

Preliminary evidence of a new microbial species capable of sustainable intracellular survival and transfer in mammalian cell lines

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Abstract

The minimisation of exposure of mammalian cell lines to potential microbial contaminants is handled by routine adherence to quality laboratory procedures. Mycoplasma, are capable of sustainable intracellular existence, are not visible in light microscopy and must be tested for, using dedicated methods. Bacterial contamination is usually detectable by relatively simple optical, spectroscopy and pH methodology. Symbiont occupation assumes an evolved mutually beneficial relationship and does occur with many eukaryote-prokaryotes, but rarely mammals. Other, purely intracellular low density, low energy and relatively stable and non-visible bacterial occupation of mammalian cytoplasm, assumes the existence of new intra-genus relationships and associated mechanisms. In this study, preliminary microscopy and sequence data has been collated implying the presence of low density *cocci* in the cytoplasm of hepatocyte lines with negligible impact on cell function and behaviour.

INTRODUCTION

Studies over multiple decades in the UK, US, Germany and Japan have indicated that up to 36% of cell cultures include a misidentified species or cell type. A mixture of different tissue cells as a false single cell line is still the major contamination issue for cell culture, with in theory, the difficulty of bacterial contamination, including mycoplasma being addressed by routine cell handling practice, (Editorial 2009).

While cell line cross contamination may represent a subtle challenge with significant impact on integrity, function and behaviour, routine contamination is more closely affiliated with bacteria, fungi, viruses, mycoplasma, and rare protozoa and invertebrates. Viral and mycoplasma contaminants of course don't generate visible media turbidity and lower pH, are more tolerated by cells and are only exempted from cell lines if they are regularly tested and subject to appropriate processing and manipulation standards, (Merten. 2002). Viral contamination usually requires termination of the affected cell line. Mycoplasma, also have a significant impact on cell metabolism, gene expression profile, apoptosis and turnover. Levels vary, but mycoplasma contamination of mammalian cells has been estimated to range between 15 and 35% of cultures,

(Hans G. Drexler 2002), if extended to embrace all Mollicutes, then this could imply an even higher percentage, (Lehmann D 2010).

Various cell lines of multiple species in Middle Eastern cell banks, were evaluated for contamination over two years of cell culture handling after which, a proportion of 39% contamination was determined, with mycoplasmas accounting for 19%, followed by mixed infection (8%), fungi (8%) and bacteria (4%), (Mirjalili A 2005). There are four main sources of contamination, cells themselves, associated labware, cell media, and air and laboratory environment. All cell culture laboratories must implement effective practices to ensure these four domains are compliant with necessary quality practice, (Ryan 2005).

Media incorporating antibiotics are efficient against many bacterial species, but they cannot protect cultures against all bacterial contaminants, especially mycobacteria or corynebacteria, (Lelong-Rebel IH 2009). Some bacteria and other pathogenic microbes bind to the host cell surface and then become internalized via microbial invasion. Intracellular bacteria can resist treatment by aminoglycoside antibiotics, whereas bacteria bound to the outside of mammalian cells are rapidly killed. Cryostorage may also be a mechanism for contamination transfer, particularly, viral, (Mirabet V 2012).

Routine successful cell culture is highly dependent upon adherence to very secure and aseptic practices. Pen-strep and other antibiotics should not routinely be incorporated in media, because they will risk increased bacterial resistance, enhance mycoplasma inclusion and selected intracellular reactions such as protein and ATP synthesis, (Freshney 2000; Coecke S 2005).

There are more than 100 companies engaged in cell line development and provision in a market approaching €4.5b in annual value, but the main global R&D cell providers are obviously ATCC (American Type Culture Collection) and HPACC/ECACC (Health Protection Agency Culture Collections) embracing more than 3400 cell lines of 80 species and 40,000 cell lines and 45 species, respectively.

There have been many studies analysing the use of molecular assays for the detection of positive bacterial contamination (Marlowe 2003). PCR screening assays provide fast, dependable and cost-effective methods for quality assessment, ultimately resulting in faster product release and product optimization – detection can be based on generic primer sequences to maximize detection of difference bacterial strains, (Jimenez 2001; Lleo 2005). The highly conserved bacterial ribosomal DNA sequence has been employed in PCR-based assays to determine sterility of pharmaceutical samples, (Jimenez 2007). Nucleic acid amplification has been described as a

significant improvement in technology for microbial research laboratories and microbial diagnostic industries, due to sensitivity and capacity to be automated, (Nocker 2008).

Compliance with approved quality procedures, assumes deployment of effective aseptic techniques and adherence to clean and sterile procedures while manipulating cells in order to protect and maintain them. An additional complication occurs when researchers use lines that are not commercially available and are often irreplaceable, difficult to obtain, or need rederivation from primary cells, (Jennifer Sue Gary 2010).

While many undesirable organisms may consume nutrients from cells lines in cultures, they may also exploit cells themselves. Predatory bacteria have been shown to feed on other bacteria (KL. 2007; Blazkova H 2009), particularly in a limited nutrient environment, (Nandy SK 2006). 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, (Blazkova H 2009; Testro AG 2009; Zenk SF 2009).

