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A Single-Tube Real-Time PCR Assay for Mycoplasma Detection as a Routine Quality Control of Cell Therapeutics

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Summary
Background: Contamination of cell culture and biological material by mollicute species is an important safety issue and requires testing. We have developed a single-tube real-time polymerase chain reaction (PCR) assay for rapid detection of *Mollicutes* species stipulated by the European Pharmacopeia. **Methods:** Primers and TaqMan probes (FAM-labeled) were deduced from 16S rDNA sequence alignment of 18 mollicutes species. A synthetic internal control (IC) DNA and an IC-specific TaqMan probe (VIC-labeled) were included. The analytical sensitivity of the assay was determined on DNA dilutions from 12 mollicute strains. Specificity was proven by the use of DNA from other bacteria. **Results:** Analytical sensitivities of the PCR assay were in the range of 405–2,431 genomes/ml for 11 of the 12 tested mollicute DNA samples. The lowest sensitivity was found for *Ureaplasma urealyticum* (19,239 genomes/ml). Negative results for DNA samples from 3 different ubiquitous bacteria demonstrated the specificity of the PCR assay for *Mollicutes*. Direct testing of cell culture supernatants spiked with *Mycoplasma orale* revealed similar sensitivity compared to isolated DNA. **Conclusions:** Our single-tube real-time PCR assay with internal reaction control enables rapid and specific detection of mollicute contaminants. The test protocol is suitable for routine quality control of cell therapeutics.

Introduction

Contamination with bacteria of the *Mollicutes* class, including *Mycoplasma*, *Acholeplasma* and *Ureaplasma*, is amongst the most frequently occurring problems associated with cell cultures. Literature data offer contamination rates varying between 15 and 80% depending on cell type, cell source and culture methods [1]. The clinical use of cultured cells and cellular products in therapy of diseases makes testing for *Mollicutes* a necessity, at least for the proven pathogens *M. pneumoniae*, *M. genitalium* and *M. hominis*. Legal requirements such as those described in the European Pharmacopoeia (EP, 7th edition, Section 2.6.7.) demand the testing of cell lines and cell therapeutics for mycoplasma with a detection limit of about 100 mycoplasma in the tested product [2]. Validated PCR-based assays can be used instead of culture methods or indicator cell methods, and require detection limits of 10 or 100 colony-forming units (CFU)/ml, respectively. In our previous work we validated and compared a conventional PCR assay with a culture method, i.e. color-changing units (CCU) [3]. The analytical sensitivity of our PCR was the CCU equivalent of 100 for *M. orale* and *M. pneumoniae*.

Microbiological testing by incubating cell culture supernatants in specific media and subsequent determination of viable bacteria still represents the ‘gold standard’. Viable *Mycoplasma* can be determined as CFU on agar plates or CCU using liquid indicator media. Hence, determination via CFU is sensitive enough to detect at least the demanded 100 mycoplasma. However, the quality of CFU data may be inaccurate because of the non-standardized, subjective interpretation of the results [4]. CCU on the other hand is not sensitive enough, and determination provides higher estimates of cell

