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Molecular detection and characterization of sustainable intracellular contaminants in commercially used cell cultures for pre-clinical studies

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ABSTRACT

Microbial contamination in cell and tissue culture is a constant problem, which can compromise development and applications of cell lines. An immediate consequence of cell culture contamination is loss of researcher time, money and effort spent developing cultures and setting up experiments. There are adverse effects detected on cultures suffering from undetected biological contamination. This hidden contamination can potentially achieve high densities altering the growth and characteristics of the cultures. The objective of this study was to assess the molecular detection and characterization of sustainable intracellular contaminants in commercially used cell cultures use for regulatory pre-clinical studies for developing new chemical entities. This study was prompted by a series of observations by multiple researchers that cell lines were harbouring visible black particulate contaminants, capable of intracellular mobility with secondary impacts on cell adherence and lyses. This was initially limited to human liver cells, HepG2, C3A and $CaCO_2$ cell lines, but recently similar evidence has been found in a series of other cell lines as well. Giemsa staining did show many spore-like entities in the cytoplasm mainly of 0.5-1 µ diameter, rounded and transparent in colour. Electron microscope examination of C3A infected cell line revealed the presence of numerous intracellular bacteria located in vacuoles or free in the host cytoplasm. In addition, the interaction of this bacterium with epithelial cells was associated with the elongation of micro-villar extension that extruded from the host cell membranes and engulfed the bacteria. This internalization mechanism strongly resembles *Salmonella*- or *Shigella*-induced macro-pinocytosis. The strain was characterized using the 16S RNA sequence that amplified the gene from many genera. The closest phylogenetic relative was |HQ877772.1| *Escherichia sp.* A94 with 88% 16S ribosomal RNA gene sequence similarity. It was proposed that unidentified strain be assigned as type strain of species of the bacteria origin based on the 16S rRNA gene sequence search in Ribosomal Database project, small subunit rRNA and large subunit rRNA database together with the phylogenetic tree analysis.

Keywords: Intracellular contaminants, cell cultures, bacteria culture, pre-clinical studies.

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INTRODUCTION

Microbial contamination in cell and tissue culture is a constant problem, which can compromise development and applications of cell lines. An immediate consequence of cell culture contamination is loss of researchers' time, money and effort spent developing cultures and setting up experiments (Kim, 2007; Fogh et al., 2015) Apart from mycoplasma, microbial contamination of cell cultures is usually very detectable and largely concerns media and can be addressed by sanitation procedures. Mammalian cells can host microbes in addition to mycoplasma, in a form that preserves cell survival but seriously damages their expressive behaviour and function is a major concern (Zenk and Hansel, 2009; Everoad et al., 2012). There are adverse effects on cultures suffering from undetected biological contamination. This hidden contamination can potentially achieve high densities altering the growth and characteristics of the cultures (Croxton et al., 2012; Usta et al., 2014).

Tissue culture or cell culture are terms used interchangeably for the process where cells are grown and maintained in a controlled environment such as a laboratory, outside their natural and original source (Gupta et al., 2014). Cell culture is a vital technique in many branches of biological research, from cancer research to vaccine development and therefore a cell culture contaminant can be defined as some element in the culture system that is undesirable because of its possible adverse effects on either the system or its use (Ajibola et al., 2017). '*Contamination cannot be totally eliminated, but it can be managed to reduce both its frequency of occurrence and the seriousness of its consequences'* (Ryan, 2005; Borman et al., 2013).

The presence of microbial contamination- bacterial, fungi, mycoplasma, or protozoa- in cell culture seriously compromises virtually all research or production work involving culture technology (Behrendorff et al., 2013). Although many contamination events are overt and readily apparent, others are insidious and more difficult to detect (Ikonomi, 2006; Ecology 2011). There are many types of contaminants, but all result in cell death and poor culture which cost the laboratory both time and money.

Improvements have been made in conventional culture methodologies, where there have been numerous studies analyzing the use of nucleic acid test for the detection of positive bacterial contamination (Marlowe, 2003; Thomas et al., 2013). Molecular biological methods for detection of nucleic acids have shown to be greater sensitivity than immunological and staining methods (Everoad et al., 2012; Mohammad et al., 2015). Molecular detection methods allows for detection of non-cultural bacteria (Lleo and Canepari, 2005). PCR assays provide fast, dependable and cost-effective methods for quality assessment, ultimately resulting in faster product release and product optimization (Jimenez, 2001; Evaroad et al., 2012). Sequence based molecular technique have been used to characterize bacteria, using 16S rRNA sequence as a common tool for detection of contamination in groundwater and industrial water systems (Grahn et al., 2003; Hay, 2010). Studies by Jamenez et al., (2007), used the highly conserved bacterial ribosomal DNA sequence in PCR-based assay to determine sterility of pharmaceutical samples, The group also reported that conserved eubacterial sequences has been used in clinical and industrial samples for PCR analysis. The study by Nocker (2008), described nucleic acids amplification as a significant improvement in technology for microbial research laboratories and microbial diagnostic industries, due to its utility to be automated.

