ARTICLE
Journal of Cellular Biochemistry 116:934–942 (2015)

Journal of Cellular Biochemistry

Sphingomonas sp. is a Novel Cell Culture Contaminant

Muhammad Tahir Asghar,[1] K Al-Ghanim,[2] Shahid Mahboob,[2,3] Muhammad Sharif,[4] Jawad Nazir,[5] and Abdul Rauf Shakoori[1,6]*

[1]*School of Biological Sciences, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan*
[2]*Department of Zoology, College of Science, King Saud University, P.O.Box 2455, Riyadh 11451, Saudi Arabia*
[3]*Department of Zoology, GC University, Faisalabad, Pakistan*
[4]*Department of Biology, Syed Babar Ali School of Science and Engineering, Lahore University of Management Sciences, Lahore, Pakistan*
[5]*Department of Microbiology, University of Veterinary and Animal Sciences, Lahore, Pakistan*
[6]*Visiting Professor, Department of Zoology, College of Science, King Saud University, P.O.Box 2455, Riyadh 11451, Saudi Arabia*

ABSTRACT

A novel contaminant was isolated from Madin Darby Bovine Kidney (MDBK) cells. The organism was unable to grow on standard microbiological media by conventional techniques, but grew well in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose concentration. The organism formed a white biofilm on the bottom without any signs of turbidity. Upon genome sequence analysis of 16 S rDNA, the contaminant was identified as *Sphingomonas* sp. Shah, a member of the group α-Proteobacteria. Neutral red dye uptake method confirmed clear cytotoxic potential of the bacterium on A-549 cells. The organism was capable of invading and infecting different mammalian cell lines: MDBK, ZZ-R, 293-T, A549, and HeLa cells. Infected cells showed a variety of cytopathic effects including vacuolation at perinuclear area, cytoplasmic granulation and membrane blebbing. Microscopic analysis of the infected cells revealed the presence of cytoplasmic vacuoles harboring motile organisms. Apparently local serum preparations seem to be the source of this contamination, which is imperceptibly passed on from one culture passage to the other and ultimately leading to serious cytopathic manifestations. J. Cell. Biochem. 116: 934–942, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: CELL CULTURE CONTAMINANT; ALPHA-PROTEOBACTERIA; HeLA CELLS; MDBK CELLS; CYTOPATHIC EFFECTS; APOPTOSIS; MEMBRANE BLEBBING

Since the last two decades, cell culture technology has become a major research tool in biomedical sciences. This technology is extensively applied in production of different health care products like bio-active compounds, pharmaceuticals, and viral vaccines. Cell culture contaminations are serious challenges as they can undermine the experimental results and may lead to wastage of time and resources. Different microorganisms (bacteria, fungi, and yeasts) can easily invade mammalian cells because the cultured cells lack immunological defense mechanism. Among these, Mycoplasmas are considered as the most common and deteriorating cell culture contaminant which were first time discovered in 1956 by Robinson [Rosengarten and Kirchhoff, 1987; Young et al., 2010]. Their smaller size and ability to pass through the membrane filters used to sterilize the cell culture reagents (media, serum, and supplements) made them ideal candidate to invade a variety of cell types. Published reports show that worldwide 5–35% of the cultures have been contaminated with various *Mycoplasma* spp [Uphoff and Drexler, 2011].

About 17 years ago, a new category of alpha-2 subgroup of Proteobacteria was initially isolated from human blood, commercially available cell lines and fetal bovine serum [Kajander and Ciftcioglu, 1998]. These organisms were named as *Nanobacterium sanguineum* referring to its small size and blood habitat [Akerman

Abbreviations: Nu, nucleus; V(s), small vacuoles; V(l), large sized vacuoles; Gr, cytoplasmic granulations.

Conflict of interest: The authors have no conflict of interest to declare.
Grant sponsor: Deanship of Scientific Research, College of Science Research Centre, King Saud University, Riyadh.

*Correspondence to: Dr. Abdul Rauf Shakoori, Distinguished National Professor and Director, School of Biological Sciences, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan.
E-mail: arshakoori.sbs@pu.edu.pk, arshsak.sbs@yahoo.com

Manuscript Received: 13 December 2014; Manuscript Accepted: 16 December 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 5 January 2015
DOI 10.1002/jcb.25044 • © 2015 Wiley Periodicals, Inc.

et al., 1993; Folk, 1993; Ciftcioglu et al., 1999; Kajander, 2006]. These organisms were 0.08 to 0.50 μm in size and were able to pass through 0.20 μm filters. They have special mechanism to invade the mammalian cells and most importantly lead to cytoplasmic vacuolation and unexpected cell lysis. Some extraordinary characteristics of nanobacteria such as very slow growth rate (approximately 3 days doubling time), small size, very thick and flexible cell wall, ability to grow in mammalian cell culture media and extremely resistant to heat and gamma radiation, highlighted their significance as a possible wide spread contaminant in cell culture [Kajander et al., 1997; Kajander and Ciftcioglu, 1998; Kumar et al., 2006]. Heavy contamination of nanobacteria-like particles has also been reported in more than 15 different cell lines such as MDBK, BHK-21, BT, HEP-2, VERO, PK15, MMTV, MDCK, TT, SP2O, PK13, SK6 [Simonetti et al., 2007]. Serum was blamed as a source of these particles. Recently, motile black dots were detected in young adult murine colon epithelial cells which were successfully cultured on bacteriological media plates. Genome amplification of the organism proved it as *Achromobacter*, a Gram negative bacterium that is commonly present in different environments and reported as cell culture contaminant [Gray et al., 2010]. With a growing implication of cell culture technology in biomedical sciences, potential risk of unusual contaminations is also increasing.

Present study describes the isolation of a novel cell culture contaminant from Madin Darby Bovine Kidney (MDBK) cells. Genome sequence data revealed it as *Sphingomonas* sp. Culture characteristics of the isolate have been studied and induction of apoptosis and cytotoxicity of the contaminant on different mammalian cell lines have been evaluated.