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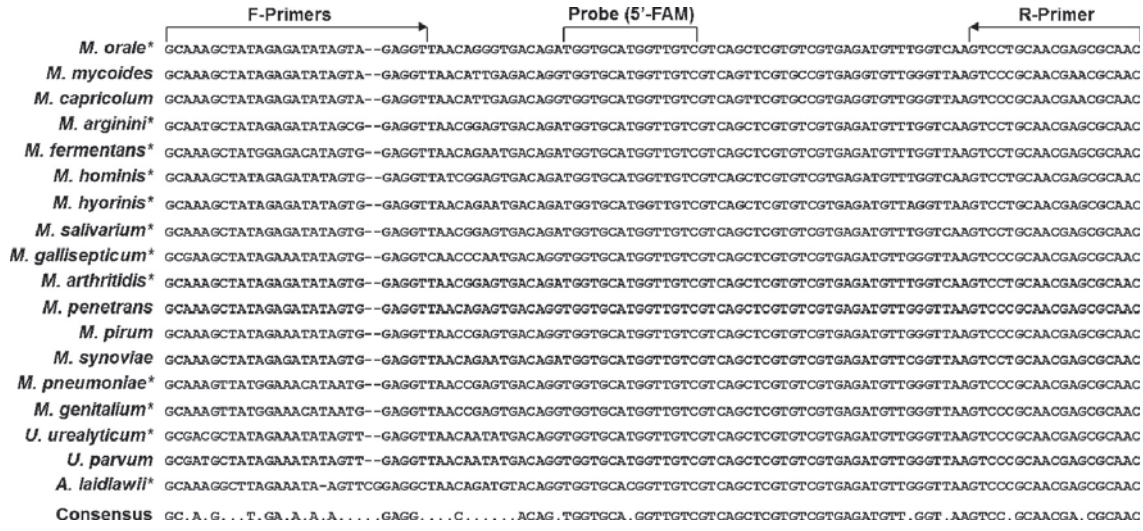

Fig. 1. DNA sequence alignment of part of the 16s rDNA genes of different mollicutes species. PCR amplification using the forward and reverse primers as indicated resulted in a 106-bp amplicon (107 bp for *Acholplasma laidlawii*). The probe matched to all sequences aligned, except a minor mismatch (pyrimidine exchange T→C) in the *A. laidlawii* DNA sequence. In the consensus sequence only the 100% identical bases are given. Most of the strains (*) were tested by PCR using corresponding DNA samples.

numbers of about 1,000 or more mycoplasma and better correlates with the DNA content [5]. Main disadvantage of both methods is the time consumption. Incubation periods of 21 days are required for the determination. Furthermore, not all mycoplasma species grow in the media [5].

We and others have developed assays to detect *Mollicutes* species based on conventional PCR [3, 6–10]. More recently, a number of assays based on real-time PCR have been introduced [11–14]. Whereas Störmer et al. [14] used conserved regions of the *tuf* gene for development of a broad-range PCR assay, most of the PCR assays target the 16S rDNA region. The 16S rDNA is characterized by a significant degree of sequence conservation, allowing a *Mollicutes*- or *Mycoplasma*-specific primer design. It also allows differentiation from other ubiquitous bacteria including *Clostridium acetobutylicum, Lactobacillus acidophilus* and *Streptococcus pneumoniae.* The analytical sensitivity of the different PCR assays (when reported) was mostly in the range of 1–10 genome copies/µl sample. However, it is still unclear whether the sensitivity of these PCR assays meets the EP criteria of 10 CFU/ml.

Based on our previously reported conventional single-tube PCR assay, we have now developed a single-tube real-time PCR assay for fast detection of the most relevant *Mollicutes* species as a quality control method for cell cultures and cell therapeutics. We designed *Mollicutes*-specific primers and a FAM-labeled probe for real-time detection based on the alignment of 16S rDNA sequences. We also included an artificial oligonucleotide as internal control (IC), which is simultaneously amplified and detected by a specific VIC-labeled probe. The specificity and sensitivity of the real-time PCR assay was estimated using DNA samples from 12 different *Mollicutes* strains and the detection limits were calculated by probit analysis. Furthermore, heat-inactivated cell culture supernatants could be used directly for PCR without DNA extraction. On the simple and fast procedure we could implement the PCR assay as a routine quality control for cell therapeutics.

Material and Methods

DNA Samples and Bacteria

DNA samples of all *Mollicutes* that according to the EP require analyzing were included: *Acholplasma laidlawii*, *Mycoplasma hyorhinis*, *M. orale*, *M. pneumoniae*, *M. fermentans*, *M. arthritidis* (similar to *M. synoviae*) and *M. gallisepticum*. Additionally, we investigated DNA samples of *M. arginini*, *M. genitale*, *M. hominis*, *M. salivarum* and *Ureaplasma urealyticum*. To demonstrate the specificity of the PCR assay we also investigated DNA samples of ubiquitous bacterial species *Clostridium acetobutylicum*, *Lactobacillus acidophilus* and *Streptococcus pneumoniae* using a negative control. All DNA samples had a concentration of 100 ng/ml (Minerva Biolabs GmbH, Berlin, Germany). In addition to the DNA samples, we used a sample of lyophilized *M. orale* with a bacterial cell number of 10^9 cells/ml (determined before lyophilization by the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The lyophilized bacteria were rehydrated in standard PBS buffer and used in spiking experiments.

