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Validation of a NAT-based *Mycoplasma* assay according European Pharmacopoiea

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ABSTRACT

Eucaryotic expression systems are widely used to produce biologicals for human use, e.g. vaccines, recombinant proteins and monoclonal antibodies. As part of the safety testing the current U.S. Food and Drug Administration (FDA) regulatory guidelines as well as several European Pharmacopoiea monographs requests the demonstration of the absence of *Mycoplasma* in the cell culture in the bioreactors prior to harvest and further downstream processing. In recent years progress has been made in the development of a sensitive NAT-based method for the detection of *Mycoplasma* species in CHO cells, e.g. Eldering et al. This method is based on a nucleic acid amplification technique using a very sensitive touch-down PCR-profile. The presence of mollicutes DNA in the test specimens is determined by an approx. 450 bp target sequence which is amplified and this amplicon is finally detected by polyacrylamide gel electrophoresis. Based on this method a ready-to-use test kit was developed. In this report the validation of both method variants according the European Pharmacopoiea monograph 2.6.7 "*Mycoplasmas*" is described. The validation demonstrated the robustness and precision as well as a sufficient specificity of both assay formats. The validated sensitivity fulfills the requirements of the European Pharmacopoiea for a PCR-based method proposed as an alternative to the time consuming indicator cell culture and the culture method for the detection of *Mollicutes* (requested sensitivity of at least 10 colony-forming-units/mL).

1. Introduction

Species of the class *Mollicutes* (the trivial name for this group of bacteria is '*Mycoplasma*'; this name will be used in the following) can cause various problems in living organisms and in *in vitro* cell cultures [1–3]. Some species are pathogenic, saprophytic or commensal contaminants introducing changes in cell metabolism and phenotype. *Mycoplasma* infections can be asymptomatic or of subclinical nature and therefore they are insidious and sometimes hardly detectable. In addition, due to their physical nature - they are bacteria without cell wall - they can pass through conventional 0.2 μm filters often used in cell culture technology resulting in high contamination rates in research laboratories [4].

For these reasons and to minimize the risk for patients mammalian cell cultures which are often used for the production of biologicals such as vaccines, recombinant proteins or monoclonal antibody products needs to be tested to verify the absence of viable *Mycoplasmas* [5–9]. The testing protocol includes the testing of the cell banks (master cell bank and working cell bank) as well as testing of the cell culture prior to harvest and further downstream processing – the so called 'unprocessed bulk' or 'harvest'. Two different methods are requested: The culture method [9–14] is performed by inoculating cell culture samples onto a solid agar medium and in parallel into a liquid enrichment medium to allow growth of a low level of *Mycoplasmas*. At defined time steps small aliquots of the enrichment culture are subcultured onto solid agar medium. After incubation the solid agar media are checked for the growth of *Mycoplasmas* indicated by typical colony morphology. This method is quiet sensitive (the European Pharmacopoiea states a sensitivity of 10 cfu/mL [10]) but is lengthy (28 days) and several *Mycoplasma* species are known which do not grow under the described culture conditions. Some isolates belongs to the so called 'noncultivable', fastidious *Mycoplasmas* or to a genus which can proliferate only under different growth conditions. One example is the genus *Spiroplasma* which grows at lower incubation temperatures and needs a different growth medium. To overcome this safety gap, a second method, the indicator cell culture method was introduced by the national authorities [9,10,13,14]. In this case the sample is co-cultured with an indicator cell line susceptible for *Mycoplasma* contaminations. After some days a fluorochrome stain specific for DNA is applied to the fixed indicator cells and this sample is checked under the microscope for fluorescence typical for *Mycoplasmas*. Unfortunately the indicator cell culture method is less sensitive compared to the culture method (the European Pharmacopoiea states a sensitivity of 100 cfu/mL [10]).

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Although the combination of the two *Mycoplasma* testing methods is accepted by the authorities great effort was made to overcome the disadvantages of the traditional *Mycoplasma* testing methods. Polymerase chain reaction (PCR) was identified as an useful tool to replace the traditional methods since this technology is faster and less expensive. Several methods were described [15–19] but all approaches were not sensitive enough to fulfill the requirements of the authorities. In 2004 a PCR-based procedure was published [20] with sufficient sensitivity and specificity for the detection of *Mycoplasmas* in CHO cell cultures used for the production of biologicals. In the meantime the authorities accepted – after thorough validation – the use of a PCR-based method for the detection of *Mycoplasmas* as an alternative to the traditional methods [9,10,14]. Based on the publication of Eldering et al. [20] a ready-to-use test kit (MycoTOOL PCR *Mycoplasma* detection kit) was developed. This report describes the complete validation according to the European Pharmacopoiea [10] for the 'Eldering'-method as well as for the MycoTOOL PCR *Mycoplasma* detection kit.

