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IDENTIFICATION OF BACTERIA CONTAMINATION IN ARPE-19 CELL CULTURE USING 16S rRNA AMPLICON SEQUENCING

Eunice Cheah Yi Theng, Yam Hok Chai, Okechukwu Patrick Nwabueze, Tan Yong Hui*

Department of Biotechnology, Faculty of Applied Science, UCSI University, 56000 Cheras, Kuala Lumpur, Malaysia

*Corresponding Author: tanyh@ucsiuniversity.edu.my

SHORT COMMUNICATION

Keywords:

16S rRNA; Antibiotic treatment; ARPE-19; Cell culture contamination; Sphingomonas sp.

Abstract

ARPE-19, a spontaneously arising human retinal pigment epithelium cell line serves as a useful model for a visual-related study. While maintaining healthy and viable APRE-19 is challenging, researchers worried most is the culture contamination. Appropriate antibiotic treatment could be carried out to save the cells and time for an experiment. However, many times the contaminants in the cell culture were not identified. Hence, the present study identifies the microorganism that contaminated the ARPE-19 cells via culture media changes and 16S rRNA amplicon sequencing. Upon cell revival from cryovial, the cells were observed daily under an inverted microscope. On day 3, white translucent biofilm and turbidity were observed in the culture media coupled with interesting observation where media became viscous and molten form. The culture media was collected and centrifuged to obtain a cell pellet for DNA extraction using the Macherey-Nagel NucleoSpin Microbial DNA Mini kit. The 16S rRNA amplicon sequencing was run on the Illumina MiSeq platform using 300 PE. Microscopic observation revealed the contamination was from a rod-shaped bacterium. This result was compatible with the NGS data that the contaminant was *Sphingomonas* sp. This paper serves as a reference for quick identification of *Spingomonas* sp contamination in cell culture. To our knowledge, this is the first report of *Sphingomonas* sp. contamination in ARPE-19 cells upon shipment arrival.

INTRODUCTION

Human retinal pigment epithelial cell line, ARPE-19, plays an important physiological role in retinal homeostasis from forming the blood-retinal barrier, nutrient delivery, waste disposal, phagocytosis of photoreceptor outer segments, synthesis and release of growth factors, and isomerization of retinal during the visual cycle [1,2]. This made it a useful cell model for *in vitro* studies of cellular and molecular mechanisms related to diabetic retinopathy and age-related macular degeneration [3]. Researchers fear most in cell culture work is none other than mycoplasma and bacteria contaminations. A common practice to counteract bacteria contamination in cell culture is by using antibiotic treatment to prevent cell death. However, antibiotics must be used with caution to avoid morphological and gene expression changes that could result in discrepancies to their original state, hence influencing assay results [4]. Common bacterial contaminants in cell culture are *Escherichia coli*, *Bacillus* sp. *Enterococcus sp., Staphylococcus sp.*, and *Kluyveravulneris* [5]. So far, there have been limited articles on *Sphingomonas* sp. as a cell culture contaminant. The only study was by Asghar *et al.* [6], who found the presence of *Sphingomonas* sp. as a cell culture contamination in Madin Darby Bovine Kidney (MDBK), ZZ-R 127, HeLa 293-T, and A-549 cells. This article features the rapid identification of *Sphingomonas* sp based on changes in the culture media and 16s rRNA amplicon sequencing.

MATERIALS AND METHODS

Materials

ARPE-19 (CRL-2302™) cells, DMEM: F12 media and foetal bovine serum (FBS) were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, United States). Trypsin-EDTA solution and Penicillin-Streptomycin Solution were obtained from ThermoFisher Scientific (Waltham, MA, United States). Gentamycin Sulphate was sourced from Sigma-Aldrich (St. Louis, MO, United States).