DESIGN of the Single-Tube Real-Time PCR Assay

DNA sequence information for the 16S rDNA gene region of all bacterial strains was retrieved from sequence databases (NCBI and MolliGen [15]) and aligned (fig. 1). From the alignment we identified a sequence region that is highly homologous among the strains of *Mollicutes*, and that is significantly different in the ubiquitous bacterial strains. We deduced 7 different forward primers, a single reverse primer and a single probe to detect the bacterial strains of interest (table 1). The probe was used with an FAM label at the 5' end and a minor groove binding (MGB) modification at the 3' end (Applied Biosystems GmbH, Darmstadt, Germany). As IC, we designed a synthetic DNA molecule of 95 nucleotides (Myco16sQIC) that included binding sequences for

Table 1. Primers, probes and synthetic internal control (IC) DNA for the *Mycoplasma* single-tube real-time PCR assay

Name	Sequence (5′–3′)	Specificity
Primers		
Myco16sQF1	gcaaagctatagagatatagtagaggt	*Mycoplasma orale**, *M. mycoides*, *M. capricolum*, and IC
Myco16sQF2	gcraagctatagaratatagtggaggt	*M. arthritidis**, *M. gallisepticum**, *M. hominis**, *M. hyorhinis**, *M. penetrans*, *M. pirum*, *M. salivarium**, *M. synoviae*
Myco16sQF3	gcaatgctatagagatatagcggaggt	*M. arginini**
Myco16sQF4	gcaaagctatggagacatagtggaggt	*M. fermentans**
Myco16sQF5	gcaaagttatggaaacataatggaggt	*M. pneumoniae**, *M. genitalium**
Myco16sQF6	gcgacgctatagaaatatagttgaggt	*Ureaplasma urealyticum**, *U. parvum*
Myco16sQF7	gcaaaggcttagaaataagttcggaggc	*Acholplasma laidlawii**
Myco16sQR	gttgcgytcgttgcrggac	all *Mollicutes* and IC
Probes		
Myco16sQProbe	FAM-tggtgcatggttgtc-MGB	all *Mollicutes*
Myco16sQICProbe	VIC-cacgccgtaaacga-MGB	IC
IC-DNA		
Myco16sQIC**	ggcaaagctatagagatatagtagaggttagtccaccgccgtaaacgatatgctcgcaagagtaaccttcgcaaagctatagtcctgcaacgagcgcaacc	–

*DNA samples of these bacterial species were used in PCR tests.
**The synthetic IC-DNA Myco16sQIC contained binding sites (underlined) for primers Myco16sQF1 and Myco16sQR and the VIC-labeled probe Myco16sQICProbe.

primers Myco16sQF1 and Myco16sQR, as well as the binding sequence for a VIC-labeled probe (Myco16sQICProbe). Combining all primers, probes and the Myco16sQIC DNA in 1 mixture (PPIC mix) we could achieve a single-tube real-time PCR assay for the detection of relevant *Mollicutes* species.

Specificity and Sensitivity of the PCR Assay

PCRs were carried out in a total volume of 20 µl, including 5 µl DNA or sterile water (as a control without template), 5 µl PPIC mix (2 µmol/l each forward primer Myco16sQF1–7, 4 µmol/l reverse primer Myco16sQR, 0.8 µmol/l each probe, 0.4 pg/µl Myco16sQIC) and 10 µl QuantiTect Mastermix (Qiagen, Hilden, Germany). PCR was performed on a real-time PCR cycler (ABI 7000; Applied Biosystems) with a cycling protocol as follows: 10 min initial denaturation at 95 °C, followed by 40 cycles with 15 s at 95 °C and 60 s at 60 °C. Results were evaluated on the basis of the C_t (cycle threshold) value. The results were classified as positive when the FAM fluorescence signal exceeded the threshold within the 40 PCR cycles (< 40 C_t). A negative result was recorded when only the VIC signal and not the FAM signal exceeded the threshold (VIC < 40 C_t; FAM > 40 C_t).

