Simple and sensitive detection of mycoplasma by visual PCR technology

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ABSTRACT

Mycoplasma is a threat to mammalian cell culture for academic research, vaccine manufacture, and antibody production. 5~35% culture cells are mycoplasma contaminated and 80% in some countries. PCR is very sensitive and widely accepted as a standard way to test mycoplasma contamination. However, laborious steps such as sample treatment, DNA extraction and electrophoresis are routinely needed. Visual PCR technology is a novel technology in which PCR amplification is visually monitored by naked eye. There is no post-amplification operation such as pipetting or electrophoresis. Recently, a visual PCR based assay to detect mycoplasma contamination was developed. Its usefulness and sensitivity were verified in this study. In this assay, crude samples such as active cultures, frozen cells stock, medium, FBS, technician throat swabs were directly used as template. It had the desirable high sensitivity (ten copies per PCR). Therefore, this assay involves the least hand-on steps, and represents the simplest PCR procedure to sensitively detect mycoplasma.

INTRODUCTION

Mycoplasma is the smallest bacteria without cell wall[1]. It has long been recognized as common contamination of cell culture and represents a major problem in basic science research. Up to 35% of cell cultures all over the world are contaminated with mycoplasma[1], and 80% in some countries[2]. Reasons for the prevalence of mycoplasma contamination includes: (1) mycoplasma does not cause visible changes of growth medium in turbidity and pH (medium color don't change); (2) it is easily transmit by air (sneeze, cough, aerosol droplets by pipetting, etc.) and 80% of lab technician's throat swabs are mycoplasma positive[3]. Human-sourced species account for 50% of contaminated cultures[1]; (3) it is invisible under microscope even at very high concentration(>10^7cfu/ml); (4) it is resistant to antibiotics such as ampicillin and streptavidin; (5) it does not kill the cells outright; (6) it cannot be removed by 0.2um filter sterilization. Therefore, mycoplasma infected cells may go undetected even for years. It affects various cellular parameters[4-11]. Infected cells still looks healthy and has unapparent change of growth rate and cell morphology, but hundreds of host protein expressions change and NFkB signaling pathway is activated.

There are many mycoplasma detection methods, such as broth culture, bioluminescence, ELISA, fluorescence staining, and PCR. Among them, PCR is the highly sensitive and specific method[12]. Many popular mycoplasma detection products are PCR based, such as: MycoSEQ from Thermo-Fisher, MycoQuick from Systembio, LookOut from Sigma, VenorGem from Sigma, Universal from ATCC, etc. However, all of these PCR kits need DNA extraction or sample pretreatment. Post PCR agarose gel electrophoresis is also required except MycoSEQ (real time PCR). Therefore, all of the current kits has until now been a long and laborious process.

To analyze PCR, agarose gel electrophoresis or real time PCR instruments are employed by all the above kits. Visually detection of PCR amplification is a novel method recently developed by GM Biosciences. In visual PCR, PCR reaction results in color change which is read by naked eyes in visible light without any instrument[13]. There are no post-amplification operations such as pipetting or electrophoresis except observing the color of tubes by unaided eyes. A visual PCR based mycoplasma testing kit is then developed[14]. The purpose of this

study is to verify its usefulness, crude sample resistance and sensitivity.

MATERIAL AND METHODS

Samples: 1ul of cell culture of HEK293, NIH3T3, Jurkat, frozen HeLa cells and complete DMEM culture medium were directly used as template for PCR without any treatment (e.g. DNA extraction, centrifuge, boiling, etc.). The adherent cells of HEK293 and NIH3T3 were not trypsinized, 1ul of cell culture was taken from culture plates directly as PCR templates. The cell culture of non-adherent Jurkat was directly used without spin to remove cells. Frozen HeLa cell was in 90% FCS with 10% DMSO and frozen in liquid nitrogen. Complete DMEM culture medium contains 10% FBS, 1% penicillin-streptomycin and 1% non-essential amino acid. Throat swabs of two technicians were dipped several times by pipette tip, and the tip carried almost invisible (<0.1ul) solution as PCR templates.