MATERIALS AND METHODS

MAMMALIAN CELL LINES

MDBK, ZZ-R (foetal goaty tongue), and A-549 (*Homo sapiens*, human, 58 year old Caucasian male lung carcinoma epithelial cell line) were taken from Department of Microbiology, University of Veterinary and Animal Sciences, Lahore. HeLa (human cervix adenocarcinoma epithelial) cells and 293–7 (Human embryonic kidney epithelial cells) were obtained from Veterinary Research Institute, Lahore. The cells were grown in T-75 cell culture flasks using growth medium: Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (Lot No. 125080, Reference 21063–029, Gibco) supplemented with 10% FBS (Lot No. 1082776, Gibco, USA) and 1% antibiotic-antimycotic solution (Anti-Anti 100X, Lot No. 116260, Reference 15240–062, Gibco). Following trypsinization with trypsin versene solution (Lot No. 1171645, Gibco) using standard protocol [Phelan, 2007], the cells were cultured in a seeding density of 3–4 million cells per flask. The flasks were incubated at 37 °C and 5% CO_2. Confluent monolayer of the cells was achieved within 48–72 h after which growth media were replaced with the maintenance medium: DMEM supplemented with 2% FBS.

PRIMARY ISOLATION OF THE CONTAMINANT

The novel cell culture contaminant was isolated from a previously contaminated MDBK cell line. These cells have shown impaired growth properties. Spent medium from the flasks containing MDBK cells was taken as a source of the contaminant. In a separate vented T-25 flask, 0.5 ml of the inoculum was added to 5 ml of the DMEM without FBS. The flask was incubated at 37 °C and 5% CO_2 for 3 weeks. After this medium was removed and the flask was washed twice with sterile PBS; pH 7.40. The layer (biofilm) formed by the contaminant at the bottom of the flask was scraped with cell scraper. Material thus obtained was pelleted by centrifugation at 12,000x*g* and washed with the PBS to remove residual medium. The pellet was dislodged in 2 ml of the PBS and 100 μl of the material was re-suspended in 5 ml of DMEM taken in a new T-25 flask and incubated under similar conditions for another 3 weeks.

MICROBIOLOGICAL ANALYSIS

The contaminant was allowed to grow in a number of bacteriological media: trypticase soy broth, fluid thioglycolate broth, nutrient agar, blood agar under aerobic and anaerobic conditions. Inoculated plates and tubes were incubated for a maximum of 14 days. For mycoplasma detection separate, PPLO broth and agar was also inoculated with the sample and positive culture.

MOLECULAR CHARACTERIZATION

Pure culture of the contaminant (biofilm formed on the bottom of the flask) was scraped with cell scraper, washed with sterile PBS, re-suspended in 100 μl Milli-Q water and collected in 1.5 ml eppendorf tube and heated at 70 °C for 10 min. One μl of the sample was used as PCR template and added to PCR reaction mixture: Phusion DNA polymerase, 5X Phusion™ HF buffer (Phusion High-Fidelity PCR Kit, Cat No. F-530S, 100 U, Finnzymes, NEB, MA, USA), dNTPs, the conserved primers 8 F (5′AGAGTTTGATCCTGGCTCAG-3′) and 1492 R (5′GGTTACCTTGTTACGACTT-3′) [Weisburg et al., 1991; Amann et al., 1995] and nuclease free water added in the reaction tube to make the final volume up to 20 μl. The reaction was run using the cycling steps: 98 °C for 4 min; 35 cycles each of 12 s at 98 °C, 30 s at 51 °C and 70 s at 72 °C, with a final extension step of 10 min at 72 °C, prior to hold at 4 °C. PCR amplified products (5 μl) were electrophoresed on 1% agarose gel prepared in 1X tris-acetate-EDTA (TAE) buffer and 1 kb ladder (NEB, MA). Amplified PCR products were purified by QIAquick Gel Extraction Kit (Qiagen). The purified product was quantified by NanoDrop (Thermoscientific). The samples having approximate concentration of 30 ng/μl were submitted for DNA sequencing to Macrogen Korea, along with 10 pico-moles of each primer (8F and 1492 R). The sequences obtained from 1492 R primers were converted into its complementary strand sequence through online program (http://reverse-complement.com). The sequence obtained from 8 F primers and reverse complement sequence of 1492 R primers were aligned using NCBI BLAST program (http://blast.ncbi.nlm.nih.gov/) and common sequence was obtained from all samples, which was used for further queries by using Integrated Microbial Genomes (IMG) (http://img.jgi.doe.gov/cgi-bin/w/main.cgi) and Ribosomal Database Project (RDP) http://rdp.cme.msu.edu/), a web-based server containing only 16 S rDNA sequences and results were submitted to NCBI (the GeneBank/NCBI/EMBL/DDBJ accession number is KF420393).

CYTOPATHIC EFFECTS (CPE) ON MAMMALIAN CELLS

Various mammalian cell lines – MDBK, ZZ-R, 293-T and HeLa cells were grown to 80–90% confluency in T-25 flasks. The flasks were fed with 7 ml of the maintenance medium inoculated with 0.5 ml of the purified scraped biofilm (turbidity adjusted to 0.5 McFarland standard units) and placed in a humidified incubator at 37 °C and 5% CO_2. The cultures were examined under phase contrast microscope at 24, 48, and 72 h for morphological changes and CPE. Digital phase contrast images and live videos of all of the inoculated flasks were acquired by 1X-51 inverted microscope attached with CCD SP 480 H color camera (Olympus, Tokyo, Japan).

CYTOTOXICITY ON A-549 CELLS

Neutral red dye uptake assay was employed to measure the cytotoxicity of the contaminant on A-549 cells according to a previously described protocol [Repetto et al., 2008]. A-549 cells were grown in 96-well cell culture plates (Orange Scientific, Braine-l'Alleud, Belgium) to attain 90% confluency within 24 h. In each plate the wells were inoculated with 200 µl of the scrapped contaminant (turbidity adjusted to 0.5 McFarland standard units after diluting in maintenance medium) while the negative control wells were fed with the maintenance medium. Spent medium was discarded and 200 µl of the neutral red medium (DMEM mixed with 50 µg/ml neutral red dye) was added and plates were incubated for another 3 h. After this, the cells were washed with the PBS and destained with 150 µl of the destaining solution (50% H_2O, 49% C_2H_5OH, and 1% CH_3COOH). The plates were shaken on microtiter plate shaker at room temperature for 10 min. Optical density was measured at 540 nm in ELISA plate reader.

