elimination of mycoplasma contamination in virus strains. The contaminated virus strains were first filtered, treated with Mycokill AB for three hours and repeatedly passaged in its presence in the cultivation medium in pure cell lines. Three passages in the presence of Mycokill AB were invariably followed by three passages without Mycokill AB. The effectivity of purification was then checked by PCR. Twenty-four out of 28 tested virus strains became free of mycoplasma after a first or second cycle of the treatment with Mycokill AB. The other four strains remained positive even after repeated passages. In these cases of a likely resistance to Mycokill AB, we managed to eliminate the mycoplasma contamination through a subsequent treatment with the antibiotic combination BM-Cyclin. Mycokill AB was shown in the elimination of mycoplasma from virus suspensions as successful as other known most effective antibiotics.

Keywords: Mycokill AB; BM-Cyclin; sparfloxacin; virus stock contamination

Mycoplasma contamination is frequent and serious problem in virological laboratories, where they degrade the virus strains propagated in contaminated cells and reduce their replication. The contaminated virus strains can then lead to unrealistic experimental results and also represent a great risk during production of biological preparations. It is therefore necessary to keep cell cultures, as well as virus strains free of contamination. While contaminated cell lines can be relatively easily replaced with mycoplasma-free lines, virus strains are often unique. When a virus strain is contaminated it is usually necessary to apply elimination methods. Several techniques for elimination of mycoplasmas from virus suspensions were described. These techniques are, however, often impractical and difficult to perform (e.g. solvent extraction or ultracentrifugation; Ikoev et al., 1973; Linn et al., 1995). Ideally, an elimination method should be simple, fast and effective. In the case of virus suspensions, it is also indispensable to consider the level of cytotoxicity of a substance applied on eucaryotic cells in which the virus is propagated. Physical methods like filtration are not applicable for a complete elimination of mycoplasmas since they reduce greatly the virus titre with a risk of its inactivation. Some decrease of mycoplasma titre may, however, be achieved by filtration providing the filter size is adjusted not to affect the virus titre too much (Gemende et al., 1992; Cronholm et al., 2009). The most promising method is a treatment with antibiotics, being also cheap and fairly rapid. Unfortunately, mycoplasmas are resistant to most antibiotics commonly used during cultivations of cell cultures and viruses. On the other hand, macrolids, tetracycline and quinolon have proved to be mostly effective (Fleckenstein and Drexler, 1996; Drexler and Uphoff, 2002; Kazemiha et al., 2009). In recent years, several commercial antibiotic kits have been, therefore, designed for elimination of mycoplasmas from cell lines which are also useful for virus suspensions.

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In this study, we tested the effectivity of a commercial antibiotic kit Mycokill AB for the elimination of mycoplasmas from virus suspensions combined with a microfiltration through a 0.22 µm filter. We found out that this kit can be effectively used for decontamination of viral strains from mycoplasma contaminations.

MATERIAL AND METHODS

Virus strains

We used 28 virus strains contaminated with mycoplasmas. The contamination was ascertained by the PCR described below. A list of the virus strains and the cell lines in which the viruses were propagated is given in Table 1. DMEM with a 3% fetal bovine serum was used as a cultivation medium in the mycoplasma elimination experiments. The cell lines and the serum used in the tests were PCR negative for the presence of mycoplasmas.

Antibiotic treatment of mycoplasma contamination

For mycoplasma eradication from virus strains, the antibiotic kit Mycokill AB (PAA Laboratories, Pasching, Austria), was tested. Ingredients of Mycokill AB are not specified by the manufacturer. Mycoplasma contaminants resistant to Mycokill AB were subsequently treated by Sparfloxacin (fluoroquinolone antibiotic, Sigma-Aldrich) and the antibiotic kit BM-Cyclin (macrolide and tetracycline antibiotics, Roche).