For testing the specificity of the PCR assay for *Mollicutes*, all bacterial DNA samples were used at a concentration of 10 ng/ml. To determine the sensitivity of the PCR assay, we tested dilution series of DNA samples from 12 *Mollicutes* species. We prepared 12-step linear dilution series with a DNA concentration range from 10 ng/ml (highest) to 5 pg/ml (lowest). Each dilution was tested in 3 independent PCR experiments each with 5 replicates to obtain a total of 15 measures per sample.

PCR Testing for M. orale *in Spiked Experiments*

To compare the sensitivity of the PCR assay on isolated DNA samples to the sensitivity in spiked cell culture supernatants, we used mesenchymal stromal cells (MSCs) cultured for 5 days under standard conditions. MSC cultures were spiked with defined numbers (10^6 to 10^{-1}) of *M. orale* cells/ml that had been rehydrated in PBS buffer from a lyophilizate. Aliquots of 20 µl of each cell culture supernatant were subjected to heat inactivation (95°C for 10 min) followed by a short centrifugation of 5 s at 1,000 × *g*. 2 µl of heat-inactivated supernatants together with 3 µl sterile water (to adjust volume) were used in PCR as described above, and testing was carried out in 3 replicates for each sample.

Probit Analysis

Based on the estimation that a *Mollicutes* genome is approximately 10^6 bp in size, 1 ng DNA corresponds to approximately 10^6 genomes. The number of genomes was calculated for each DNA dilution tested by PCR, and this was used for calculations of the detection limits for each bacterial strain. As a model of non-linear regression, the probit (predicted proportion positive) analysis was performed using a SPSS software package (SPSS 12.0; SPSS Inc., Chicago, IL, USA). Detection limits were calculated for the 50, 90, 95 and 99% probability, including a continuous 95% confidence interval (95% CI) of the probability to achieve a positive PCR result.

Results

Specificity of the Single-Tube Real-Time PCR Assay

According to the alignment of 16S rDNA sequences (fig. 1), we designed 7 different forward primers, 1 reverse primer and 2 fluorescent probes, 1 with a *Mollicutes*-specific sequence (FAM-labeled) and 1 with an IC-specific sequence (VIC-labeled). Together with the synthetic IC-DNA molecule, we combined all primers and probes into a single reagent mix (PPIC mix) for the real-time PCR. Testing of 12 different *Mollicutes* DNA samples and the DNA samples from 3 different ubiquitous bacteria demonstrated the *Mollicutes* specificity of the PCR assay (fig. 2A). As expected, the VIC fluorescence signal for IC detection was positive, and was independent of the bacterial species (fig. 2B). At high concentrations of