RESULTS

CELL CULTURE CONTAMINANT IDENTIFIED AS *SPHINGOMONAS* SP. SHAH

The cell culture contaminant formed a white biofilm in the form of small coccoidal particles on the bottom of flask. It started appearing as small white dots after 48 h of incubation, became clearly visible after 5 days and formed a dense layer after approximately 14 days as shown in the Figure 1. The medium did not show any signs of turbidity after 14 days of incubation but became opaque after scrapping the biofilm formed at the bottom of the flask (Fig. 2). The contaminant did not grow on any of the liquid or solid media used to culture bacteria and mycoplasma species.

After PCR amplification, only a single product of about 1500 base pairs was obtained (Supplementary Figure 1). Sequence results were submitted to NCBI under assigned accession number KF420393. The sequence homology checked with reference to other known sequences using NCBI's BLAST and the RDB showed that it shared a significant level of identity (97%) with unculturable *Sphingobium* (Accession Number: JN860414). According to IMG data base it shared highest identity (98%) with *Sphingomonas* sp. S17 (Accession Number: NZ_AFGG01000052). The organism was denoted as *Sphingomonas* sp. Shah according to SeqMatch from RDP data base with following taxonomic position Phylum: Proteobacteria, Class: α-Proteobacteria, Order: Sphingomonadales, Family: Sphingomonadaceae and Genus: *Sphingomonas*.

The 16 S rRNA gene of cell culture contaminant displayed highest similarity to various unculturable environmental bacteria (more than 95%). Among previously identified and studied bacteria, it exhibited highest homologies of 98% (identity) to both *Sphingomonas* sp. S17 (Accession Number: NZ_AFGG01000052) and to *Sphingomonas paucimobilis* (Accession Number: HF558376.1). Two phylogenetic trees were constructed by neighbor-joining methods (Supplementary Figures 2 and Figure 3).

SPHINGOMONAS SP. SHAH INDUCES APOPTOSIS IN MAMMALIAN CELLS

CPE observed through phase contrast microscopy after 24, 48, and 72 h post-infection unveiled the dramatic modifications in cellular morphology. In the case of MDBK cells, small vacuoles started appearing at the perinuclear regions after 24 h of infection (Fig. 3a), which increased in size and merged after 48 h (Fig. 3b). This resulted into robust growth of the organism in the large sized vacuoles that occupied the entire cell cytoplasm and pushed the nucleus towards

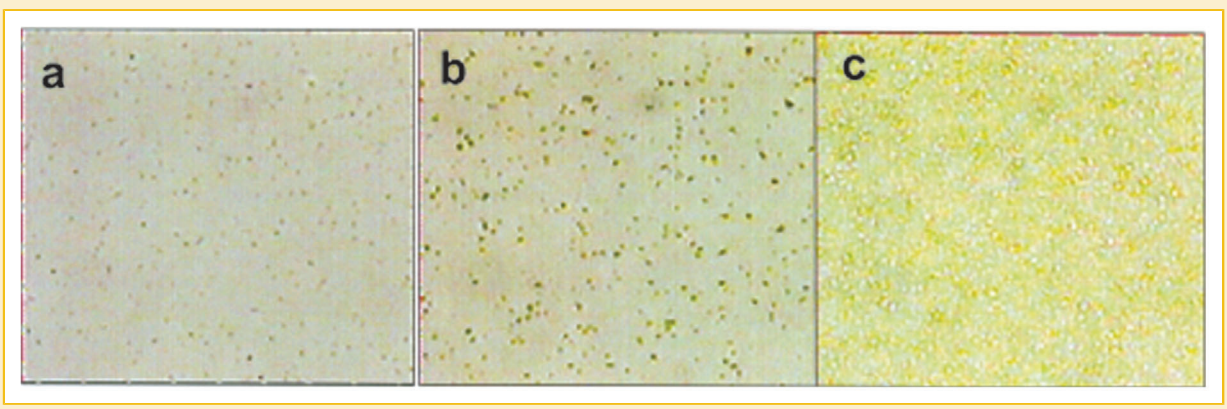

Fig. 1. Microscopic examination of cell culture contaminant: Few intermediate images of the growth phases of Cell Culture Contaminant on day 2 (a) 5 (b) and 14 (c) at 400 × magnification.