Mycokill AB

The virus suspension was first filtered through a 0.22 µm filter. One ml suspension was mixed with 40 µl Mycokill AB (solution 1 : 25) and incubated for three hours under 37 °C. The cultivation medium was removed from the flask with a correspondent cell line and a virus was pipetted into it. After one hour cultivation under 37 °C, the inoculum was removed and the virus infected cell line was cultivated in the presence of DMEM with 3% fetal bovine serum and Mycokill AB (solution 1 : 50). The cells were cultivated until a marked cytopathic effect was achieved and then frozen at –80 °C. A cell line cultivated with no virus and the same concentration of Mycokill AB was used as a control of cytotoxicity. Altogether, we performed three virus passages in the presence of Mycokill AB. After the third passage, the efficacy of elimination was tested with PCR. In case of a negative result, we performed another three passages of the virus in the absence of antibiotics and the virus strain was then re-tested again with PCR. In case of a positive result, we performed up to six another passages of the virus in the presence of Mycokill AB.

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

CAPM Number	Virus	Cell line
CAPM V-315	BVDV	MDBK
CAPM V-438	BVDV	MDBK
CAPM V-514	BVDV	MDBK
CAPM V-515	BVDV	MDBK
CAPM V-19	*Bovine herpesvirus 1*	MDBK
CAPM V-25	*Bovine herpesvirus 1*	MDBK
CAPM V-132	*Bovine herpesvirus 1*	MDBK
CAPM V-133	*Bovine herpesvirus 1*	MDBK
CAPM V-184	*Bovine herpesvirus 1*	MDBK
CAPM V-255	*Bovine herpesvirus 1*	MDBK
CAPM V-256	*Bovine herpesvirus 1*	MDBK
CAPM V-257	*Bovine herpesvirus 1*	MDBK
CAPM V-261	*Bovine herpesvirus 1*	MDBK
CAPM V-317	*Bovine herpesvirus 1*	MDBK
CAPM V-10	*Bovine enterovirus*	MDBK
CAPM V-326	*Bovine coronavirus*	MDBK
CAPM V-490	PRRSV	MARC 145
CAPM V-502	PRRSV	MARC 145
CAPM V-503	PRRSV	MARC 145
CAPM V-504	PRRSV	MARC 145
CAPM V-506	PRRSV	MARC 145
CAPM V-537	PRRSV	MARC 145
CAPM V-538	PRRSV	MARC 145
CAPM V-482	*Canine coronavirus*	A-72
CAPM V-320	*Canine adenovirus*	A-72
CAPM V-477	*Canine adenovirus*	A-72
CAPM V-465	*Encephalomyocarditis virus*	VERO
CAPM V-238	*Feline calicivirus*	CRFK

CAPM = Collection of Animal Pathogenic Microorganisms; BVDV = *bovine viral diarrhea virus*; PRRSV = *porcine reproductive and respiratory syndrome virus*; MDBK = Madin Darby bovine kidney cell line; MARC 145 = monkey kidney cell line; A-72 = canine tumor cell line; VERO = African Green monkey kidney cell line; CRFK = cat kidney cell line

Sparfloxacin (fluoroquinolone antibiotic, Sigma-Aldrich)

The virus suspension was treated with the same method as in the previous case except for using sparfloxacin instead of Mycokill AB. Sparfloxacin was used in the concentrations of 20 µg/ml for a treatment of the virus before the cultivation on cell lines and 10 µg/ml as an addition into the medium during the virus cultivation.

BM-Cyclin

BM-Cyclin is a two-component kit. In the first passage, BM Cyclin 1 was applied in concentrations of 25 µg/ml for a treatment of a virus before the cultivation and 10 µg/ml during the cultivation. The second passage was performed with the addition of BM Cyclin 2 in the concentrations of 12.5 µg/ml for a treatment of a virus before the cultivation and 5 µg/ml during the cultivation. The whole procedure was repeated twice, i.e. four passages of a virus were performed in total, alternating BM Cyclin 1 and 2.

PCR

Five hundred µl virus suspension was centrifuged under 13 000 g and the pellet was washed out twice and resuspended in 200 µl PBS. The isolation of DNA from the samples was performed using QIAamp DNA Mini Kit according to the recommendations of the manufacturer (Qiagen). The mycoplasma DNA was detected by the PCR kit Venor *GeM* (Minerva Biolabs), specific for a spectrum of contaminants of cell lines and their biological derivates belonging to the genera *Mycoplasma* and *Acholeplasma*. The kit was applied following the recommendations of the manufacturer. The resulting PCR products were separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide, visualized under UV light and documented by photography.