Scientists must maintain careful aseptic technique and adhere to proper clean and sterile procedures while manipulating cells in order to keep them alive and healthy. Research take multiple steps, including

aseptic culture handling, filter sterilization, purchasing of liquid media and solutions, and inclusion , and inclusion of antibiotics in the growth media, to keep the cultures free of unwanted organism (Degelling et al., 2012; Blazkova et al., 2015). An additional complication arise when researchers use lines that are not commercially available and are often irreplaceable, difficult to obtain, or need re-derivation from primary cells (Jennifer et al., 2010; Jerome et al., 2014). In case of non-commercially available lines, all attempts are usually taken to clean up the culture by selectively killing the contamination without harming the cell line. Often the culprit of culture contamination is mycoplasma, intracellular and bacteria that can be almost undetectable in culture. Approximately 20% of 460 human cell lines surveyed contained mycoplasma (Uphoff and Horn, 2011; Kly, 2013). Numerous commercial kits are available to identify mycoplasma contamination (Garner and Charkraborti, 2000; Fogh et al., 2015) and antibiotic solutions are commercially available to rid cultures of them. Unknown bacterial contamination can be transient, and contamination is common with poor aseptic technique and can be devastating in a research setting (Croxton et al., 2012). In the laboratory contaminants can come from different sources and in some cases from the laboratory coats of researchers, medical personal poor hygiene work conditions, and non-labeling of materials on work surfaces (Zenk and Hansel, 2009; Awodiran et al., 2014).

While many undesirable organisms may steal nutrients from cells lines in cultures, they may also prey on the cells themselves. Predatory bacterial have been shown to feed on other bacteria (Klausegger et al., 2010; Kly, 2013), particularly in a limited nutrient environment (Nocker, 2008; Vimlesh et al., 2009). Experimental results may also be altered due to unwanted activation of cells. Different cellular functions, including those triggered by Tool-like receptors, can be activated by variety of bacterial components (Testro, 2009; Zenk and Hansel, 2009; Blazkova et al., 2015).

The highly biologically reactive molecules have major influences *in vivo* on humoral and cellular systems. Studies of endotoxins using *in vitro* systems have shown that they may affect the growth or performance of cultures and are a significant source of experimental variability (Case Gould, 1984; Ryan, 2005; Weids, 2007). Furthermore, since the use of cell culture produced therapeutics, such as hybridomas and vaccines, are compromised by high endotoxin levels, efforts must be made to keep endotoxin levels in culture systems as low as possible. Biological contamination can be subdivided into two groups based on the difficulty of detecting them in culture. The first concern those that are easy to detect such as bacteria, molds and yeast and secondly those as that are more difficult to detect, and as a result potentially more series culture problems, such as viruses, protozoa, mycoplasmas and other cell lines (Fogh ,1971; Incorporated, 2002).

The objective of this study was to assess the molecular detection and characterization of sustainable intracellular contaminants in commercially used cell cultures use for regulatory pre-clinical studies for developing new chemical entities.

MATERIALS AND METHODS

This was a prospective analytic cross sectional study, conducted at the centre for Bio-molecular and Biopolymer laboratory, at the Athlone Institute of Technology**.,** Republic of Ireland

In vitro culture

C3A cells were cultured in Dulbecco's modified Essential medium (DMEM) with the addition of 10% bovine serum (FBS).

Cells Lysate preparation

The medium for contaminated cells were removed and the cells washed with PBS buffer. Trypsin was used to detach cells from the flask, and cells were collected in universal

tube for centrifugation. The cells were plated and were exposed to the freeze and thaw process using liquid nitrogen at 60 ºC. The DNA isolation Kit from Sigma Aldrich was used for the isolation of DNA.

DNA Extraction

The cell pellets were obtained by centrifugation and the concentrated samples were suspended in a 500 µl of TE buffer(10 mM Tris-HCL, 1 mM EDTA, pH 7.5) .The efficiency of the DNA extraction and purification protocol was tested by including in each series of experiments and before DNA extraction, a control consisting of distilled de-ionized water sample was added. The DNA concentration was determined by Picodrop (Testro, 2009).

DNA amplification

DNA was quantified using Picodrop, 59 ng of DNA was added to a 50 µl of PCR reaction using Bioline Kit. The primers of 16 s gene listed in Table 1.