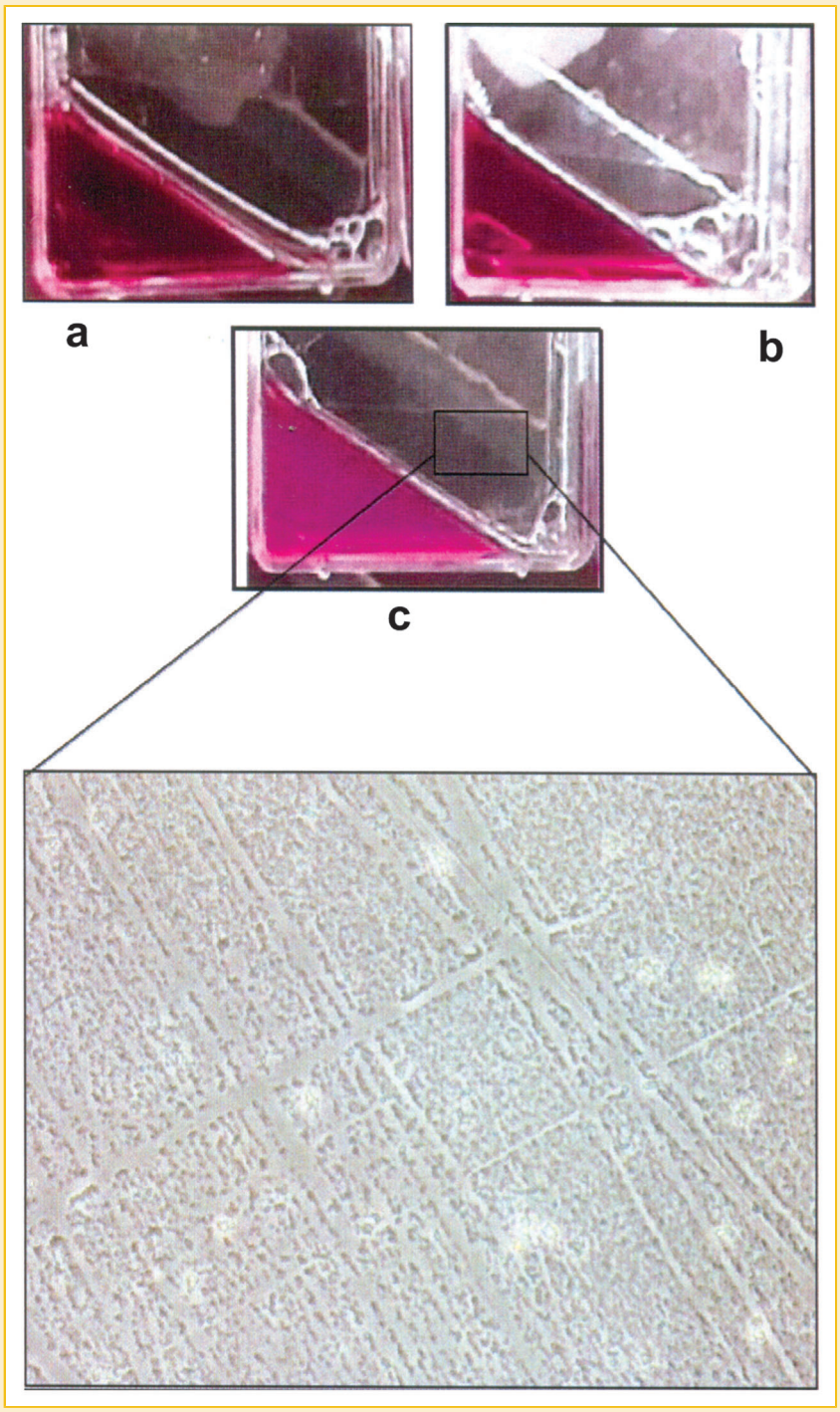

Fig. 2. Growth of cell culture contaminant: Control DMEM medium (a) Cell culture contaminant growing in DMEM after 2 weeks of culture before scraping (b) while after scraping revealed turbidity (c) Micrograph (400 × magnification) of bottom of cell culture flask after scraping.

periphery. Motility of the organisms can be clearly visualized after 48 h within the vacuole (see video S1 in the supplementary material, http://youtu.be/UKee1OIdWeg). Similar morphological changes were observed in the human kidney cell line 293 T (Fig. 4a,b). ZZ-R cells inoculated with *Sphingomonas* sp also developed similar cytopathic changes (Fig. 5a,b; see also video S2 and S3 in the supplementary material, http://youtu.be/QKyoqKFJfUY, http://youtu.be/mCxTtcu_nbA). In few cells, cytoplasmic granulation was also observed that occupied the entire cytoplasm on later stages and disturbed the nuclear boundary and the cells passed on

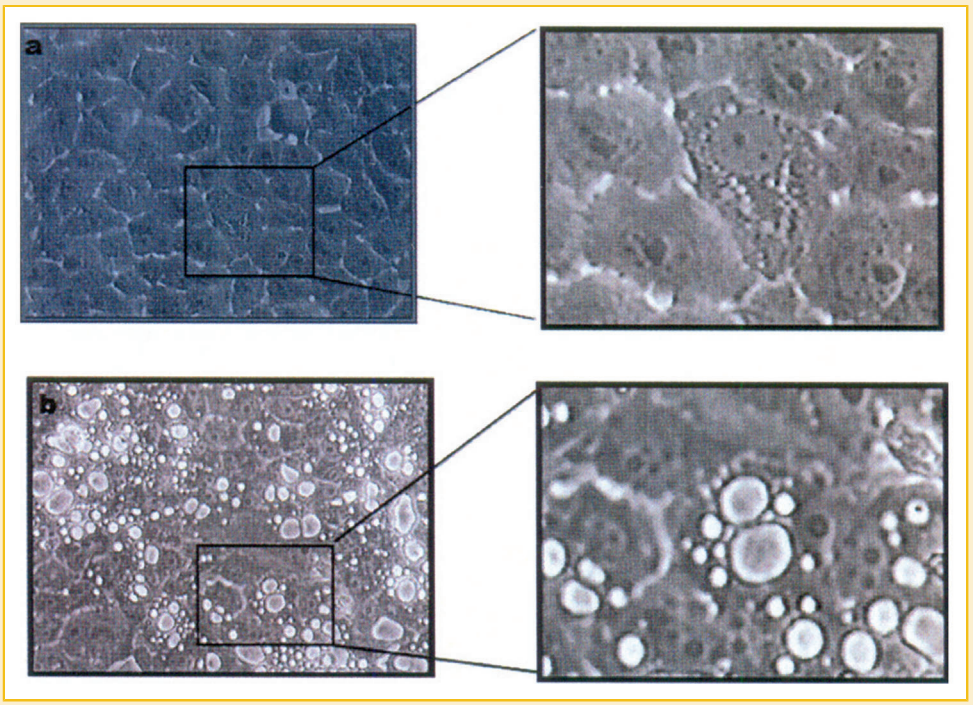

Fig. 3. Cytopathic effects of cell culture contaminant on MDBK Cells: After 24 h of infection, small vacuoles appeared at perinuclear area (a, 200 × magnifications). After 48 h these vacuoles merged and grew in size (b, 200 × magnification).

into the death phase (Fig. 5c). Motility of the organism can be clearly seen within the granulated cytoplasm (see video S4 in the supplementary material, http://youtu.be/egTvU5oEkto).