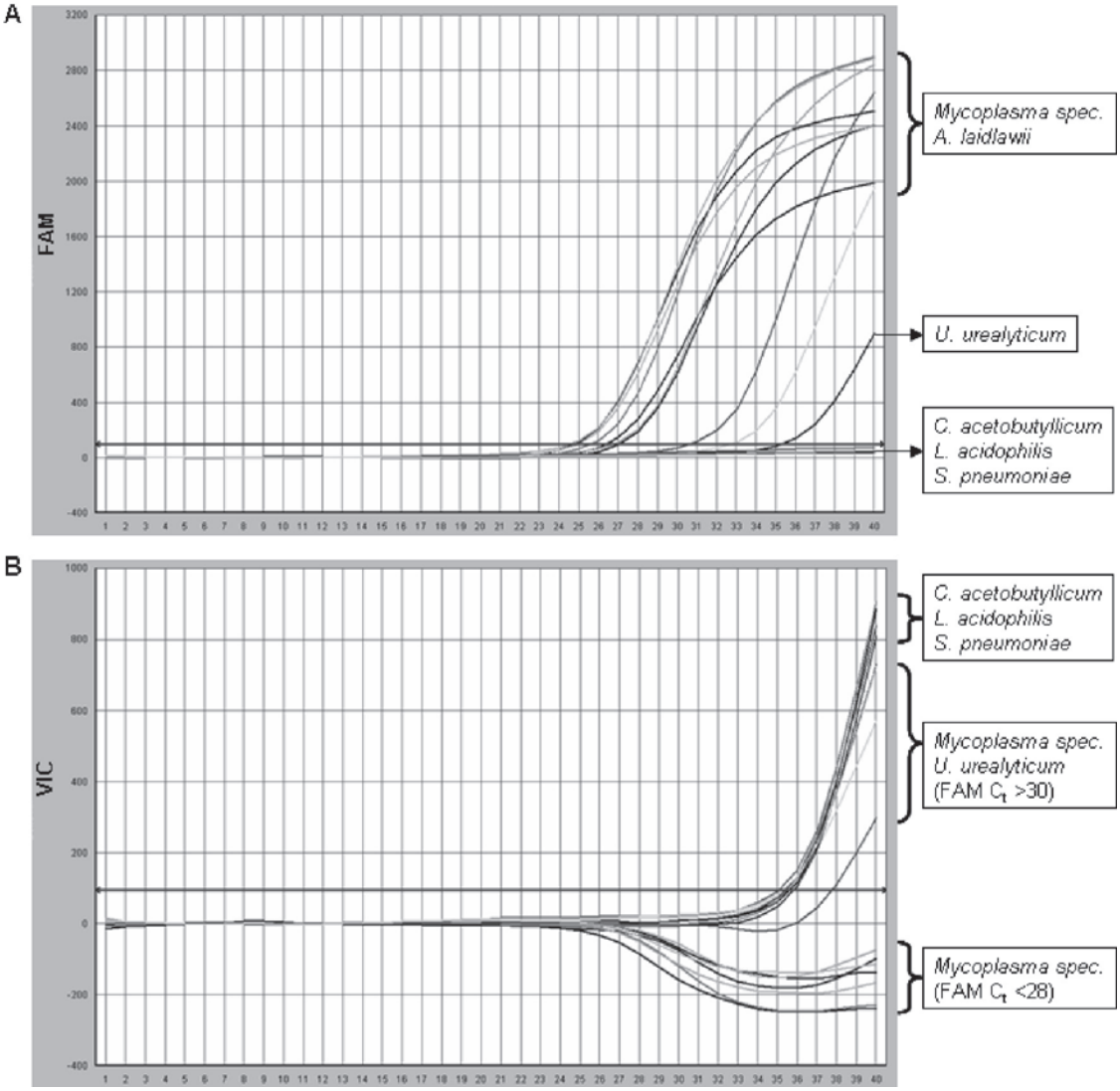

Fig. 2. Results of the single-tube real-time PCR using 50 pg/ml DNA of *Mollicutes* species. **A** All *Mollicutes* samples revealed positive PCR results (FAM cycle threshold (C_t) values < 40) with the lowest sensitivity for *U. urealyticum*. DNA samples from other bacteria such as *Clostridium acetobutylicum, Lactobacillus acidophilis* and *Streptococcus pneumoniae* showed negative results (FAM C_t values > 40). **B** Co-amplification and detection of the internal control by VIC fluorescence was positive for all samples, except those which showed strong amplification of *Mycoplasma* DNA (FAM C_t values < 28).

Mollicutes DNA we observed strong amplification (C_t values < 28), which frequently caused inhibition of IC amplification (fig. 2B). However, to classify a PCR result as positive, the fluorescence signal of the IC can be neglected when the FAM signal is clearly positive.