The reaction were run using the following cycling parapeters:95 ºC for 4 min, 30 cycles of 30 second at 95 ºC, 30 second at 55 ºC and 45sec at 72 ºC , with final elongation step of 10 min at 72 ºC before a 4 ºC hold. (RoboCycler® Gradient 96, Stratagene: Cambridge). A 10 µl of the reaction mixture were visualized on 1% TEA agarose with ethidium bromide at 8 V/cm and the reaction product was visualized under Gel doc/UV trans-illuminator (Syngene). The PCR product was purified by Qiagen gel extraction kit using the following protocol described below. The DNA fragment was excised from the agarose gel with a clean sharp scalpel. Then the gel slice was weighed in an eppendorf. A 3 volumes of buffer QG was then added to 1 volume of gel (100 mg ~ 100 µl). The mixture was then incubated at 50°C for 10 minutes. The gel was dissolved by vortexing the tube every 2-3 mins during the incubation until the mixture colour was uniformly yellow. A 1 gel volume of Iso-propanol was then added to the sample and mixed. A QIAquick spin column was then placed in a 2 ml collection tube provided. The sample was applied to the QIAquick column followed by centrifugation for one minute to bind the DNA to the column. The flow-through was discarded and the QIAquick column was placed back in the collection tube. This was followed by adding 0.75 ml of buffer PE to QIAquick column and centrifuged for 1 minute to wash. The flow-through was again discarded and the QIAquick column centrifuged for an additional 1 minute at 10,000 × *g*. The QIAquick column was then placed into a clean 1.5 ml eppendorf. A 50 µl of buffer EB (10mM Tris- Cl, pH 8.5) was added to the centre of the QIAquick membrane and the column centrifuged for 1 min to elute the DNA.

After quantitation of the PCR product, with Picodrop, DNA (all samples amount were around 60 ng) and was submitted for sequencing at the Bioscience Ltd, St. James Hospital, Dublin, Ireland, along with 100 pmoles primers. For the two sequences, one originated from the first primer (universal general), and the other one from the second Primers (U16S-staph). The two sequences were aligned using NCBI's BLAST. Sequences of around 450 base pairs were obtained for each of the two samples.

The rRNA based analysis is a central method in microbiology used not only to explore microbial diversity but also to identify new strains. A Sigma Aldrich kit for genome extraction (Gen Elute Bacterial Genomic DNA Kit) was used to ensure the isolation of the microbe genome. To process the freeze and thaw the liquid nitrogen was used and a bath at 60 °C was used to lyse the spore like microbe. The PCR produced different bands compared to the single band of positive control of *Staphylococcus aureus* genomeand the different bands wer compared with the single band of positive control of *Staphylococcus aureus*.

Table 1: Universal primers used in PCR reactions to amplify the 16 s region in contaminated cells.

Primer	Forward primer	Reverse primer
Universal-general	5' TGAGCTCAAGCTTCAGCMGTCCGCGGTAATWC-3'	5'-TTTTGGATCCTCTAGAACGGGCGGTGTGTRC-3
U16S-staph	5' GGAATTCAAAKGAATTGACGGG-3'	5' CGGGATCCCAGGCCCGGAACG-3'

Giemsa Stain

Cells were seeded at cell density of $5x10^3$ (µl/ml), measured with the aid of a haemocytometer, on cover slips, for 72 h at 37 ^oC. The cells were cultured without changing medium, and were washed with phosphate-buffered saline (PBS), and fixed with methanol for 10 min at room temperature. The fixed cells were immersed in a Giemsa solution (10%) for 15 min at room temperature. Staining was followed by rinsing the cover-slips for two to five minutes in phosphate buffer, air-dried, and mounted on microscope slides in DPX (1:1 glycerol: PBS) and then examined under an oil-immersion objective microscope at 1000 x magnification.

Transmission Electron Microscopy (TEM)

The **c**ross sections of the C3A cells were prepared as follows. The cells were fixed with 3% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) at 4 °C for 2 h and then post fixed in 1% OsO_4 in cacodylate buffer at 4 °C for 1 h. After dehydration in a graded series of ethanol, the cell cultures were embedded in a 2-mm-thick Epon coating, in the tissue culture well and polymerized for 3 days at 60 °C. Suitable areas were reoriented either parallel or perpendicular to the cell layer surface on Epon blocks with an Epon mixture. Ultra-sections were contrasted with uranyl acetate and lead citrate.