PCR: The Visual-PCR™ Mycoplasma Detection kits were from GM Biosciences (Catalog# GM7048). The kits contains 3 vials of premix including all necessary components (buffer, primers, dNTP, DNA polymerases) to perform PCR, and a vial of synthesized 16s RNA gene of Mycoplasma (*M. orale*) for positive control. A complete-PCR-cocktail was prepared by mixing the 3 vials of premix together, and it gave a desired purple color. After aliquoting 24ul of complete-PCR-cocktail for each reaction, 1ul sample or positive control was added into each reaction respectively. The PCR was performed at 95°C 2 min, 60 cycles of 95°C 20 sec and 70 °C 30 sec. After PCR completion, the colors of samples were visually checked and photos were taken.

RESULTS

Visual-PCR based method can sensitively detect mycoplasma. The PCR mixture gives original purple color before initiation of PCR. After PCR completion, positive PCR reaction displayed a blue

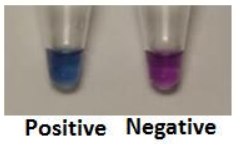

Figure 1: ***Typical color change of Visual-PCR reaction.*** PCR reactions was performed with (positive) or without (negative) template. Synthesized 16s RNA gene of Mycoplasma was used as template.

color whereas negative reaction kept its purple color (Figure 1). It is reported that a typical mycoplasma contaminated cell culture contains 10^6-10^8 copies/ml mycoplasmas[1]. To test whether the Visual-PCR can detect such mycoplasma amounts, a serial of reactions were prepared with different copies of synthesized mycoplasma genes. As shown in Figure 2, the visual-PCR is very sensitive, and as low as 10 copies of spiked mycoplasma 16s genes were detected successfully. The sensitivity is high enough to detect cell cultures for mycoplasma contamination.

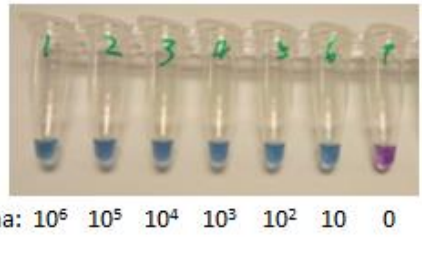

Figure 2. ***Visual-PCR is very sensitive to detect mycoplasma.*** Different copies of synthesized 16s RNA genes of mycoplasma were added into complete-PCR-cocktail, and then PCR was performed. Photo was taken after PCR reaction.

Visual-PCR is tolerant to various culture reagents. Cell culture medium such as DMEM and RPMI 1640 contains phenol red as pH indicators, which may interfere with the color development of visual PCR. Serum, such as fetal bovine serum (FBS)

may also inhibit PCR amplification. To see whether visual PCR is tolerant to phenol red and serum, the cell culture medium DMEM, RPMI 1640 and FBS were spiked with 1000 copies of mycoplasma 16s genes and directly used as PCR templates. As shown in Figure 3, striking color changes were developed in each group between positive samples and negative samples. Samples with templates were blue color, whereas non-template controls were purple color. The visual-PCR was resistant to cell culture media and FBS, and successfully detected 1000 copies of mycoplasma genes which is equivalent to a typical contaminated cell culture containing 10^6 copies mycoplasmas per ml[1].

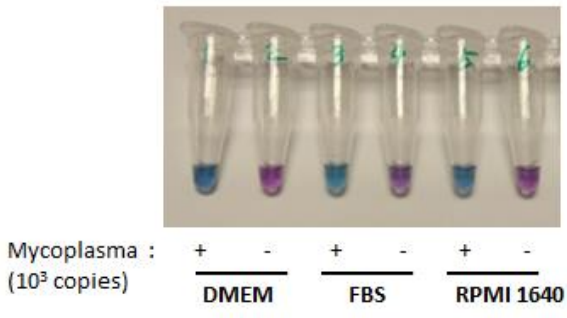

Figure 3. ***Visual-PCR is tolerant to cell culture reagents.*** Culture medium DMEM, RPMI-1640 and FBS (fetal bovine serum) spiked with or without mycoplasma 16s genes were directly used as PCR templates. 1ul of DMEM, FBS or RPMI-1640 was added into 24ul complete-PCR-cocktail. PCR was then performed. Post-PCR tubes were pictured.

