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Cross comparison of rapid mycoplasma detection platforms

Bill Lawrence*, Houman Bashiri, Houman Dehghani

Biosafety Development Laboratory, Cellular Resources, Amgen, 1201 Amgen Court West, Seattle, WA 98119, USA

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ABSTRACT

The use of animal and plant derived raw materials in mammalian cell culture processes may provide a possible route of entry for adventitious contaminants such as mycoplasma. Mycoplasma contaminations of cell culture represent a serious challenge to the production of biotechnology derived therapeutics. The slow growing nature of mycoplasma can disguise their infection of cultures since cells may continue to proliferate, though at reduced levels and with lesser output of engineered protein. Rapid identification of *mycoplasma* contaminated cell cultures and materials enables a faster response time to prevent the spread of the contamination. We describe here the comparison of different mycoplasma detection methods: two nucleic acid-based technologies, the standard mycoplasma culture procedure, and a hybrid culture-quantitative PCR assay. In this study, a cell line infected with two species of mycoplasma was used to compare the different detection methods. Our data demonstrates that the two nucleic acid-based techniques are robust methods for detection of mycoplasma and have similar detection capability. In contrast, no mycoplasma was detected in the standard culture assay or in a hybrid culture-quantitative PCR assay. This shows a potential limitation of the culture assay that relies on the ability of mycoplasma to grow in broth media.

1. Introduction

Among the measures taken to assure safety of biotechnology derived therapeutics produced in mammalian cell culture systems is testing for pathogens using orthogonal measures. These measures include comprehensive characterization and testing of the cell substrates from which such therapeutics are derived. Lot-to-lot quality control testing for detection of adventitious microbial contaminants and process characterization, for inherent capacity to remove or inactivate adventitious contaminants such as viruses are two additional measures [1]. Further safety assurance in the production of biotechnology derived therapeutics is provided through strict adherence to current good manufacturing practice (cGMP) guidelines.

The standard culture method for the detection of mycoplasmas in protein therapeutics produced in mammalian cell culture systems is a requirement for the release of biotechnology derived therapeutics (biotherapeutics) and is defined in various regulatory guidelines [2–4]. This broth-culture testing method requires twenty-eight days and several passages for mycoplasma determination. This length of time for detection of potential mycoplasma contaminants can lead to further spread of the contamination to other cell lines or exposure of down-stream purification processing equipment and suites. The use of rapid nucleic acid-based detection technologies provides the ability to quickly respond to a mycoplasma contamination. Hence, this study focused on two rapid nucleic acid-based mycoplasma detection platforms compared to the standard culture-based assay and a hybrid culture-quantitative polymerase chain reaction (PCR) assay. The first nucleic acid-based method, a SYBR green quantitative PCR technique, enables the detection of more than ninety mycoplasma species (including the *mollicutes Spiroplasma citri* and *Acholeoplasma laidlawii*) with a final output that can be quantitative but is not species specific. The second nucleic acid-based method utilizes a combination of end-point PCR using multiple primer sets followed by electrospray ionization mass spectrometry (ESI-MS) to determine the base composition of the PCR amplicons and analysis of the base composition using proprietary software to give a final genus/species determination and quantification of the *mycoplasma* species. The latter platform is also capable of distinguishing multiple species in a single sample. Both of these nucleic acid-based techniques were compared to the standard culture assay and a hybrid culture-quantitative PCR assay method.

Abbreviations: cGMP, Current good manufacturing practice; CHO, Chinese Hamster Ovary cells; CRO, Contract Research Organization; C_T, Cycle Threshold; EP, European Pharmacopeia; ESI-MS, Electrospray ionization mass spectrometry; FDA, Federal Food and Drug Administration; NAT, Nucleic Acid Technology; PCR, Polymerase Chain Reaction; qPCR, Quantitative Polymerase Chain Reaction.

* Corresponding author. Tel.: +1 (206) 265 8644; fax: +1 (206) 217 0492.
E-mail address: bill.lawrence@amgen.com (B. Lawrence).

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2. Materials and methods

2.1. Cell lines

2.1.1. Mycoplasma contaminated human natural killer-like cell line (NKm)

Human NKm cells banked from an original vial of YTN17 cells [5,6] were used in this study. YTN17 cells were contaminated at some point during routine maintenance and were later confirmed by qPCR to be *mycoplasma* contaminated. These *mycoplasma*-contaminated cells were cryopreserved as a positive control source for mycoplasma detection method development. *Mycoplasma* contaminated NK cells (NKm) cells were propagated in RPMI 1640-based media supplemented with appropriate growth promotion factors, and were grown without antibiotic. The NKm cells were grown to a density of 1×10^6 cells/mL at 37 °C and 5% CO_2.

2.1.2. Chinese hamster ovary cell line (CHO)

CHO cells were propagated in DMEM-based media supplemented with appropriate growth promotion factors and were grown without antibiotic. For use in the growth kinetics study (Section 2.4.4) and dilution of NKm cells (Section 2.2), the CHO cells were grown to a density of 1×10^6 cells/mL at 37 °C and 5% CO_2. CHO cells were tested and confirmed to be negative for *mycoplasma* contamination by each of the nucleic acid-based methods being compared.