RESULTS

PCR amplification

The PCR products ranged in size from 1200 base pair to 500 bp. Five bands were excised and cleaned using a kit (Qiagen, QiAquick Gel extraction Kit, Ltd). The samples were sent to the Bioscience Company in Dublin for analysis. After sequencing; the resulting sequences were checked for similarity to other known sequences using NCBIs Blast and ribosomal Database project (RDP). As indicated in Figure 1 the PCR amplification as indicated on band lane 1 to 5 showed PCR Bioline® 100 bp ladder, line 1&2, PCR product of sample genome using 16S primer giving two product of 450 and 650 bp; line 3 & 4 and line 5 using 16S primer for bacteria genome gave only one product of ~ 500bp.

PCR amplification of *Staphylococcus aureus* DNA genome compare to multiple bands in contaminated culture cells sample

The gel images in Figure 2 showed four lanes; +ve, S-1, S-2, S-3. The four gel images depicted one band amplified in the positive control of *Staphylococcus aureus* DNA genome compared to multiple bands contaminated culture cells sample. The Bioline® 100 bp ladder was used for the PCR amplification. The S-1, S-2 and S-3 gel bands compared to the positive bands showed the presence of contaminants. The base pair for the contaminants were different from the positive.

Neighbour-joining tree of 16S rRNA PCR product gene sequence homology to the unknown obtained from BlAST search

A phylogenetic tree view of a second PCR product with less homology to genome database indicated that the sequence was unknown as shown in Figure 2, and as

indicated on the phylogenetic tree analysis. Phylogenetic tree base on the PCR product sequences 16S RNA primers Blastn were searched from NCBI databases. The unknown sequence in this work is highlighted in yellow and the homology Bacteria name are indicated from the NCBI blast search.

A more detailed phylogenetic output indicating the clade for the different homology of bacterial sequences are shown in Figure 3. The unknown bacteria sequence is highlighted in yellow and needed further advance blast search for possible identification for bio-informatics analysis.

We characterized the strain using 16S RNA sequence that amplified the gene from many genera. The closest phylogenetic relative was |HQ877772.1|. *Escherichia sp*. A94 with 88% 16S ribosomal RNA gene sequence similarity. It was proposed that the unidentified strain be assigned as a typed strain of species of the bacteria origin based on the 16S rRNA gene sequence search in Ribosomal Database project, small subunit rRNA and large subunit rRNA database, together with the phylogenetic tree analysis. In the different main clades there was the Enterobacter, Salmonella, Escherichia, Enterobactaceae main families.

Transmission electron microscope (TEM) micrograph of C3A cells infected with unknown bacteria

The cross section of the cells monolayer as shown in Figure 4 had numerous intracellular bacteria. Micrograph showed membrane ruffling upon contact with bacteria. The bacteria were engulfed by elongated microvilli from infected epithelial cells. High magnification showed partially lysed vacuole membrane containing bacteria, indicating the ability of bacteria to escape from the endocytic vacuoles. The photomicrograph in Figure 4 also clearly showed the presence of numerous bacteria inside the cells. Most of the bacteria observed were enclosed by endocytic vacuoles through Giemsa stain observation. In addition, some bacteria were free in the cytoplasm, perhaps as a result of an escape from endocytic vacuoles by bacterium-induced lyses of the vacuole membrane.

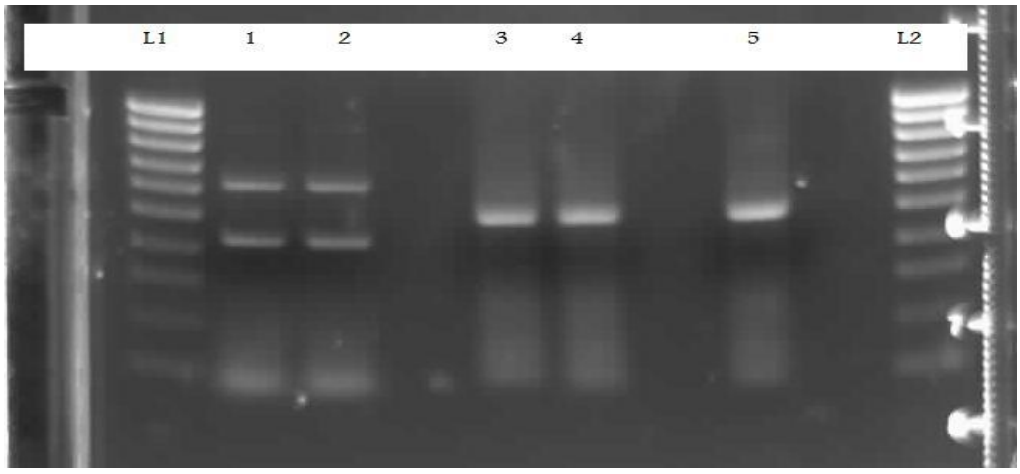

Figure 1: PCR L1 AND L2 Bioline® 100 bp ladder, Line 1&2 PCR product of sample genome using 16S primer giving two product of 450 and 650 bp; line 3 & 4 and 5 using 16S primer for bacteria genome giving only one product of ~ 500bp.