The *Sphingomonas* sp. Shah was capable of invading and infecting HeLa cells. After 72 h of infection, cells showed blebbing of plasma membrane along with the perinuclear vacuolation. Single or multiple small transluscent bleb(s) containing numerous organisms with visible motility can be seen (Fig. 6a, and Video S5 in the supplementary material, http://youtu.be/wwRmFgxvS6c). At terminal stages these blebs either ruptured to release out the organisms (Fig. 6a/c), or detached from the infected cells (Fig. 6b). Some cells were also sloughed off from the flask surface and found floating in the medium.

CYTOTOXICITY OF *SPHINGOMONAS* SP ON A549 CELL LINE

Cytotoxic effects of *Sphingomonas* sp. on A549 cells were screened by neutral red assay. Loss of cellular viability was evident over time. Ability of the contaminant inoculated A549 cells to uptake neutral red in relation to the un-inoculated cells at various time intervals in shown in Figure 7.

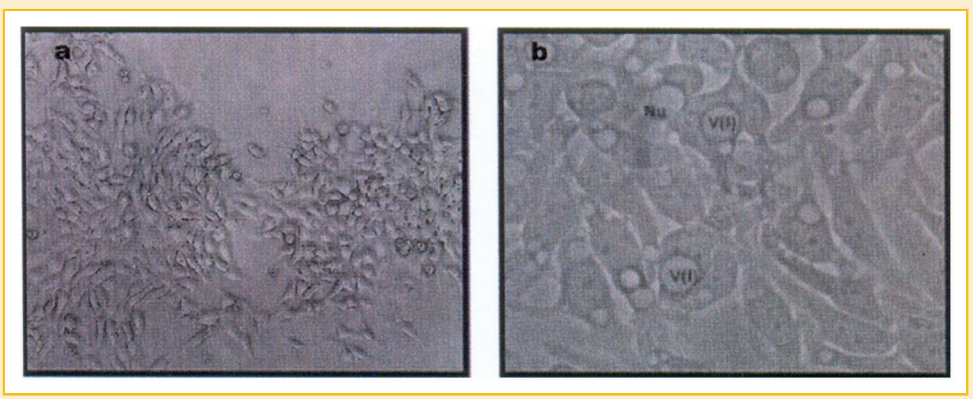

Fig. 4. Cytopathic effects of cell culture contaminant on 293 T Cells: Vacuolation in the cells (a, 100 ×, b, 400×) (Nu: nucleus, V(l); large sized vacuoles).

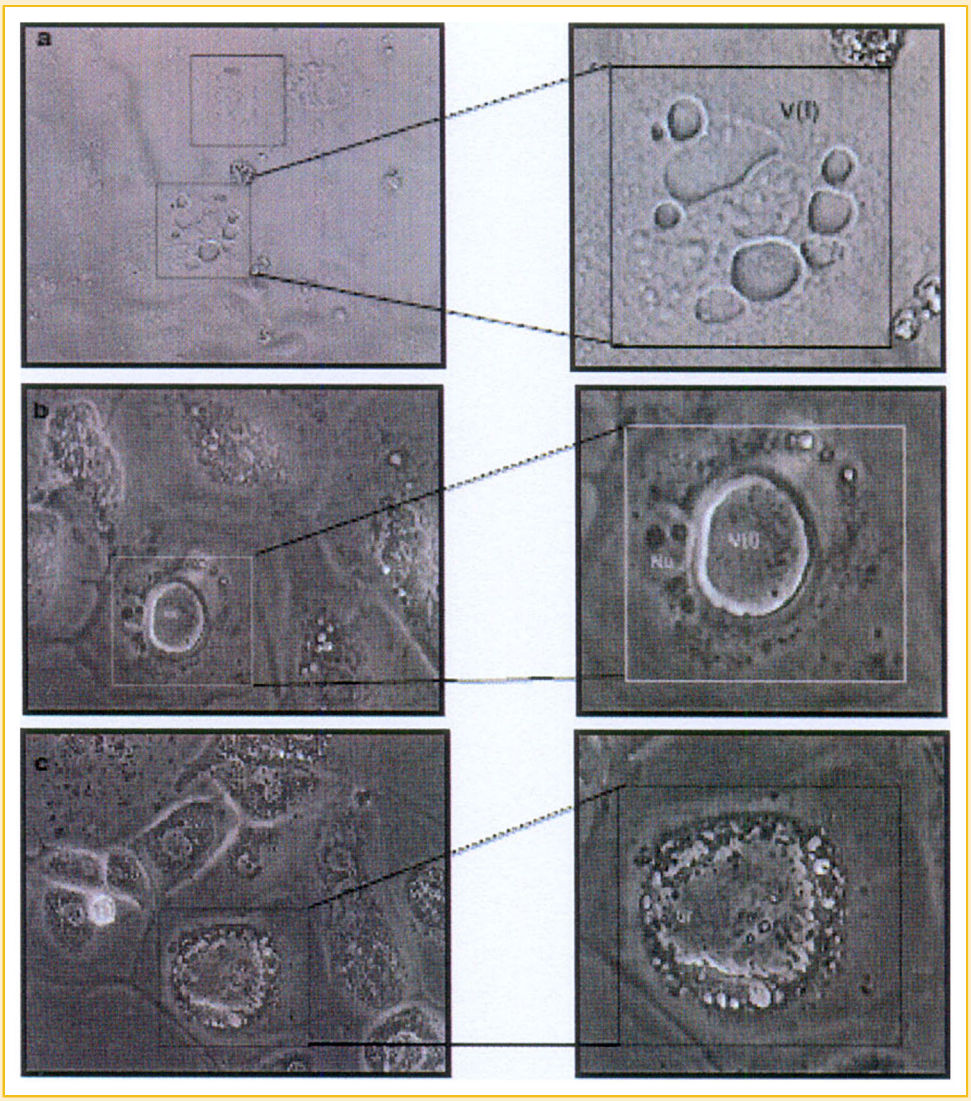

Fig. 5. Cytopathic effects of cell culture contaminant on ZZR Cells*:* After 24 h of infection cell acquired small perinuclear vacuoles (a, taken at 100 × magnification) which become bigger compartments after 48 h and push the nucleus aside (b, captured at 200 × magnification). In some cells the cytoplasm granulation was observed (c, taken at 200 × magnification).