2.2. Preparation of serially diluted NKm cells in CHO cells

To ascertain relative detection limits, NKm cells were serially diluted ten-fold (Fig. 1) in *mycoplasma*-free CHO cells, and 1 mL aliquots were immediately flash frozen in a dry-ice ethanol bath and transferred to a −70 °C freezer for a minimum of 24 h before DNA extraction or shipment to a contract research organization (CRO) for the twenty-eight day culture assay and the hybrid culture-quantitative PCR assay. Ten-fold serial dilutions were labeled "neat" (undiluted NKm cells) to 9 (the 9th and final serial dilution). Serial dilution of contaminated cells into clean cells was performed to maintain a constant amount of genomic DNA in all samples tested. Excess host genomic DNA is known to potentially interfere with amplification associated with nucleic acid-based detection techniques [7].

2.3. DNA extraction

DNA extraction was performed with the Qiagen (Valencia, CA) QIAamp DNA Mini Kit (Catalog #51306) according to manufacturer's instructions. Briefly, 20 μL of proteinase K was added to 200 μL of cell suspension, followed by the addition of 200 μL of AL lysis buffer. The solution was vortexed briefly and incubated at 56 °C for 10 min. After incubation, 200 μL of 100% ethanol was added to the digestion, and this mixture was loaded into a QIAamp DNA Mini Column. The column with mixture was centrifuged at 8000 RPM for 1 min, and the filtrate was discarded. 500 μL of AW1 wash buffer was added to the column, centrifuged at 8000 RPM for 1 min, and the filtrate discarded. 500 μL of AW2 wash buffer was added to the column, centrifuged at 8000 RPM for 1 min, and the filtrate discarded. The column was then transferred to a new 2 mL collection tube, and spun at 13 000 RPM for 1.5 min; the column was rotated and spun again for 1.5 min at 13,000 RPM. The column was then placed in a new 1.5 mL tube, and allowed to air dry with the top open in a biological safety cabinet for 5 min to remove trace amounts of ethanol. DNA was eluted from the column by adding 200 μL of sterile nuclease-free water directly to the column membrane and was incubated at room temperature for 1 min, and centrifuged at 13,000 RPM for 1 min. The extracted DNA was immediately transferred to −20 °C freezer until ready to proceed with testing.

2.4. Mycoplasma detection methods

2.4.1. Mycoplasma real-time detection kit

The *Mycoplasma* Real-Time Detection Kit (Applied Biosystems, Foster City, CA) is a SYBR green-based quantitative PCR assay using multiplex primers targeted to unique sequences in the *mycoplasma* genome in a single well [8] and was analyzed on an Applied Biosystems 7900 Sequence Detection System according to manufacturer's instruction. SYBR green is a fluorescent dye that intercalates with double stranded DNA. Fluorescence is achieved when the dye is both intercalated with double stranded DNA and is excited by an appropriate light source. As double stranded PCR product accumulates during amplification, so does the fluorescence, allowing for a quantitative measurement of DNA. With the Mycoplasma Real-Time Detection Kit there are three parameters required to reach a positive result: (i) a cycle threshold (C_T) value less than 36, (ii) a dissociation melting temperature between 75 °C and 85 °C, and (iii) a derivative value greater than 0.1. Because the SYBR green dye intercalates with any double stranded DNA, it is

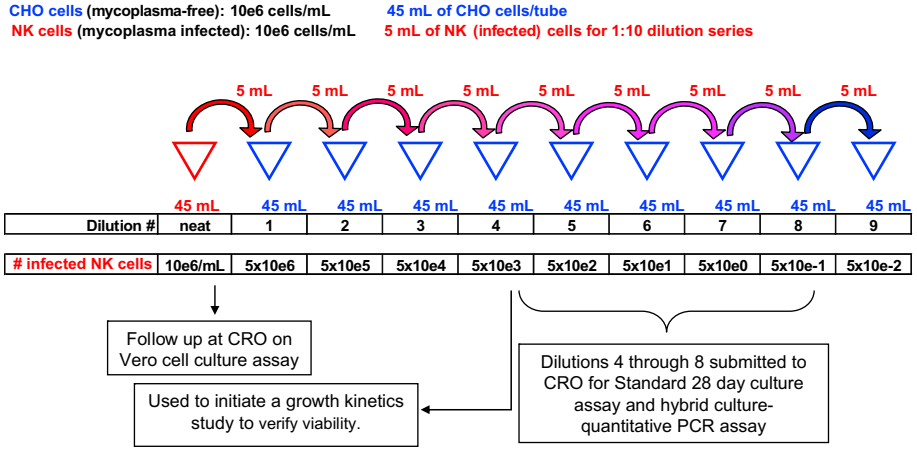

Fig. 1. Dilution series for comparison of mycoplasma DNA extraction techniques.