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Development and validation of a multiplex quantitative polymerase chain reaction assay for the detection of Mollicutes impurities in human cells, cultured under good manufacturing practice conditions, and following European Pharmacopoeia requirements and the International Conference on Harmonization guidelines

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Abstract

Background aims. The clinical applications of *in vitro* manipulated cultured cells and their precursors are often made use of in therapeutic trials. However, tissue cultures can be easily contaminated by the ubiquitous Mollicutes micro-organisms, which can cause various and severe alterations in cellular function. Thus methods able to detect and trace Mollicutes impurities contaminating cell cultures are required before starting any attempt to grow cells under good manufacturing practice (GMP) conditions. *Methods.* We developed a multiplex quantitative polymerase chain reaction (qPCR) assay specific for the 16S–23S rRNA intergenic spacer regions, for the Tuf and P1 cytoadhesin genes, able to detect contaminant Mollicutes species in a single tube reaction. The system was validated by analyzing different cell lines and the positive samples were confirmed by 16S and P1 cytoadhesin gene dideoxy sequencing. *Results.* Our multiplex qPCR detection system was able to reach a sensitivity, specificity and robustness comparable with the culture and the indicator cell culture method, as required by the European Pharmacopoeia guidelines. *Conclusions.* We have developed a multiplex qPCR method, validated following International Conference on Harmonization (ICH) guidelines, as a qualitative limit test for impurities, assessing the validation characteristics of limit of detection and specificity. It also follows the European Pharmacopoeia guidelines and Food and Drug Administration (FDA) requirements.

Key Words: *clinical trials, European Pharmacopoeia, Food and Drug Administration, good manufacturing practice, Mollicutes, mycoplasmas, acholeplasmas, quantitative polymerase chain reaction, tissue culture contaminants*

Introduction

Mollicutes are physically small (0.2–0.3 μm) free-living prokaryotic organisms, being without a cell wall and difficult to detect by microscopy and/or conventional microbiologic methods (1–3). In tissue cultures their presence is awkward to prevent because the contamination may be operator-induced or linked to cell-culture medium recipes. Mollicutes are unresponsive to antibiotics that target cell wall synthesis, and their eradication in *in vitro* cell culture is not easy (4,5). Mollicutes are known to induce dramatic cellular changes, such as chromosome aberrations, metabolism and cell growth alterations. Severe infections may lead to the loss of cell lines, thereby hindering the production of biologic materials (6,7).

From a clinical point of view, several pathologic conditions benefit from the use of either autologous or allogeneic bone marrow transplantation by *in vitro*-manipulated cultured cells or their precursors (8). Moreover, adoptive immune reconstitution has proven to be a life-saving procedure for controlling complications caused by cytomegalovirus (CMV) and Epstein–Barr virus (EBV) infection after hematopoietic stem cell transplantation (HSCT). Thus patients carrying malignancies or hematopoietic disorders, upon CMV or EBV infection, may follow clinical trials that need re-infusion of manipulated and/or expanded virus-specific lymphoid cells (9). In general, re-infused material needs to be grown in a good manufacturing practice (GMP) environment, with careful surveillance for microbiologic contamination, following the European Directive, Food and Drug Administration (FDA) requirements and International Conference on Harmonization (ICH) guidelines (10–13).

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Mycoplasmas and acholeplasmas belonging to the Mollicutes genera represent the most common tissue culture contaminants and, without doubt, are the most challenging to highlight (14). Among the Mollicutes, the species frequently involved (> 95%) are: *Acholeplasma laidlawii*, *Mycoplasma fermentans*, *Mycoplasma pneumoniae*, *Mycoplasma orale*, *Mycoplasma arginini* and *Mycoplasma hyorhinis* (6,15). To detect these contaminants, different techniques are used, including molecular assay, enzyme immunoassay, broth–agar culture and direct/indirect DNA staining (16–18). According to European Pharmacopoeia (EuPh) guidelines 2.6.7 Mycoplasmas (19), nucleic acid amplification techniques (NAT) may be used as an alternative detection system if their sensitivity, specificity and robustness are comparable with either the cell culture or indicator cell culture method (18). NAT methods have greater analytical sensitivity, simplicity and time advantages compared with classical mycoplasma testing. Therefore their application may improve the efficiency of mycoplasma detection in biologic products.