Sensitivity of the Single-Tube Real-Time PCR Assay

We used linear dilution series of DNA from 12 different *Mollicutes* species in a concentration range of 10 ng/ml to 5 pg/ml to estimate the detection limits of the single-tube real-time PCR assay. Each dilution of each DNA sample was tested 15 times and the PCR results were classified according the C_t values: FAM C_t < 40 = positive; FAM C_t > 40 and VIC C_t < 40 = negative. Based on the PCR results, we determined the detection limits for each *Mollicutes* species by probit analysis (table 2). The PCR assay showed the highest sensitivity for detection of *M. pneumoniae* with a 95% probability to detect 405 genomes/ml. Furthermore, *M. arthritidis, M. gallisepticum, M. salivarium, M. arginini* and *M. genitalium* also revealed detection limits < 1,000 genomes/ml at 95% probability. The PCR assay showed the lowest sensitivity for detection of *U. urealyticum* (19,239 genomes/ml at 95% probability).

Direct Testing of Spiked Cell Culture Supernatants

We were interested in establishing a simple and fast protocol for PCR testing of cell culture supernatants that circumvents DNA extraction. We, therefore, performed spiking experiments using a rehydrated sample of lyophilized *M. orale*, and compared the sensitivity of this protocol to the use of DNA samples as described above. Human MSC cultures were used as a model and were spiked with decreasing numbers of *M. orale* cells. Aliquots of the cell culture supernatants were heat inactivated and directly used for PCR testing. We found strong correlation (R^2 = 0.9918) of the FAM C_t values with the *M. orale* cell numbers (fig. 3A). Positive results were obtained with MSC culture supernatants spiked with ≥ 10^3 *M. orale* cells. This is very similar to the detection limit determined with the use of extracted DNA of *M. orale* (1,673 genomes/ml at 95% probability).

Table 2. Detection limits for the different *Mollicute* species calculated by probit analysis on the basis of PCR testing of linear DNA dilution series. Each dilution was tested 15 times (3 separate PCR experiments each with 5 replicates) and the results were classified positive ($C_t < 40$) or negative ($C_t > 40$). Detection limits are given for the 50, 90, 95 and 99% probability

Bacterial strain	Probability, %	Detection limit, genomes/ml (95% confidence interval)
M. orale	50	875 (702–1,125)
	90	1,497 (1,222–2,020)
	95	1,673 (1,359–2,283)
	99	2,004 (1,612–2,781)
M. arthritidis	50	297 (210–423)
	90	715 (550–1,074)
	95	834 (638–1,268)
	99	1,056 (801–1,632)
M. gallisepticum	50	344 (260–474)
	90	701 (548–1,020)
	95	802 (624–1,180)
	99	992 (765–1,482)
M. hominis	50	1,107 (853–1,519)
	90	2,139 (1,677–3,121)
	95	2,431 (1,895–3,590)
	99	2,979 (2,300–4,475)
M. hyorhinis	50	1,095 (855–1,485)
	90	2,019 (1,594–2,914)
	95	2,280 (1,790–3,333)
	99	2,772 (2,154–4,121)
M. salivarium	50	317 (246–432)
	90	593 (467–850)
	95	671 (526–972)
	99	817 (635–1,202)
M. arginini	50	169 (121–237)
	90	377 (292–569)
	95	436 (335–667)
	99	547 (416–854)
M. fermentans	50	807 (545–1,187)
	90	2,030 (1,531–3,220)
	95	2,377 (1,782–3,825)
	99	3,027 (2,246–4,967)
M. pneumoniae	50	215 (173–274)
	90	363 (298–486)
	95	405 (331–548)
	99	483 (391–667)
M. genitalium	50	274 (220–358)
	90	455 (368–629)
	95	506 (407–708)
	99	603 (480–858)
U. urealyticum	50	7,559 (3,394–16,976)
	90	16,659 (10,911–47,508)
	95	19,239 (12,571–56,634)
	99	24,079 (15,571–73,867)
A. laidlawii	50	864 (698–1,099)
	90	1,450 (1,191–1,931)
	95	1,616 (1,322–2,176)
	99	1,928 (1,562–2,641)

