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Multi-primer qPCR assay capable of highly efficient and specific detection of the vast majority of all known *Mycoplasma*

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ABSTRACT

Mycoplasma bacteria are able to pass through sterilizing grade filters due to their small size and lack of a cell wall, making them a common contaminant of biopharmaceutical productions. The classical method for detecting *Mycoplasma* is described in the European Pharmacopeia (Ph.Eur) 2.6.7. The method takes 28 days to perform, due to the slow growing nature of some *Mycoplasma* species. The Ph.Eur has described Nucleic Acid Testing (NAT) as a rapid alternative to the classical method. Here we present the development of a quantitative polymerase chain reaction (qPCR) assay capable of unambiguous detection of *Mycoplasma* with high sensitivity and specificity. The broadness of detection and the specificity towards *Mycoplasma* has been investigated by *in silico* analysis of the primer sequences followed by testing on purified *Mycoplasma* DNA as well as DNA from closely related genera. The assay will in all probability detect at least 356 species and strains of *Mycoplasma*, *Spiroplasma* and *Acholeplasma* with high sensitivity. To our knowledge this assay has the most uniform amplification efficiency over the broadest range of species and it is extremely specific towards *Mycoplasma*. With appropriate validation, the assay can be applied as a powerful tool for rapid *Mycoplasma* detection in the biopharmaceutical industry.

1. Introduction

The bacterial class of *Mollicutes* includes the families *Mycoplasma*, *Spiroplasma* and *Acholeplasma*. Certain species from these families are common contaminants in mammalian biopharmaceutical productions due to their ability to penetrate sterilizing grade filters [1,2].

The current standard method for detecting *Mycoplasma* is a classical growth based method, that takes 28 days to perform due to the fastidious nature of some *Mollicute* species [3]. The method is described in the European Pharmacopeia (Ph.Eur) 2.6.7 [4]. The increased number of biopharmaceuticals, some of which have very short shelf lives, has created a need for a rapid, sensitive, and specific *Mycoplasma* test for release of these products. The Ph.Eur has recognized this need and has proposed nucleic acid based testing (NAT) as a substitute for the classical method. NAT by quantitative polymerase chain reaction (qPCR) is attractive because the analysis time can be reduced to a few hours. The Ph.Eur mentions seven *Mycoplasma* species and two related species of *Mollicutes* – *Spiroplasma citri* (*S. citri*) and *Acholeplasma laidlawii* (*A. laidlawii*) – which the NAT method must be able to detect. Furthermore the NAT method must be able to detect *Mollicute* species down to a concentration of 10 colony forming units (CFU) per mL, and it should be specific for *Mycoplasma*, i.e. the assay primers should not detect contaminants from closely related genera such as *Clostridium*, *Streptococcus* and *Lactobacillus*.

Previous attempts to design a non-commercial PCR that detects broadly and specifically within the *Mycoplasma* genus have been reported. Some of these [5–7] are based on a set of primers designed by Wong-Lee and Lovett [8] in 1993. Although these primers may detect broadly, they lack specificity and have been shown to detect at least *Clostridium* and *Streptococcus* DNA [5,6].

Störmer et al. [9] developed a qPCR assay based on *tuf* gene sequences and showed *in vitro* that it was capable of detecting 32 *Mollicute* species and did not cross react with non-*Mollicute* DNA. However, the specificity of the assay was only shown *in vitro* on 17 non-Mollicute species and only two of these species belonged to *Streptococcus*. No species belonging to the *Clostridium* or *Lactobacillus* genera were included in this study. Janetzko et al. [10] also published a *Mycoplasma* qPCR assay based on 16S rRNA sequences, but the sensitivity of the assay was not uniform among the tested species and the sensitivity for all the tested species could be

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improved. Common for these studies is that the broadness of detection and the specificity of the primers are largely unknown since the primer sequences have mostly been investigated *in vitro* and not thoroughly by *in silico* PCR.

Several commercial kits are available and studies investigating the broadness of detection and specificity of the assays *in vitro* have been published [11,12]. However, since the primer sequences of these kits are not publicly available only *in vitro* analyses can be performed which greatly limits the number of species that can be investigated. The manufacturers may have done *in silico* analyses but the full extents of these studies are not publicly available. Therefore we developed a *Mycoplasma* qPCR assay which detects *Mycoplasma* and related species with the best possible sensitivity, furthermore the assay has been thoroughly investigated in terms of broadness of detection and specificity both by *in vitro* and by *in silico* analyses.

The assay is a SYBR® Green based multi-primer qPCR assay that targets the 16S rRNA gene which is present in all *Mollicute* genomes in one or two copies [13]. The assay detects the vast majority of all known *Mycoplasma* species as well as selected species from the closely related genera *Spiroplasma* and *Acholeplasma* with high efficiency and a high degree of specificity.

The assay fully meets the requirements given in the Ph.Eur in terms of broadness of detection and specificity. However we cannot claim that the assay meet the limit of detection (LOD) requirement of 10 CFU/mL, since the LOD is very much dependent on the DNA extraction procedure applied prior to qPCR. Different product matrices may require different DNA extraction procedures and it is therefore the task of the individual laboratories to choose an appropriate DNA extraction procedure and to show the LOD for the entire procedure for each individual product matrix. However we have shown that the LOD of the qPCR assay described here is close to the theoretical lowest, and the qPCR assay itself will therefore not be the limiting factor when determining the LOD of the entire procedure. If validated appropriately and if suitable controls are included, this assay, combined with a suitable DNA extraction procedure, can substitute the time consuming classical *Mycoplasma* detection method.