Methods

Cell Culture Thawing and Maintenance

The culture protocol was according to the ATCC recommendation, where ARPE-19 cells were cultured in complete growth media containing DMEM: F12 basal media and 10% FBS. Cells were incubated in a humidified incubator at 37 °C and 5% CO_2. The cell was thawed immediately after arriving in the lab. The vial of cryopreserved ARPE-19 cells was thawed for 2 minutes in a 37 °C water bath until ice crystals disappeared. The vial was sprayed with 70% ethanol before being brought into the Level 2 laminar hood. The contents of the vials were transferred into a centrifuge tube containing 9 ml of complete growth media and centrifuged at 125 x g for 10 minutes to remove the cryoprotectant agent. The cells were resuspended in 2 ml of complete media and transferred into a T-25 flask containing 3 ml of complete growth medium and mixed by gentle rocking. The condition of the cells and media was monitored every day under an inverted microscope. Subculturing of the cell was done when cell reached 70%-80% of confluency. For subculturing, the flask was first rinsed with 0.05% trypsin-EDTA. Then, the cells were dissociated by incubation at 37 ºC with 0.25% of typsin-EDTA for 15mins. Complete growth media was added to the cell suspension and centrifuged at 125 x g for 10 mins. After centrifugation, the supernatant was discarded, and new complete growth media were used to resuspend the cells. The cell suspension was divided into a ratio of 1:3.

Antibiotic Treatment

Antibiotics of penicillin-streptomycin and gentamicin sulfate were used up to a concentration of 5% and 2% respectively. Treatment with 1% Penicillin-streptomycin began immediately after bacteria contamination was discovered on day 3 of culture and increased to 2% on day 14. In the meantime, while new fresh media containing 2% pen-strep were regularly replaced, an additional 5% pen-strep treatment which is a combination of basal media with 5% pen-strep were carried out on day 28. After discarding spent media, the 5% pen-strep basal media were added into the flask to immerse the cells for 1 minute before discarding and adding in fresh complete growth media. On day 40, a switch between a choice of antibiotics was made. A complete growth media of 1% gentamicin sulfate were first introduced to the culture followed by an increase to 2%.

DNA Extraction and Quality Control

Contaminated spent growth media on day 3 and day 23 were kept for bacteria identification using 16S rRNA amplicon sequencing. They were labelled as cell culture 1 and 2, respectively. The bacteria DNA was extracted using the Macherey-Nagel NucleoSpin Microbial DNA Mini kit (Düren, Germany). Extraction methods were performed according to the manufacturer's guidelines. Extracted samples were kept in a -20ºC freezer before being delivered for 16S rRNA amplicon sequencing, a service provided by Apical Scientific Sdn Bhd, Malaysia. Quality control of the genomic DNA samples was conducted using 1% TAE agarose gel. The concentration of the DNA was measured using Implen NanoPhotometer N60/N50 spectrophotometer and fluorometric quantification using iQuant Broad Range dsDNA Quantification Kit.

16S rRNA V3 and V4 Amplicon PCR Quality Control and Library Construction

The genomic DNA samples were proceeded to amplicon PCR QC using locus-specific sequence primers namely: 16S rRNA V3-V4 Forward: CCTACGGGNGGCWGCAG and Reverse: GACTACHVGGGTATCTAATCC. PCR reactions were carried out with the 1st BASE REDiant 2x PCR master mix (Selangor, Malaysia). The amplicon library was prepared using 2-Step PCR according to Illumina's 16S metagenomic library preparation guideline using KOD-Multi & Epi (Toyobo) and 16S rRNA V3-V4 locus-specific sequence primers with overhang adapters:
Forward:
5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG3'
reverse:
5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACG3'
In the 2nd stage of PCR, dual indices were attached to the amplicon PCR using Illumina Nextera XT Index kit v2 and the quality of the libraries was measured using Agilent Bioanalyzer 2100 System (Santa Clara, California, United States) by Agilent DNA 1000 kit and quantified fluorometrically by Helixyte Green Quantifying reagent. The libraries were then subjected to next-generation sequencing using the Illumina MiSeq platform. (San Diego, California, United States).

Bioinformatic

Sequence adapters and low-quality reads were removed from the paired end reads before the first 200 000 raw reads were extracted out using BBTools (DOE Joint Genome Institute). The forward and reverse reads were merged using QIIME. The error reads, low quality regions, and chimera errors were removed and corrected with the DADA2 pipeline (https://benjjneb.github.io/dada2/). The resulting data was in the form of amplicon sequence variant (ASV) and was proceeded with the taxonomic classification by using scikit-learn (https://scikit-learn.org/stable/) and Naive Bayes classifier against database SILVA (release 132). Statistical analysis was performed in R Studio version 3.6.2 by using phyloseq (https://www.bioconductor.org/packages/release/bioc/html/phyloseq.html).

RESULTS AND DISCUSSION

Observation

Contamination was observed with a visible layer biofilm and media turn molten on day 3 after cell revival from cryovial. A slippery flask surface with a turbid orange-pink colour culture media was observed (Figure 1). The antibiotic treatment was deemed futile. Under an inverted microscope, the presence of rod-shaped bacteria as shown in Figure 2, was visibly swimming actively. After two months of persistent antibiotics treatment, cells were only able to be cultured up to four passages. Eventually, a slow decline in cell growth where a diminishing number of epithelial cells and an increase of visible cell debris (Figure 3) was observed before complete cell death.