DISCUSSION

A novel cell culture contaminant isolated in the present study showed highest homology to unculturable Sphingomonads (NCBI-BLAST) and *Sphingomonas* sp. S17 (IMG Data Bank) and identified as *Sphingomonas* sp. Shah. *Sphingomonas* are aerobic Gram-negative, oxidase positive and non-fermentative bacteria that are ubiquitous in nature [Ryan and Adley, 2010]. These bacteria have a unique sphingoglycolipid with long chain base dihydrosphingosine, ubiqinon-10 and 2-hyroxymyristic acid [Kawahara et al., 1990]. Inability of the newly identified organism to grow on various bacteriological media and preference to grow on DMEM confirms the fastidious nature of the organism. Ability of the members of the genus *Sphingomonas* to pass through filters of 0.20 μm porosity [Ryan and Adley, 2010] as well as power to infect a number of mammalian cells as evident in present study highlights the significance of the organism as an evolving cell culture contaminant. Currently, *Mycoplasma* spp are supposed to be the most notorious cell culture contaminants that can pass through the membrane filters.

A dense white biofilm at the bottom of the flask (Fig. 2) that can be scraped from the surface indicates sticky nature of the organism which shows its homology with nanobacteria that makes unique calcifying nano-particles after growth on serum free DMEM [Mathew et al., 2008]. *Sphingomonas* sp is prevalent in a variety of water types including ultrapure water and are known to make biofilm in the water distribution system [Ryan and Adley, 2010], while some of the species are recognized as human (*S. paucimobilis*) and plant (*S. meloni*) pathogens [Buonaurio et al., 2002; Maragakis et al., 2009; Meric et al., 2009]. *S. alaskensis* is an ultramicroorganism that is resistant to

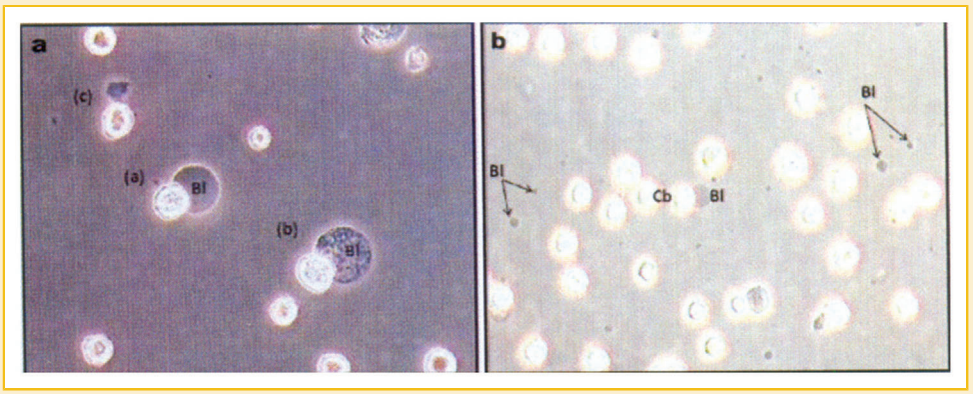

Fig. 6. Cytopathic effects of cell culture contaminant on Hela Cells: Membrane blebbing is prominent feature (a), blebs in progressive phase (a/a) advanced phase (a/b) and ruptured bleb through which the organisms exudes out (a/c), multiple small sized blebs can be visualized in the culture flasks (b) which may detach from the cells. (Cb, cell body; Bl, bleb). All images were taken at 200 × magnification.

various stress inducing agents like UV irradiation, hypersalinity, drastic temperature changes, desiccation, and alkaline pH [Farias et al., 2011]. Certain *Sphingomonas* spp have been involved in death of coral reefs off coast of Florida [Richardson et al., 1998].

Sphingomonas produced a variety of CPE including peri-nuclear vacuolation and membrane blebbing on HeLa cells. After getting entry inside the host cells through unknown mechanism like phagocytosis, endocytosis, receptor mediated endocytosis, and engulfment, the organism affects cellular viability. Initially, nascent vacuole appeared in the peri-nuclear region, containing motile organisms. Some intracellular pathogens (*Listeria monocytogenes* and *Shigella flexneri*) after getting entry into the host cells escape from their resident vacuoles and stay in the cytosol for proper growth and replication [Hybiske and Stephens, 2008]. It was observed that unlike those pathogens, *Sphingomonas* sp persisted in membrane bound vacuoles, which shows its ability to thrive inside the cytoplasmic vacuoles, like *Coxiella burnetii* [Baca et al., 1994]. Enlargement of vacuoles with time suggests progressive addition of membranes, which is consistent with the alteration of membrane trafficking along the endocytic-endosomal pathways [Charras et al., 2006].

The phenomenon of blebbing was much prominent in HeLa cells. Small and large highly transparent blebs showing quick motility of the organism within them (Video S5, http://youtu.be/wwRmFgxvS6c)