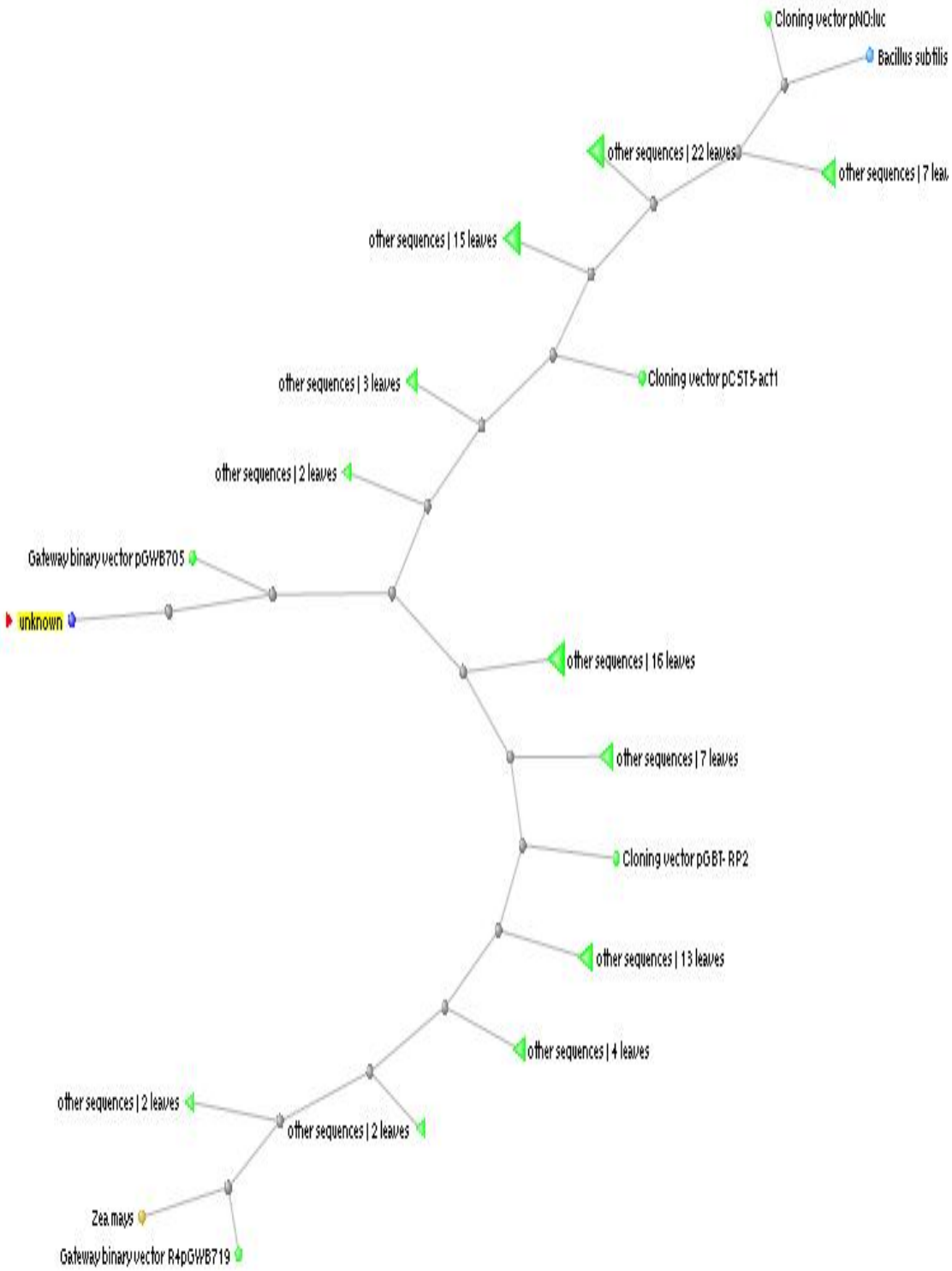

Figure 2: A phylogenetic tree containing all available homology bacterial sequence was constructed from a multiple sequence alignment with the neighbour-joining method using Blast nucleotide search. Scale bar 0.006 changes per site.