RESULTS

The experiments to eliminate mycoplasmas using Mycokill AB were performed with all 28 contaminated virus strains. In 21 strains, we recorded the absence of mycoplasma DNA after three passages with Mycokill AB. All these strains remained negative even after the repeated passages without any addition of antibiotics. Seven strains remained positive for mycoplasma DNA after the first three passages with Mycokill AB and were then subject to additional three passages with Mycokill AB. After these repeated passages and three subsequent passages with no antibiotics, additional three strains became PCR negative (*Bovine enterovirus* – CAPM V-10, *Bovine herpesvirus 1* – CAPM V-25, *Bovine coronavirus* – CAPM V-326). Four strains of PRRSV, however, remained positive even after six passages with Mycokill AB, and we had to treat them with other antibiotics.

Sparfloxacin turned out as totally ineffective in the elimination of the mycoplasmas contaminating the strains of PRRSV (CAPM V-490, V-506, V-502, and V-504) which were resistant to Mycokill AB. No elimination of mycoplasmas was detected even after six passages of the virus in the presence of sparfloxacin.

To eliminate the mycoplasmas resistant to Mycokill AB and sparfloxacin, we finally applied BM-Cyclin. Following the recommendations of the manufacturer, we performed four passages: two with BM Cyclin 1 and two with BM Cyclin 2. After the treatment, none of the strains (CAPM V-490, V-506, V-502 a V-504) was detected as mycoplasma DNA positive. The results were confirmed also by three subsequent passages performed in the absence of antibiotics.

DISCUSSION

In our study, we tested the effectivity of the antibiotic kit Mycokill AB for the elimination of mycoplasma contaminations of virus strains deposited in a virus collection. The contaminations originate with the greatest probability from the cell lines on which the viruses had been propagated. Mycokill AB was selected for its favourable price and simple use.

We ascertained the elimination of the mycoplasmas using Mycokill AB in 24 virus strains out of 28 tested in our study, i.e. in nearly 86% cases. Similar results in the effectivity were achieved with sparfloxacin and BM-Cyclin for decontamination of cell lines (Drexler et al., 1995; Fleckenstein and Drexler, 1996; Drexler and Uphoff, 2002; Uphoff et al., 2002; Gopalkrishna et al., 2007). The problem of cytotoxicity appeared in our case as unimportant. In literature, a cytotoxicity leading to death of cell lines caused by an antibiotic elimination of mycoplasmas has been reported between 3–18%

(Uphoff et al., 2002; Kazemiha et al., 2009). In our experiments, the control cell lines lacking virus inoculum but containing Mycokill AB did not show any cytopathic modifications. The low level of cytotoxicity of Mycokill AB in our tests could also be a result of a shorter time of exposure of cell lines to the effects of antiobiotics during a virus propagation. In repeated passaging of cell lines in a constant presence of antibiotics which is necessary for a decontamination of cell lines, the risk of cytotoxicity would be much greater.

Because of the already reported effectivity of sparfloxacin, we tried to eliminate the mycoplasmas resistant to Mycokill AB using this antibiotics in a well-proven concentration (Drexler and Uphoff, 2002; Uphoff et al., 2002). Nevertheless, sparfloxacin was shown as totally ineffective for the elimination of mycoplasmas resistant to Mycokill AB.

The mycoplasmas resistant to Mycokill AB were, on the other hand, successfully eliminated with BM-Cyclin. A high cytotoxicity of BM-Cyclin (up to 18 %) to cell lines was reported as the main disadvantage of this otherwise very effective antibiotic combination (Uphoff et al., 2002; Kazemiha et al., 2009). Similarly to Mycokill AB, we detected no apparent toxic effects of BM-Cyclin on cells during the elimination of mycoplasmas from our virus suspensions.

Mycokill AB thus seems to be a relatively effective and practical mean for the elimination of mycoplasma contaminations of virus suspensions. Its effectivity, however, has to be taken with caution as cases of resistance may appear. In these cases, BM-Cyclin was shown as a good supplementary antibiotic kit. Sparfloxacin was unsuitable for this purpose; it cannot be excluded that Mycokill AB is an antibiotic on a similar base as sparfloxacin.

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