Mycoplasma, *Spiroplasma* and *Acholeplasma* will be collectively referred to by their common class name – *Mollicutes* – from here on.

2. Materials and methods

2.1. Sequence alignments

Mollicute and non-*Mollicute* 16S rRNA gene sequences were downloaded from the European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena) and from the Nucleotide database provided by National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/nucleotide/). Multiple sequence alignments were performed in DNA Dynamo V.1.423 (Blue Tractor Software Ltd.).

2.2. Primer design

Primers capable of broad and specific detection within the class of *Mollicutes* were designed by aligning *Mollicute* 16S rRNA gene sequences and 16S rRNA gene sequences from closely related non-*Mollicute* genera such as *Clostridium*, *Streptococcus* and *Lactobacillus.* Conserved *Mollicute* regions showing sequence variation to the related genera were identified and primers were chosen from these regions. A single primer pair capable of detecting all *Mycoplasma* including *S. citri* and *A. laidlawii* was not possible to design. The *Mollicute* species, except for *A. laidlawii*, were therefore divided into three groups based on 16S rRNA gene sequence similarity. Each group was aligned with 16S rRNA gene sequences from closely related non-*Mollicutes*. Three primer pairs were chosen, each one capable of detecting all of the species in one of the three groups. Since *A. laidlawii* exhibits a high degree of sequence variation when compared to the *Mycoplasma* and *Spiroplasma* genera, a fourth primer pair was designed to detect *A. laidlawii* and a few additional *Acholeplasma* species (Table 1). Primers were carefully chosen to contain mismatches in the 3′ ends to the sequences of non-*Mollicute* genera since the detrimental effect of primer to template mismatch becomes greater the closer the mismatch is located to the 3′ end of the primer [14].

The primers were chosen to yield a minimum of self-dimers, hetero-dimers and hairpin structures, all of which can have a detrimental influence on the sensitivity of the assay. The suitability of the primers was evaluated by their ability to form these structures by using the online tool Oligo analyzer 3.1 provided by Integrated DNA Technologies (http://eu.idtdna.com/calc/analyzer). From here on the position of the terminal nucleotide at the 3′ end of a primer is designated n, the following nucleotide positions within the primer from the 3′ end is designated n-1, n-2, n-3 etc.

2.3. Preparation of template DNA

Purified *Mollicute* genomic DNA stocks were obtained from *Mycoplasma* Biosafety Services GmbH. The genomic copy (GC) numbers were calibrated by the supplier to 100,000 GC / μL by Pico Green measurement of the DNA concentration. DNA from the following species were purchased: *M. pneumoniae FHT* (*M. pneumoniae*), *Mycoplasma gallisepticum* PG31^T (*M.* gallisepticum), *Mycoplasma fermentans* PG18^T (*M. fermentans*), *Mycoplasma arginini* G230^T (*M. arginini*), *Mycoplasma hyorhinis* BTS7^T (*M. hyorhinis*), *Mycoplasma orale* CH19299^T (*M. orale*), *Mycoplasma synoviae* WVU 1853^T (*M. synoviae*), *Acholeplasma laidlawii* PG8^T (*A. laidlawii*) and *Spiroplasma citri* R8-A2^T (*S. citri*).

Purified non-*Mollicute* genomic DNA was obtained from LGC Standards GmbH. The GC number was determined in house by measuring the DNA concentration using the Qubit® 3.0 fluorometer (Life Technologies) and calculating the copy number from the genome weight of the respective species. DNA from the following species were purchased: *Bacillus subtilis* ATCC-23857D-5 (*B. subtilis*), *Bacillus cereus* ATCC-14579D-5 (*B. cereus*), *Streptococcus agalactiae* ATCC-BAA-611D-5 (*S. agalactiae*), *Streptococcus pyogenes* ATCC-700294D-5 (*S. pyogenes*), *Clostridium sporogenes* ATCC-11437D-5 (*C. sporogenes*), *Clostridium* perfringens ATCC-13124D-5 (*C.* perfringens), *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC-BAA-365D (*L. delbrueckii*) and *Escherichia coli* strain ATCC-8739D-5 (*E. coli*). Linear double stranded DNA gene fragments representing a 1000 basepair sequence of the 16S rRNA gene from *Clostridium* sp. *DMHC10* (*C. DMHC10*) and *Lactobacillus plantarum strain LS8 (L. plantarum)* were synthesized by Eurofins Genomics (Germany), since we were unable to obtain genomic DNA from these particular species. For *C. DMHC10* and *L. plantarum* the 16S rRNA gene sequences were copied from the GenBank accession numbers GU727851 and JQ236618 respectively. The gene fragment copy number was determined in house by measuring the DNA concentration using the Qubit® 3.0 fluorometer and calculating the copy number from the weight of a single gene fragment. The primer target sequences were contained within the 1000 base pairs.

2.4. qPCR

qPCR was performed using the LightCycler® 480 system (Roche), using SYBR® Select Master Mix (Life Technologies). The following temperature profile was used: one initial step of 2 min at 50 °C and