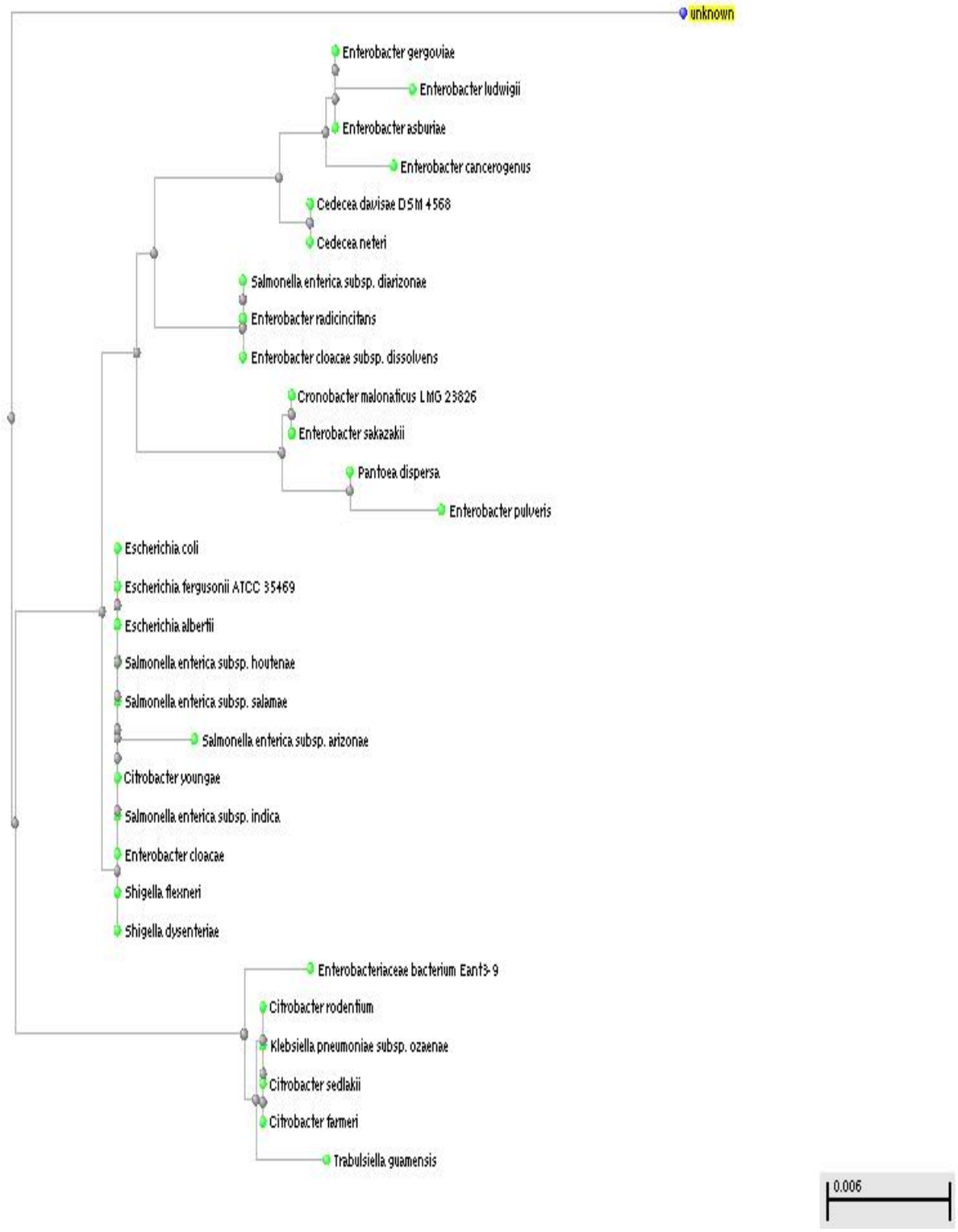

Figure 3: A more detailed phylogenetic output indicating the clade for the different homology of bacterial sequences.