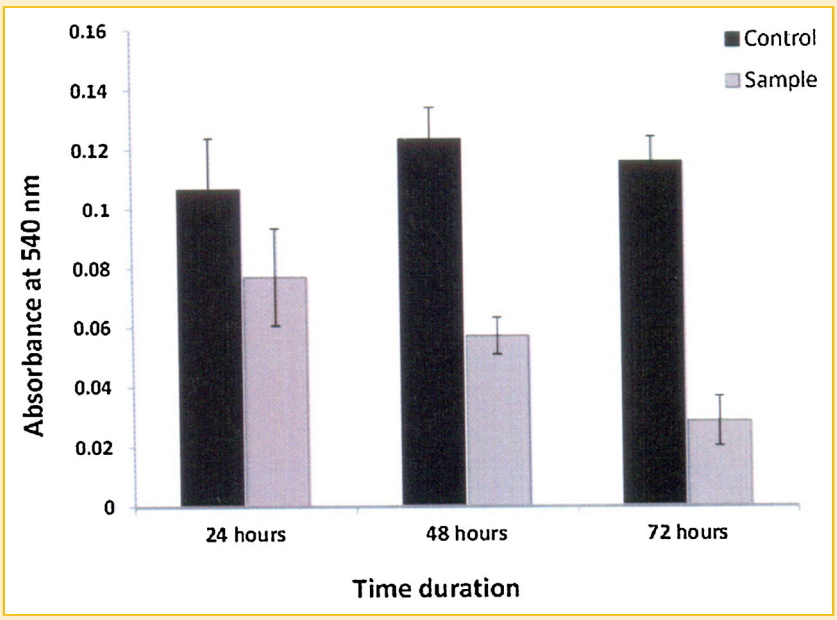

Fig. 7. Kinetics of viability of A549 cells through neutral red dye uptake method following exposure to cell culture contaminant: Mean absorbance of the inoculated and negative control groups recorded at various time intervals using 540 nm filter.

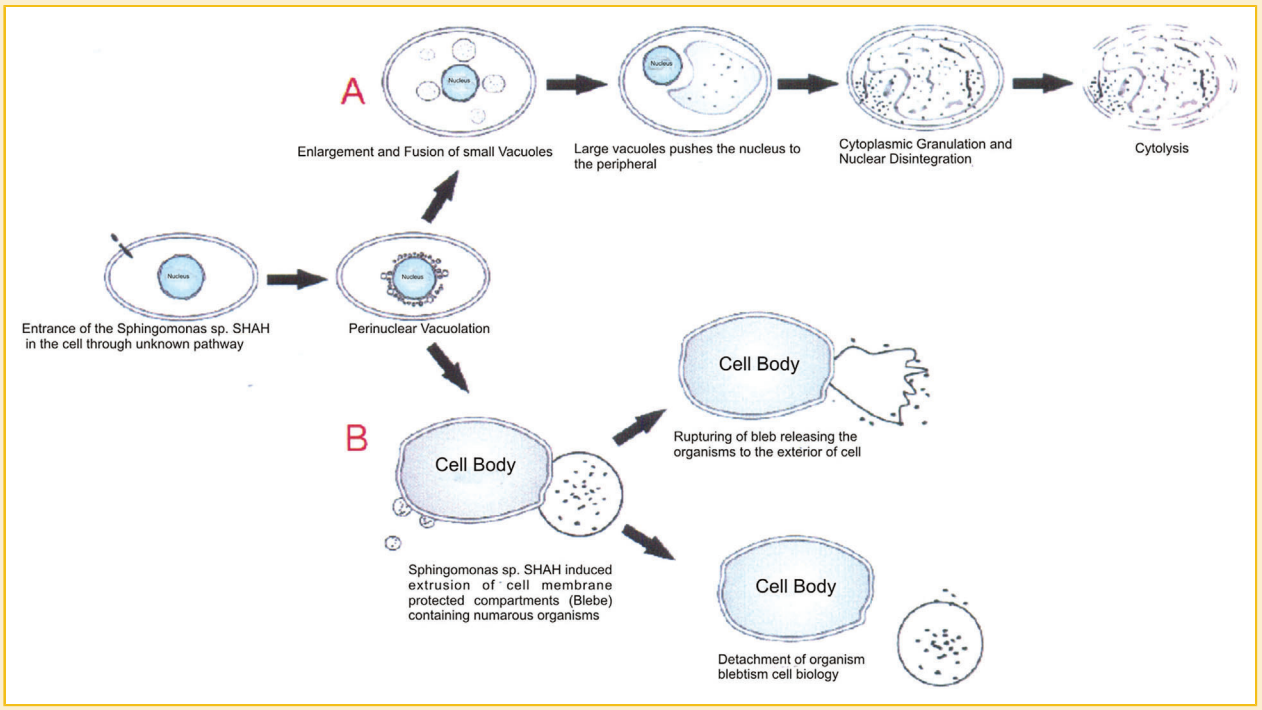

Fig. 8. Mechanisms used by *Sphingomonas* sp Shah to invade and exit from the host cells: cell culture contaminant adopts dual exit strategy from the cell, (A) destructive mode of release (lysis pathway). (B) Packaged release process (blebbing or extrusion). The cytolysis was general phenomenon in MDBK, 293T and ZZ-R cell line, on invasion with cell culture contaminant, However blebbing (extrusion) was prominent in HeLa cell line.

suggested that these blebs lack cytoplasmic cytoskeleton. Visible motility in the real time video excludes the possibility of actin-based motility that is extremely slow and only detectable with the usage of time-lapse video recording [Gaillard et al., 1987; Southwick and Purich, 1998]. Membrane blebbing is also a normal physiological phenomenon as a sequel to apoptosis, but such blebs are relatively small in size and retract rapidly [Charras et al., 2006]. Evidences suggest that type 3 secretary system of *Pseudomonas aeruginosa* can induce the formation of organism containing blebs [Angus et al., 2008]. Likewise, blebbing of HeLa cells was supposed to be induced by *Sphingomonas* sp. to facilitate the exit of the organism from the infected cells and the dispersal of the organism. A proposed mechanism for the invasion of cells by the *Sphingomonas* sp. and release of the organism is shown in Figure 8.

CONCLUSIONS

A novel contaminant isolated from Madin Darby Bovine Kidney (MDBK) cells, which could grow in DMEM containing high glucose concentration, and formed a white biofilm on the bottom without any signs of turbidity was identified as *Sphingomonas* sp. The contaminant had cytotoxic effect on A-549 cells and was capable of invading and infecting different mammalian cell lines: MDBK, ZZ-R, 293-T, A549, and HeLa cells. Microscopic analysis of the infected cells revealed the presence of cytoplasmic vacuoles harboring motile organisms.