Discussion

As previously shown [11–14], real-time PCR provides a highly sensitive and rapid technique for mycoplasma detection in cell culture or clinical samples. Based on the concept of a conventional PCR assay described in our previous study [3], we now present a single-tube real-time PCR assay with less hands-on time suitable for routine testing. The assay was found to be robust because the use of different PCR cyclers and different reagent charges did not affect the results (data not shown). We determined the detection limits of our assay for 12 relevant bacterial strains. Detection limits between 405 (*M. pneumo-*

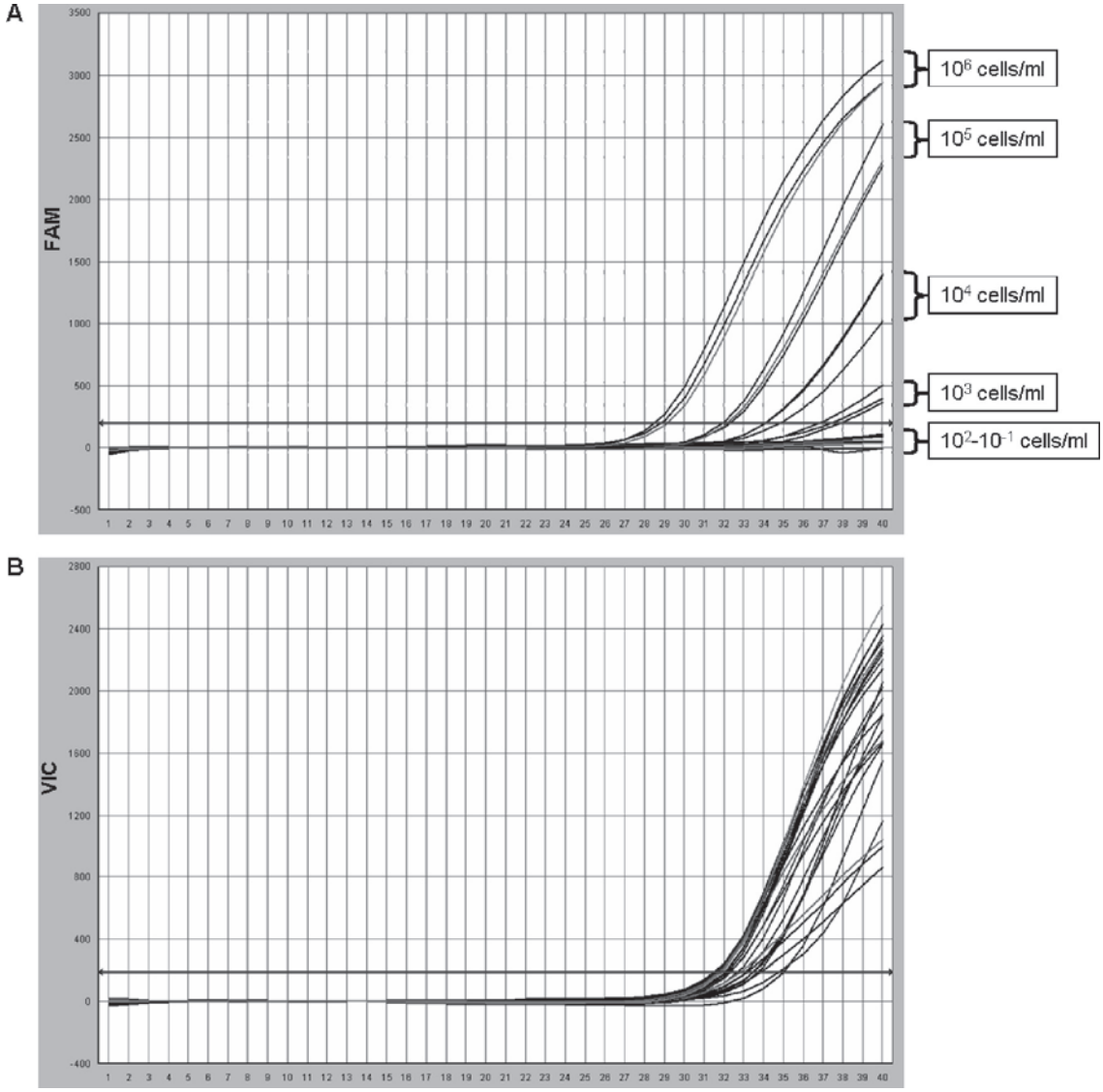

Fig. 3. PCR results from testing human mesenchymal stem cell culture supernatants spiked with defined numbers of *M. orale* cells. **A** The FAM fluorescence revealed strong correlation of the C_t values with the *M. orale* cell number. Cell numbers of 10^2–10^{-1}/ml were below the detection limit of the PCR assay. **B** Co-amplification and detection of the internal control by VIC fluorescence was positive for all samples.

niae) and 19,239 genomes/ml (*U. urealyticum*) at 95% probability were found. As demanded by the EP [2], our real-time PCR assay is also able to discriminate other common bacteria such as *C. acetobutylicum, L. acidophilus* and *S. pneumoniae*.

According to EP [2], a sensitivity of 10 CFU/ml is required for a PCR assay if it is to be used as an alternative to mycoplasma culture assays. Based on the assumption that 1 CFU or CCU corresponds to 1 mycoplasma cell containing 1 genome copy with 1 16S rDNA gene locus, the sensitivity of our and other published PCR assays is too low (approximately 1,000 DNA copies/ml). However, whether 1 CFU or CCU corresponds to 1 copy of the mycoplasma genome is unclear. Determination of CFU is inaccurate because it depends highly on culture conditions, growth behavior of different mollicute species and differences in subjective interpretation of results [4]. Especially larger colonies could result from more than 1 mycoplasma cell. CCU determination correlates better with the total DNA content [4], although this is also erroneous. It is assumed that 1 CCU corresponds to a higher number of mycoplasma cells. In our previous study we determined the analytical sensitivity of a conventional PCR assay according to CCU for *M. pneumoniae* and *M. orale* [3]. The detection