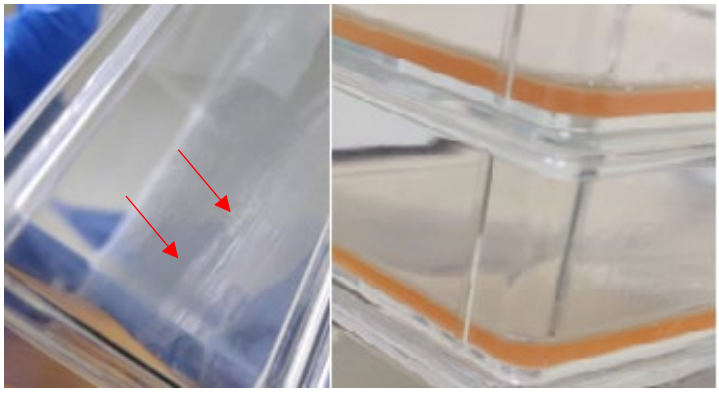

Figure 1. Observation on day 3 upon cell revival. Image on the left indicates the molten form, slippery surface of the culture flask, image on the right shows the contaminated DMEM:F12k media turn to turbid orange-pink color.

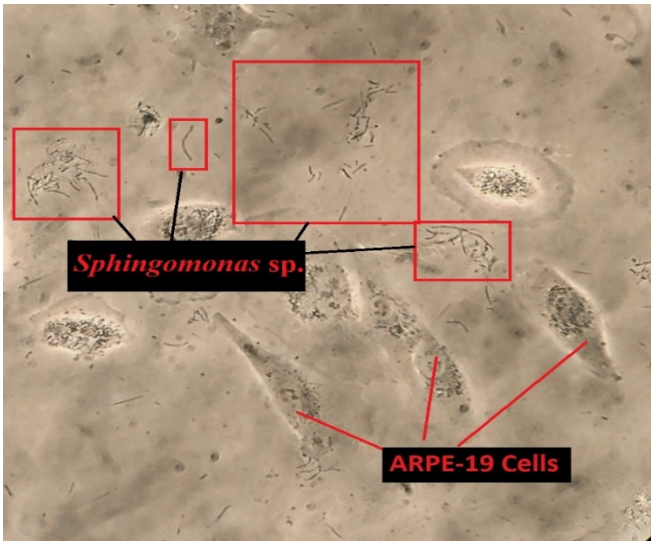

Figure 2. Rod-shaped of *Sphingomonas* sp. can be seen either in clumps or individually propelling across the cells. (400X)

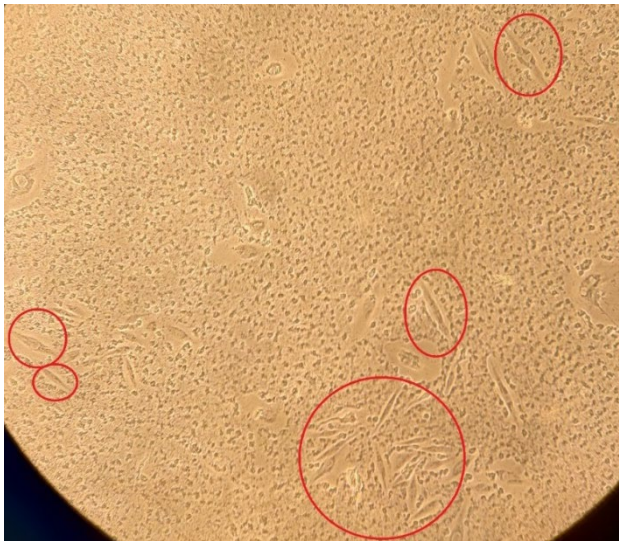

Figure 3. Culture observation on day 29. The circle in red indicates the few visible ARPE-19 cells which were surrounded by cell debris.