ACKNOWLEDGMENT

This project was supported by Deanship of Scientific Research, College of Science Research Centre, King Saud University, Riyadh, through the Research Group Project No. RGP-341.

REFERENCES

Akerman K, Kuronen I, Kajander EO. 1993. Scanning electron microscopy of nanobacteria-novel biofilm producing organisms in blood. Scanning 15:90–91.

Amann RI, Ludwig W, Schleifer KH. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169.

Angus AA, Lee AA, Augustin DK, Lee EJ, Evans DJ, Fleiszig SM. 2008. *Pseudomonas aeruginosa* induces membrane blebs in epithelial cells, which are utilized as a niche for intracellular replication and motility. Infect Immun 76:1992–2001.

Baca OG, Li YP, Kumar H. 1994. Survival of the Q fever agent Coxiella burnetii in the phagolysosome. Trends Microbiol 2:476–480.

Buonaurio R, Stravato VM, Kosako Y, Fujiwara N, Naka T, Kobayashi K, Cappelli C, Yabuuchi E. 2002. *Sphingomonas melonis* sp. nov., a novel pathogen that causes brown spots on yellow Spanish melon fruits. Int J Syst Evol Microbiol 52:2081–2087.

Charras GT, Hu CK, Coughlin M, Mitchison TJ. 2006. Reassembly of contractile actin cortex in cell blebs. J Cell Biol 175:477–490.

Ciftcioglu N, Bjorklund M, Kuorikoski K, Bergstrom K, Kajander EO. 1999. Nanobacteria: An infectious cause for kidney stone formation. Kidney Int 56:1893–1898.

Farias ME, Revale S, Mancini E, Ordonez O, Turjanski A, Cortez N, Vazquez MP. 2011. Genome sequence of *Sphingomonas* sp. S17, isolated from an alkaline, hyperarsenic, and hypersaline volcano-associated lake at high altitude in the Argentinean Puna. J Bact 193:3686–3687.

Folk RL. 1993. SEM imaging of bacteria and nannobecteria in carbonate sediments and rocks. J Sediment Petrol 63:990–999.

Gaillard JL, Berche P, Mounier J, Richard S, Sansonetti P. 1987. *In vitro* model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect Immun 55:2822–2829.

Gray JS, Birmingham JM, Fenton JI. 2010. Got black swimming dots in your cell culture? Identification of *Achromobacter* as a novel cell culture contaminant. Biologicals 38:273–277.

Hybiske K, Stephens RS. 2008. Exit strategies of intracellular pathogens. Nat Rev Microbiol 6:99–110.

Kajander EO, Ciftcioglu N. 1998. Nanobacteria: An alternative mechanism for pathogenic intra and extra cellular calcification and stone formation. Proc Natl Sci USA 95:8274–8279.

Kajander EO, Kuronen I, Akerman K, Pelttari A, Ciftcioglu N. 1997. Nanobacteria from blood, the smallest culturable autonomously replicating agent on earth. Proc SPIE 3111:420–428.

Kajander EO. 2006. Nanobacteria–propagating calcifying nanoparticles. Lett Appl Microbiol 42:549–552.

Kawahara K, Matsuura M, Danbara H. 1990. Chemical structure and biological activity of lipooligosaccharide isolated from *Sphingomonas paucimobilis*, a gram-negative bacterium lacking usual lipopolysaccharide. Jap J Med Sci Biol 43:250.

Kumar V, Farell G, Yu S, Harrington S, Fitzpatrick L, Rzewuska E, Miller VM, Lieske JC. 2006. Cell biology of pathologic renal calcification: Contribution of crystal transcytosis, cell-mediated calcification, and nanoparticles. J Investig Med 54:412–424.

Maragakis LL, Chaiwarith R, Srinivasan A, Torriani FJ, Avdic E, Lee A, Ross TR, Carroll KC, Perl TM. 2009. *Sphingomonas paucimobilis* bloodstream infections associated with contaminated intravenous fentanyl. Emerg Infect Dis 15:12–18.

Mathew G, McKay DS, Ciftcioglu N. 2008. Do blood-borne calcifying nanoparticles self-propagate. Int J Nanomed 3:265–275.

Meric M, Willke A, Kolayli F, Yavuz S, Vahaboglu H. 2009. Water-borne *Sphingomonas paucimobilis* epidemic in an intensive care unit. J Infect 58:253–255.

Phelan MC. 2007. Basic techniques in mammalian cell tissue culture. In: Current protocols cell biology. 36:1.1.1-1.1.18. John Wiley & Sons, Inc.

Repetto G, del Peso A, Zurita JL. 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nat Protoc 3:1125–1131.

Richardson LL, Goldberg WM, Kuta KG, Aronson RB, Smith GW, Ritchie KB, Halas JC, Feingold JS, Miller SL. 1998. Florida's mystery coral-killer identified. Nature 392:557–558.

Rosengarten R, Kirchhoff H. 1987. Gliding motility of *Mycoplasma* sp. nov strain 169:163K–1898.

Ryan MP, Adley CC. 2010. *Sphingomonas paucimobilis:* A persistent Gram-negative nosocomial infectious organism. J Hosp Infect 75:153–157.

Simonetti AB, Englert GE, Campos K, Mergener M, de David C, de Oliveira AP, Roehe PM. 2007. Nanobacteria-like particles: a threat to cell cultures. Brazilian J Microbiol 38:153–158.

Southwick FS, Purich DL. 1998. *Listeria* and *Shigella* actin-based motility in host cells. Trans Am Clin Climatol Assoc 109:160–173.

Uphoff CC, Drexler HG. 2011. Cell culture mycoplasmas. *DSMZ-German Collection* of Microorganisms. Cell Cultures 1–18. (http://www.dsmz.de/fileadmin/Bereiche/HumanandAnimalCell-Lines/WebMycoCell Culture. pdf.).

Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. J Bact 173:697–703.

Young L, Sung J, Stacey G, Masters JR. 2010. Detection of *Mycoplasma* in cell cultures. Nat Protoc 5:929–934.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.