limits of the PCR assay corresponded to 1,222 CCU/ml for *M. pneumoniae* and 2,547 CCU/ml for *M. orale*. Assuming 1 genome copy per CCU the sensitivity of our real-time PCR assay is higher for the 2 species (405 CCU/ml for *M. pneumoniae* and 1,673 CCU/ml for *M. orale*). However, it is still below the EP demand of 10 CFU/ml. Knowledge about the correct number of genome copies per CFU or CCU for each of the relevant *Mollicutes* strains would be very helpful for the accurate determination of detection limits on the basis of DNA dilution series.

Spiking experiments were done in addition to our testing of DNA dilution series in order to compare the sensitivity of the PCR assay. We used lyophilized *M. orale* rehydrated with PBS buffer to spike MSC culture supernatants with defined

numbers of bacterial cells. We found a similar sensitivity compared to isolated DNA samples of the real-time PCR assay. Moreover, we could show that cell culture supernatants can be used directly after heat inactivation without prior DNA extraction as a source material for the PCR. However, mollicutes species tend to adhere to and colonize cells as well as to adhere to inert plastic surfaces [16]. Nevertheless, significant numbers of bacteria remain in the supernatant and can be detected by PCR methods. Cell culture supernatants have been used in other studies and revealed comparable sensitivity [14]. In our study we avoided culturing living bacteria and performing true spiking experiments with infected cells because this would hold the risk of uncontrollable spread of *Mollicutes* bacteria in the laboratory. We decided that this risk is not acceptable for our institute, which is a facility working with blood products and cellular therapeutics.

Our single-tube real-time PCR assay is suitable for detecting all mycoplasma strains demanded by the EP. Testing can be directly performed with only 2 µl of cell culture supernatant without the need for DNA isolation. Compared to conventional PCR, the real-time PCR assay requires less hands-on time and offers the potential for automation. We introduced this simplified procedure into the routine quality control of cellular therapeutics, and the rapid provision of results offers a valuable advantage over the time-consuming culture methods.

Disclosure Statement

The authors declared no conflict of interest.

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