Detection of mycoplasma in various crude samples. Encouraged by the superb sensitivity and resistance to serum, a variety of crude samples were further tested. Samples were obtained from a lab with suspicious mycoplasma contamination. For several months, the lab had suffered inconsistent data and failed to repeat data from publications. Cell cultures of adherent and non-adherent cells, frozen cell line, complete DMEM culture medium and throat swab of technicians were collected and tested for mycoplasma contamination using the Visual-PCR™ Mycoplasma Detection kit. These crude samples were directly used as PCR templates without pretreatment or DNA extraction. As shown in Figure 4, mycoplasmas were detected in three cell cultures (#1,2,3), throat swabs of two technicians (#5,6) and frozen cells (#7). Of the 7 samples tested, only the DMEM complete medium (#4) was absent of mycoplasma. This result verified that visual-PCR based assay is tolerant to various crude samples. It is very efficient in testing mycoplasma contamination in variety of crude samples. The mycoplasma contamination found in all of cell lines (3 active culture and 1 frozen) explains the reason that the lab continuously obtained inconsistent data.

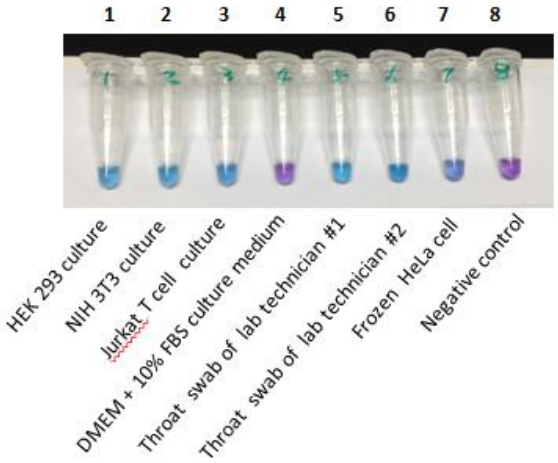

Figure 4. ***Mycoplasma testing of a variety of crude samples from a suspicious laboratory.*** A variety of samples from a suspicious laboratory were tested for mycoplasma contamination by Visual-PCR Mycoplasma Detection Kit. Crude samples were directly used as PCR templates without DNA extraction or sample pretreatment. After PCR reaction, the photo was taken. Negative control was not added any sample or template.

DISCUSSIONS

The widespread presence of mycoplasma in laboratory requires routine screening to minimize the mycoplasma contamination in cell cultures. PCR plays a pivot role in diagnostics of mycoplasma. However, current PCR based methods not only rely on laborious sample pretreatment procedure (e.g. DNA extraction, centrifuge, boiling), but also need to run agarose gel electrophoresis or expensive instrument to detect the amplified products. All of

commercial kits in current use do not work for crude samples such as serum and cultures. In these kits, either boiling/ centrifuge procedure is necessary to remove PCR inhibitions, or DNA extraction is recommended. After PCR, gel electrophoresis is generally used to detect PCR product. Therefore, the limitations of incompatible to crude samples and laborious gel electrophoresis prevent PCR from routine screening purpose.

Visual-PCR™ Mycoplasma Detection Kit was verified in this study to be a simple, quick and sensitive way to visually detect mycoplasma. It bases on GM Biosciences' proprietary Visual-PCR technology which, for the very first time, gives a simple way to analyze PCR results through sharp color-change observed by unaided eyes, and makes post-PCR agarose gel electrophoresis unnecessary. In addition, sample pretreatment or DNA extraction is not required. A variety of crude samples can be directly used as PCR template, such as adherent or non-adherent cell cultures, frozen cell lines, DMEM, RPMI-1640, FBS, frozen cell lines and throat-swab. It is verified in this study that Visual-PCR™ Mycoplasma Detection Kit can detect mycoplasmas as low as 10 copies per reaction. Benefited from the Visual-PCR technology, routine screening for mycoplasma in cell cultures becomes as simple as pipetting samples. Therefore, PCR routine screening of mycoplasma contamination, for the first time, becomes practical and feasible.

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