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Introduction of a validation concept for a PCR-based *Mycoplasma* detection assay

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Background

Mycoplasma *contamination is amongst the most frequently occurring problems associated with cell cultures. In order to meet the legal requirements (European Pharmacopoeia and FDA) for* Mycoplasma *testing of cell lines and therapeutics, we have developed a PCR-based method to detect mycoplasms and introduce a validation concept.*

Methods

The PCR assay specifically amplifies a 280-bp DNA fragment of the gene coding for the 16S rDNA. Simultaneous amplification of an artificial oligonucleotide containing primer-binding sites allowed control of the efficacy of the PCR. The validation of the PCR assay was performed with two Mycoplasma *reference strains,* M. orale *and* M. pneumoniae. *The validation concept included (i) cultivation of* M. orale *and* M. pneumoniae *in medium with an indicator for bacterial metabolism, (ii) determination of the color-changing units (CCU) in repeated dilution experiments and (iii) correlation of the PCR results with CCU values.*

Results

The detection range was found to include all Mycoplasma *species most commonly found in cell cultures. The analytical sensitivity of the PCR was the CCU equivalent of 100 for* M. orale *and* M. pneumoniae. *Probit analysis revealed a detection probability of 9% for a mean concentration of 1222 (935–1844) CCU/mL for* M. pneumoniae *and 2547 (1584–10 352) CCU/mL for* M. orale.

Discussion

The validation of the Mycoplasma *detection assay supported PCR as an attractive diagnostic tool that will help manage the important issue of* Mycoplasma *contamination of cell cultures.*

Keywords

CCU, cell culture, European Pharmacopoeia, FDA, Mycoplasma, *PCR, validation concept.*

Introduction

Mycoplasma contamination poses a recurrent threat to cell cultures and biologic materials. Contamination by members of the class *Mollicutes* (including *Mycoplasma*, *Ureaplasma* and *Acholeplasma* species) can render experimental results unreliable and biologic products defective [1,2]. Common characteristics of mycoplasmas are the complete lack of a bacterial cell wall, resistance against penicillin, osmotic fragility, colony shape and filterability through 200-nm pore diameter membrane filters [reviewed in 3]. Mollicute contamination is not always easy to detect because there is no turbidity of media as a result of bacterial growth, and cytopathic effects are also rare. Therefore the risk of overlooking a contamination is very high and 15–80% of cell cultures have been reported to be contaminated with *Mollicutes* [reviewed in 4]. Of 20 mollicute species known to cause cell culture contamination, five (*Mycoplasma arginini*, *Mycoplasma fermentas*, *Mycoplasma hyorhinis*, *Mycoplasma orale and Acholeplasma laidlawii*) account for about 95% of episodes, whereas

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Mycoplasma pneumoniae, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma parvum* and *Ureaplasma urealyticum* are most frequently found in clinical specimens [reviewed in 5 and 6].

Quality control as well as safety concepts have been developed to ensure purity and safety of biopharmaceuticals and cell therapeutics. However, despite the European Pharmocopeia [7] and the FDA [8] requirement for frequent testing for *Mycoplasma*, there is still confusion about how to transfer these agreements into practical application. A large number of methods have been described that deal with the detection of contaminant *Mollicutes* [9,10]. The microbiologic approach of incubating cell culture supernatants in specific media and subsequent plating on agar plates represents the 'gold standard' of *Mycoplasma* detection and is still the reference method in good manufacturing practice protocols and in pharmacopoeias. Viable *Mycoplasma* cells can be determined as colony-forming units (CFU) or color-changing units (CCU). However, the quality of CFU data may be inaccurate because of the subjective interpretation of the results [11]. Furthermore, this method is erroneous because not all *Mycoplasma* species grow in media, especially when using solid media [12]. CCU determination provides higher estimates of cell numbers and correlates better with the DNA content [11]. However, both techniques are time consuming because an incubation period of 21 days is required. As a consequence, non-culture methods have been developed for the detection and identification of *Mollicutes* in clinical specimens and cell culture. They comprise immunologic [13,14], DNA staining [15,16], nucleic acid hybridization [17,18] and PCR [19–23] techniques. PCR assays targeting 'universal' mollicute sequences in the 16S rRNA gene have been described elsewhere [19,20] and are components of commercially available kits [24].

We have developed and validated a PCR assay with redesigned primers targeting the mollicute 16S rRNA gene to detect the *Mycoplasma* species that are the most common cause of cell culture contaminations. To be able to monitor the PCR performance, an internal control was included consisting of a synthetic 128mer oligonucleotide with primer binding sites. We established a validation concept according to the guidelines of the European Pharmacopoeia section 2.6.7 [7] and validated the PCR system with reference species *M. orale* and *M. pneumoniae*. The determination of CCU of *M. pneumoniae* and *M. orale* achieved by serial dilutions of liquid cultures with a metabolic indicator served as reference for the PCR validation.

Methods

Microbiologic culture techniques

According to the guidelines of the European Pharmacopoeia 5.0 section 2.6.7 [7], the microbiologic testing was performed with *Mycoplasma* species *M. orale* (ATCC 23714) (LGC Promochem, Wesel, Germany) and *M. pneumoniae* (ATCC 15534). *Mycoplasma pneumoniae* tends to agglutinate and attaches to all kinds of surfaces because of its adhesive growth behavior [25]. To reduce error rates for each pipetting step, a fresh glass pipette was used at each step. The detection range of the PCR system was tested with the *Mollicutes* species listed in Table 1.

Media

Modified Hayflick liquid media was prepared by mixing two stock solutions. Solution 1 consisted of 14.7 g/L PPLO broth (Difco, BD Biosciences, Heidelberg, Germany), phenol red solution and 4.76 g/L Hepes buffer, and was stored at room temperature. Solution 2 consisted of 200 mL/L horse serum, 50% glucose solution and 100 000 U/mL penicillin G solution, and was stored at −20°C. Phenol red solution was prepared from 1 g phenol red, dissolved in 30 mL 1 n NaOH. After the volume was adjusted to 200 mL with water, the solution was incubated over night at 4°C. The pH value was adjusted to 7.5. Solid media/agar plates for the detection of *Mycoplasma* colonies by microbiologic culture were prepared according to Hayflick [26].

Determination of CCU

The color-changing test was performed in modified Hayflick medium containing a phenol red preparation according to Purcell *et al.* [27]. Bacterial metabolism leads to a pH shift that in turn causes a color change from red to orange and then to yellow. The yellow-colored media remains stable for weeks. The cultivation of *M. orale* and *M. pneumoniae* was performed under the exclusion of air. The preparatory cultures *M. orale* and *M. pneumoniae* were cultivated separately in airtight sealed test tubes in modified Hayflick medium for approximately 2 days. When the color of the media changed from red to orange, indicating that the bacteria were metabolically active, serial dilutions were prepared. Three parallel series of 1:10 dilutions of *M. orale* and *M. pneumoniae* were prepared