The detection limit needed to replace the culture method with molecular DNA amplification is specified as 10 colony-forming units (CFU)/mL, and the species that need to be tested for are *A. laidlawii*, *M. fermentans*, *M. pneumoniae*, *M. orale*, *M. arginini* and *M. hyorhinis*. Moreover the specificity of detection of these assays needs to be controlled, to avoid generating positive signals for other bacterial genera that have a close phylogenetic relationship with mycoplasmas, such as *Lactobacillus*, *Clostridium* and *Streptococcus*. Among NAT, the end-point polymerase chain reaction (PCR)-based technique displays the highest sensitivity and rapidity of detection but it requires further confirmation using an independent method of detection (15). On the other hand, the use of a quantitative (q)PCR method introduces an additional level of specificity and, once optimized, it should not require any other confirmation test. However, qPCR requires careful optimization and validation following the practical procedures for the qPCR experiment and minimal information guidelines (MIQE) (20,21). Our study aimed to develop a new multiplex qPCR detection system for the clinic that is able to identify Mollicutes species that contaminate cell cultures under GMP conditions, in order to comply with the sensitivity, specificity and robustness required by EuPh guidelines.

Methods

Micro-organisms

Genomic DNA of *M. orale* (ATCC 23714, LGC Promochem, Wesel, Germany), *M. arginini* (ATCC 23838), *M. hyorhinis* (ATCC 17981), *Mycoplasma salivarium* (ATCC 23064), *M. fermentans* (ATCC 19989), *M. pneumoniae* (ATCC 15531), *A. laidlawii* (ATCC 23206), *Spiroplasma citri* (ATCC 27556), *Streptococcus pneumoniae* (ATCC BAA-334), *Streptococcus mutans* (ATCC 25175), *Clostridium sporogenes* (ATCC 11437) and *Lactobacillus acidophilus* (ATCC 4357) were obtained directly from the American Type Culture Collection (ATCC), while genomic DNA of *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Staphylococcus haemolitycus*, *Staphylococcus saprophyticus*, *Streptococcus mitis*, *Streptococcus anginosus*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Enterobacter aerogenes* and *Escherichia coli* were collected from wild-type (WT) micro-organisms isolated directly from clinical samples. The latter isolates were identified by classical microbiologic methods and their DNA was extracted using an automated DNA extractor Magtration system 12GC (PSS BioInstruments, Pleasanton, CA, USA).

Primers and probes

Real-time PCR assays were developed to identify the elongation factor (Tuf) gene for A. *laidlawii*, the 16S–23S rRNA intergenic spacer regions for *M. fermentans*, *M. orale*, *M. arginini*, *M. hyorhinis* and *M. salivarium* (FOAHS) and finally the P1 cytoadhesin gene for *M. pneumoniae* (22). All primers, except for *M. pneumoniae* (22), were designed using a combination of software and utilities such as Oligo 4.1 (National Biosciences Inc., Plymouth, MN, USA), Primer Express 1.5 (Applied Biosystems, Foster City, CA, USA), PrimerPy v0.97 (a graphical user interface, GUI, and a quantitative PCR primer design software) and the Martin Zucker mfold web server (23). The complete list of primers, probes and their working concentrations is shown in Table I.

DNA template standard curves

Standard curves (SC) were prepared using *A. laidlawii*, *M. fermentans* and *M. pneumoniae* quantification standards (Minerva Biolabs GmbH, Berlin, Germany) as templates. These certified titrated DNA standards (1×10^6 genomes/µL) were used as calibrators to generate serial dilutions for each template DNA, alone for singleplex or in combination for multiplex assays.

In detail, to generate the SC we simulated the recovery from 1 mL of tissue culture complete medium [either Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 medium, both supplemented with 10% fetal calf serum, 2 mM L-glutamine and 100 U/mL penicillin–100 µg/mL streptomycin], spiking different serial dilutions