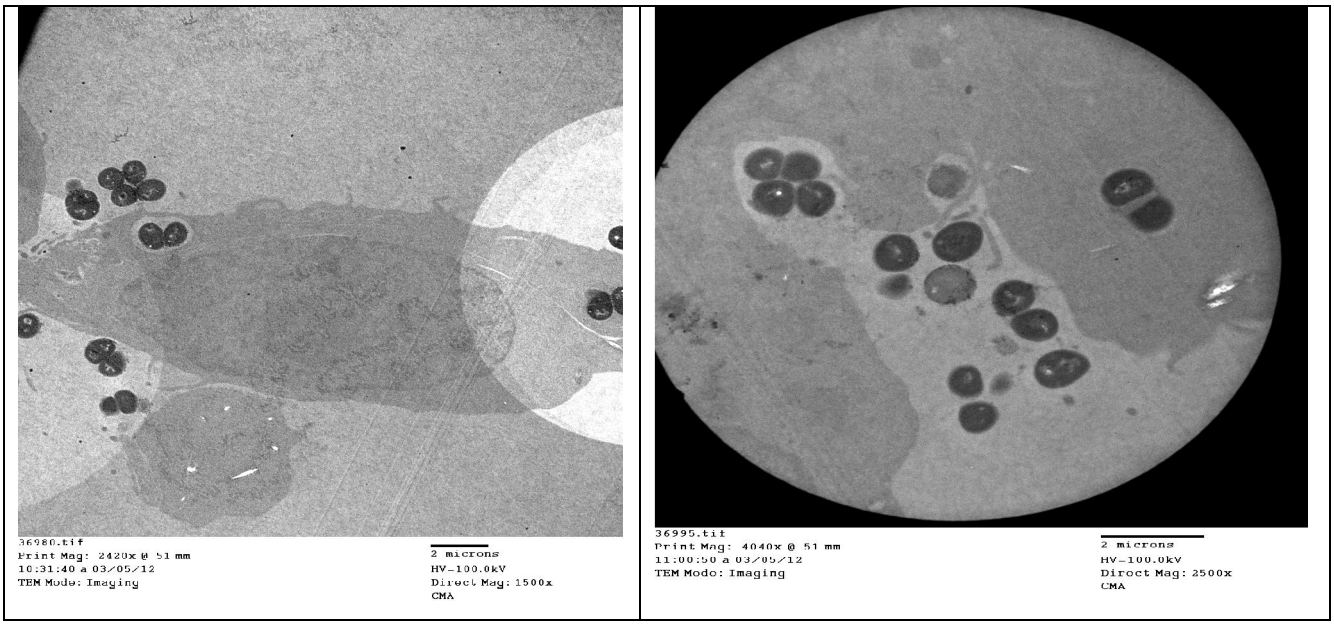

Figure 4: Transmission electron microscope (TEM) micrograph of C3A cells infected with unknown bacteria.

DISCUSSION

Microbial contamination in cell and tissue culture is a constant problem, which can compromise development and applications of cell lines. An immediate consequence of cell culture contamination is loss of researchers' time, money and effort spent developing cultures and setting up experiments (Fogh et al., 2015) Apart from mycoplasma, microbial contamination of cell cultures is usually very detectable and largely concerns media and can be addressed by sanitation procedures. Mammalian cells can host microbes in addition to mycoplasma, in a form that preserves cell survival but seriously damages their expressive behaviour and function is a major concern. There are adverse effects on cultures suffering from undetected biological contamination. This hidden contamination can potentially achieve high densities altering the growth and characteristics of the cultures (Croxton et al., 2012; Usta et al., 2014; Ajibola et al., 2017). Comparison of PCR product sequence against known sequences of nucleotide database (NCBI) showed that the gene sequence of isolate had variable 88% - 87% -95% sequence similarity (Score= bits, expect=0.0) with 16S rRNA gene sequence of *Escherichia coli* uncultured bacteria .

The characterized strain using 16S RNA sequence amplified the gene from many genera. The closest phylogenetic relative was |HQ877772.1| *Escherichia sp*. A94 with 88% 16S ribosomal RNA gene sequence similarity. It is proposed that unidentified strain be assigned as type strain of species of the bacteria origin based on the 16S rRNA gene sequence search in Ribosomal Database project, small subunit rRNA and large subunit rRNA database together with the phylogenetic tree analysis. Thus, the data showed that the isolate was a member of the genus *Escherichia*. Similarity rank program classifier (Ecology, 2011) available at ribosomal database project (Wang et al., 2007) classified the fragment (sample) as a novel genome species of genus with confidence threshold of 95%.

Most sequence shared 100 identities with Homo sapiens isolate Li110 control region, partial sequence; mitochondrial 100%, while the rest of the 2 sequences showed highest homology to sequences identity to (88% gb|HQ877772.1| such as the *Escherichia sp*, A94 16S ribosomal RNA gene, partial sequence, 88% dbj|AB609044.1| *Escherichia coli* gene for 16S rRNA, partial sequence, strain: SI-7, gb|HQ759847.1|, the uncultured organism clone ELU0045-T454-S-NIPCRAMgANb_000132 small subunit ribosomal RNA gene, gb|HQ796635.1|.Lastly, the uncultured organism clone ELU0139-T413-S-NIPCRAMgANa_000446 small subunit ribosomal RNA gene 94%. The translation of the nucleotide sequence was more likely to be more accurate than just blastn search, because the protein sequences were more evolutionary and more conserved than nucleotides sequences.