2. Material and methods

2.1. Mycoplasma species

The following *Mycoplasma* specimens were used to determine the limit of detection and robustness:

The *Mycoplasma* test microorganisms – *Mycoplasma orale* (ATCC 23714), *Mycoplasma pneumoniae* (ATCC 15531), *Mycoplasma arginini* (ATCC 23838), *Mycoplasma salivarium* (ATCC 23064) and *Mycoplasma hominis* (ATCC 23114) were obtained from the American Type Culture Collection (ATCC) (purchased by LGC Standards GmbH, Wesel, Germany) as lyophilized cultures and were grown in liquid *Mycoplasma* medium. In the early exponential growth phase the cultures were harvested. After glycerol was added as a cryoprotectant (20% v/v) the cultures were mixed to ensure uniformity and aliquoted into cryotubes. The tubes were stored at −70 °C. To determine the titers of the frozen stocks at least three vials of each stock were thawed and plated in triplicate on *Mycoplasma* agar plates in serial dilutions. The number of cfu/plate was enumerated after seven days of incubation using a microscope with a 20–80× magnification. The titer of the frozen stock was determined from the average colony count of one countable dilution. The stability of *Mycoplasma* stocks have been demonstrated over years of storage.

Mycoplasma fermentans (ATCC 19989), *Mycoplasma hyorhinis* (ATCC 17981) and *Acholeplasma laidlawii* (ATCC 23206) were obtained as reference strains from the EDQM (Strasbourg, France) [21] and examined in-house for cell density as described. The cell density determined for these isolates was within the range given in the EDQM certificate.

Spiroplasma citri (ATCC 27556) was obtained from MycoSafe (Vienna, Austria) as quantified frozen stocks.

2.2. Non-Mycoplasma species – gram-positive bacteria

In addition the following non-*Mycoplasma* specimens were used for specificity experiments: *Streptococcus bovis* (ATCC 35034), *Lactobacillus acidophilus* (ATCC 4356) and *Clostridium sporogenes* (ATCC 11437). These isolates were obtained from the American Type Culture Collection (ATCC) (purchased by LGC Standards GmbH, Wesel, Germany) as lyophilized cultures and were grown in liquid trypticase soy broth. In the early exponential growth phase the cultures were harvested. After glycerol was added as a cryoprotectant (20% v/v) the cultures were mixed to ensure uniformity and aliquoted into cryotubes. The tubes were stored at −70 °C. To determine the cell density of the frozen stocks at least three vials of each stock were thawed and plated on trypticase soy agar plates using serial dilutions.

2.3. Chinese hamster ovary (CHO) cell cultures

2.3.1. CHO-K1

CHO cells (CHO-K1 cell line, DSMZ No. ACC 110) (DSMZ, Braunschweig, Germany) used for the generic method validation and as extraction control were grown in shaker flasks in cell culture medium to a total cell count of 5×10^6 cells/mL. The cells were pooled, aliquoted in cryotubes (1 mL of cell culture suspension, each) and stored without a cryoprotectant at −70 °C. The CHO-K1 cells were tested for the absence of *Mycoplasmas* using the culture and the indicator cell culture method acc. the European Pharmacopoiea, Chapter 2.6.7 [10].

2.3.2. Recombinant CHO cell lines

Three different recombinant cell lines harvested at the end of the full scale production processes – three independent fermentation runs per recombinant cell line – were used to evaluate inhibiting or interfering substances of these unprocessed bulks.

Product A: recombinant glycoprotein (hormone)
Product B: recombinant antibody X
Product C: recombinant antibody Y

The harvested CHO unprocessed bulk samples were tested for the absence of *Mycoplasmas* using the *Mycoplasma* PCR assay as well as the culture and indicator cell culture method as described in the European Pharmacopoiea, Chapter 2.6.7 [10].

2.3.3. Preparation of the spiked CHO-samples for the method validiation

A suitable dilution of the *Mycoplasma* stocks or stocks of Gram-positive bacteria and the required amount of 1 mL CHO culture suspensions were thawed. The CHO cells were spiked with the required inoculum of *Mycoplasmas* or Gram-positive bacteria.

2.4. Positive control plasmid (pMycdel)

For the in-house-assay the positive PCR control plasmid (pMycdel), prepared in accordance to Eldering et al. [20], was provided by Genentech (South San Francisco, USA).

2.5. General cross-contamination precautions

The following precautions were taken to prevent DNA cross-contamination: the complete protocol was performed under sterile and DNA-free conditions. This included in particular: the preparation and pipetting of all solutions with nuclease- as well as DNA-free equipment (such as pipettes with aerosol-resistant, positive-displacement tips and vials) in pre-decontaminated bleach solution containing hypochloride (e.g. LTK, Biodelta, Loehne, Germany) and, in addition, UV-treated laminar airflow benches and wearing freshly laundered laboratory coats and sterile single-use gloves. Reaction vials were always closed immediately after the pipetting step.

In addition a spatial segregation of the sequential steps were applied: (A) a dedicated airflow bench for the sample preparation (B) a second, dedicated airflow bench for the master mix preparation and the spiking of the PCR negative control (C) a separate room for the PCR reaction and (D) a separate room for the detection of the amplicons (mixing of PCR-amplicons with sample buffer and running the gel).