Generally, cell culture contamination can be observed through changes in media colour, which denotes a drop in pH, and turbidity of the culture media. The movement of bacterial contaminants can be detected by a quick observation using microscopic instruments as well [7]. In the present case, turbidity in culture media was often observed though there weren't any changes to media pH. Various efforts such as changing of media daily and the increase of antibiotic concentration in hopes to eliminate the bacteria had been deemed unsuccessful. Moreover, the switch of antibiotic to gentamicin sulphate had shown not much of an improvement either. This indicates a possibility of *Sphingomonas* sp. producing resistance towards the maximum dose of 100 IU/ml penicillin, 100 µg/ml streptomycin, and 50µg/ml gentamicin sulfate [5]. Besides antibiotic resistance, the cause of cell death could be due to the increasing antibiotic dosage. It is expected that the high dose of antibiotics could cause cytotoxic effects to most mammalian cells. The bactericidal effects of antibiotics can drive cells into oxidative stress-induced apoptosis mechanism [8]. Therefore, the use of suitable antibiotics with the lowest recommended dose possible is advisable. Currently, the top effective antibiotics classes against most *Sphingomonas* sp. are tetracycline: doxycycline; aminoglycosides: amikacin, gentamicin, and carbapenems: panipenem, imipenem [9].

NGS Data Analysis

A total of 112, 719 and 108, 565 duplicated reads were obtained for day 3 and day 23 samples, respectively. Taxonomic assignment revealed that both samples harboured a high dominancy of *Sphingomonas sp* bacterium with abundancies of 0.98 and 1.00, respectively. The 16s rRNA shown a 99.7% similarity to *Sphingomonas sp* which was in good concordance with cell morphology observation. The abundancy values indicated that *Sphingomonas sp* persisted in the cell culture medium for 20 days and very likely it could be the sole contaminant in the ARPE-19 culture. The taxa classification of the bacterium is summarised in Table 1.

Table 1. Taxonomic classification of cell culture contaminants

Phylum	Proteobacteria
Class	α-Proteobacteria
Order	Sphingomonadales
Family	Sphingomonadaceae
Genus	Sphingomonas

Sphingomonas sp., is a Gram-negative, aerobic bacterium that thrives in a variety of natural resources, including diverse types of water bodies, terrestrial ecosystems, and polluted wastewater. One of few well-known contributions is the efforts as rhizobacteria and an endophyte in sustaining soil ecosystem as well as a support to plants growth [10, 11]. Their ability to hyperaccumulating heavy metals contaminants through phytoextraction serves as a potential phytoremediation mechanism in degrading harmful metals in the soil, but also act as a nutrient necessary for the activation of growth hormone. Despite that, only one had been evident as a potential pathogen risk to humans. *Sphingomonas paucimobilis*, formerly known as *Pseudomonas paucimobilis,* is an opportunistic pathogen, causing nosocomial infection to immunocompromised individuals [12]. Though the mortality rate of infected with *S. paucimobilis* is rare, rapid antibiotic treatment is required to prevent worsening of infection.

Despite the precaution taken on maintaining an aseptic condition, there is a slight possibility of contaminants present in solutions. One of it is the use of serum. The serum contains nutrients where the production of growth hormones would speed up the growth of the cell. However, several articles had pointed out animal serum as the possible source of contamination [5,6,13,14]. Thus, to minimize the risk of contamination, serum-free media may be used instead which still contains vital nutrients for cell growth. Nevertheless, the culture of ARPE-19 cells in serum containing media still provides better cell attachment and proliferation results [15]. For that reason, this became an unavoidable risk of contamination.

Adding on to the possible source of contamination is the use of DMEM: F12 media in supporting the growth of the fastidious *Sphingomonas* sp.. This is in relation to the findings of Asghar *et al.* where *Sphingomonas* sp. favours DMEM over bacteria growth media [6]. The main differences between DMEM and DMEM: F12 media is the halved nutrients concentration and the addition of HEPES buffer. Despite of the differences in terms of composition, *Sphingomonas* sp. should hypothetically be able to grow in DMEM: F12 media. Hence, we speculate that all these loopholes could provide an opportunity for *Sphingomonas* sp. to thrive. Bioinformatics analysis had also identified the culprit behind the culture contamination. Thus, preventive measurements focusing on controlling the growth of *Sphingomonas sp* would be a good strategy to mitigate bacterial contamination during ARPE-19 culturing.

Contamination can be relatively challenging and could result in the complete loss of culture. However, it can be avoided, provided the procedures are carried out in an organized and aseptic manner. All in all, the source of contamination had yet to be known. Future research on the susceptibility of *Sphingomonas* sp. to the types of antibiotics is very much needed. Furthermore, the use of mild antibiotics at the start of the culture is also recommended to prevent the outset of contamination to happen.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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