The nucleotide sequences translated into protein using blastx database, showed that the result of the search contained same results from the Blastn search. The highest homology was two hypothetical protein HMPREF9553_00243 for [*Escherichia coli* MS 200-1], with an 87% homology. The rest of the sequences gave two different strains of *Escherichia coli* (MS-198-1, 182-1, 187-1), as well as gave a high similarities to conserved hypothetical protein [*Escherichia coli* UTI89], *Escherichia coli* APEC O1 with 84% identities (Jerome Boudeau, 1999; Vimlesh Yadav, 2009). The translation of the nucleotide sequence is more likely to be more accurate than just blastn search, because the protein sequences are more evolutionary and more conserved than nucleotides sequences (Wieds, 2007). This study evaluates the surface changes and effects on *in vitro* cell attachment and spreading brought about on prepared commercially pure titanium by multiple exposures to common sterilization methods. Information of contamination of cells is very relevant in pre-clinical *in vitro* cell cultures as a regulatory compliance for drug discovery high throughput screening (Eteme et al., 2015).

Mycoplasma contamination in mammalian cell cultures is often overlooked yet is a serious issue which can induce a myriad of cellular changes leading to false interpretation of experimental results (Ajibola et al., 2017). A simple and sensitive assay was used in this study to monitor mycoplasma contamination (mycosensor) based on degradation of the Gaussia luciferase reporter in the conditioned medium of cells. This assay proved to be more sensitive as compared to a commercially available bioluminescent assay in detecting mycoplasma contamination in seven different cell lines. The Gaussia luciferase mycosensor assay provides an easy tool to monitor mammalian cell contaminants in a high-throughput fashion (Awodiran et al., 2014). The electron microscopic examination of contaminated C3A cells was used to identify the presence of mico-organism TEM performed on cells monolayer. Bacterial was observed to adhere closely to C3A cells. The adhered bacteria strikingly induced the elongation of microvilli from the cell surface. At the site of close contact between the bacteria and the epithelial cell, the elongated microvilli surrounded the adherent bacteria

Cell culture is one of the most common methods used to recapitulate a human disease environment in a laboratory setting. Cell culture techniques are used to grow and maintain cells of various types including those derived from primary tissues, such as stem cells and cancer tumours (Kim 2007; Zenk and Hansel, 2009). However, a major confounding factor with cell culture is the use of serum and animal (xeno) products in the media. The addition of animal products introduces batch and lot variations that lead to experimental variability, confounds studies with therapeutic outcomes for cultured cells, and represents a major cost associated with cell culture. Here we report a commercially available serum-free, albumin-free, and xeno free (XF) media (Neuro-Pure(TM)) that is more cost-effective than other commercial media. Neuro-Pure was used to maintain and differentiate various cells of neuronal lineages, fibroblasts, as well as specific cancer

cell lines; without the use of contaminants such serum, albumin, and animal products. Neuro-Pure allows for a controlled and reproducible cell culture environment that is applicable to translational medicine and general tissue culture.

Conclusion

Microbial contamination in cell and tissue culture is a constant problem, which can compromise development and applications of cell lines. This study showed a molecular approach in detecting the cell contaminant bacterial and gene sequence alignments. The closest phylogenetic relative was |HQ877772.1| *Escherichia sp.* A94 with 88% 16S ribosomal RNA gene sequence similarity. It was proposed that unidentified strain be assigned as type strain of species of the bacteria origin based on the 16S rRNA gene sequence search in Ribosomal Database project, small subunit rRNA and large subunit rRNA database together with the phylogenetic tree analysis. Bacterial species were shown to have at least one copy of the 16S rRNA gene containing highly conserved regions together with hyper variable regions. This study has also shown the important use of 16S rRNA gene sequence to characterize the bacterial isolate from different cell lines. The Blastx database did confirm the nucleotide Blastn search result by suggesting that the unknown sequence belong to the bacteria group indicated very high homology with *Escherichia coli.*

The electron microscopic examination of contaminated C3A cells was used to identify the presence of micro-organism TEM performed on cells monolayer. Bacterial was observed to adhere closely to C3A cells. The adhered bacteria strikingly induced the elongation of microvilli from the cell surface. At the site of close contact between the bacteria and the epithelial cell, the elongated microvilli surrounded the adherent bacteria. In addition, dense area of staining, possibly related to an accumulation of cytoskeleton components were observed beneath the sites of intimate contact.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS CONTRIBUTIONS

CFN, ETF, SB contributed in the conception of the protocol, laboratory analysis and statistics, JF, GL, FAK, BN and DG, participated in manuscript writing and data mining. PT the principal investigator and project sponsor. All the authors participated in the review of the manuscript.

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