Highly biologically reactive molecules have major influences *in vivo* on humoral and cellular systems. Endotoxin residues affect the growth or performance of *in vitro* cultures and are a significant source of experimental variability, (Case Gould 1984; Ryan 2005).

Frequency and absence of formal contaminant detection is reflected in several online science blogs that describe cell culture contaminants that resemble black specks in the cell culture (<http://www.youtube.com/watch?v=lo96fcXfcVs> ,<http://www.scientistsolutions.com/t11616-black+dots+in+cell+culture.html>, http://www.researchgate.net/post/Human_cancer_cell_line_culture_contamination).The researchers on these blogs describe black dots that are mobile and resemble rods and dots in more detail, implying *bacilli* and *cocci*. More recently, intracellular *Achromobacter* have been shown to represent a cell culture problem due to scale, some cell tolerance and antibiotic resistance, but which are confirmatory detectable by 16S rDNA analysis, (Jennifer Sue Gary 2010). The latter paper is expressing intracellular mobile 'black dots or rods', is in agreement with numerous cell culture on line questions and blogs, which tend to lack appropriate technical descriptions and analysis. They also tend to fail to indicate whether the contaminants were intrinsic in the cells on receipt or were a consequence of handling error.

This study took cognisance of prior findings, but was stimulated by a period of apparent intracellular contaminant presence in selected hepatocyte cell lines, detected within 24 hrs of culture following cryo storage, that was compliant with approved quality

procedures. The full evidential origin of this contamination has yet to be confirmed, whether a unique and rare mammalian endosymbiont, a new mode of external transfer, or sub-optimal sera, but current sequence evidence at least implies a novel bacterial strain.

Materials and Methods

In vitro culture

Good cell culture practice, embracing staff training, cell line sourcing, passage records, media, instrumentation, environment, contamination testing, approved cell release and storage was applied to all elements of cell handling and research. Bacterial and mycoplasma testing was performed regularly on batch production and culture samples.

C3A cells were cultured in Minimum Essential Medium Eagle (MEM) with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% non-essential amino acids, at 37 °C and 5% CO₂. All experiments were conducted using cells between passage 7 and 25. 100 U/ ml penicillin/ streptomycin could be added transiently to media for new set-up cultures or when a contamination risk was perceived all media constituents were sourced from Sigma Aldrich Ireland.

Cell Lysate preparation

Media in which contaminated and control cells were grown was removed separately and securely and the cells washed with PBS and then treated with trypsin to detach them from the flask surface, and then collected in a universal tube for centrifugation at 300g. Tube pelleted cells were exposed to a freeze-thaw process using liquid nitrogen and 60° C water for 3 min of each stage, comprising 3 – 5 cycles. A DNA isolation kit (Sigma Aldrich) was subsequently used for the extraction of cell DNA.

DNA Extraction

To ensure the isolation of the microbe genome and process of freeze-thaw using liquid nitrogen and 60 C bath to lyse the spore-like microbe, and a Sigma Aldrich kit for genome extraction (Gen Elute Bacterial Genomic DNA Kit), was applied. Centrifuged pellets were suspended in a 500 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The

DNA concentration was determined by A260/280 nm absorbance spectroscopy of 2 µl samples using a *Picodrop* spectrophotometer.

DNA amplification

59 ng of DNA was added to 50 µl of PCR reaction mixture using a Bioline Kit. The primers of 16S genes are listed in Table 1. The reaction was run using the following cycling parameters: 95 °C for 4 min, 30 cycles of 30 sec at 95 °C, 30 sec at 55 °C and 45 sec at 72 °C , with final elongation step of 10 min at 72 °C before a 4 °C hold. (RoboCycler® Gradient 96, Stratagene). 10 µl of the reaction mixture was separated on 1% TEA agarose with ethidium bromide at 8V/cm and the reaction product was visualized and scanned by a Gel doc/UV trans-illuminator (Syngene). The PCR product was purified with a Qiagen gel extraction kit. Several quality control steps, including negative control for masteries used in preparing cell culture and positive control such as Bacterial genome, were included which conducting this experiments.

After quantitation of PCR products, with Picodrop, all DNA (samples ~60 ng) were submitted for sequencing (Source Bioscience Ltd), plus 100 pM of primers. The two sequences, one originated from the first primer (universal general), and the other from the second primer (U16S-staph) were aligned using NCBI's BLASTN to identify the most similar 16S rDNA sequences. Sequences of ~ 1400 base pair were obtained for each of the two samples.

Differential Giemsa staining

Cells were seeded at 5×10^3 per 50 µl on cover slips and cultured for 72- 96 h at 37 °C without changing medium. The cells were then washed with phosphate-buffered saline (PBS), and fixed with 3:1 methanol/acetic acid for 10 min at room temperature. Cells were immersed in a Giemsa solution (10% v/v) 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 examined under an oil-immersion objective at overall 1000x magnification (Leica SP5 & Leica Diaplan).

TEM

Cross sections of 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 post fixed in 1% OsO₄ in cacodylate buffer at 4°C for 1 h. After dehydration in a graded series of ethanol concentrations, the cultures were embedded in a 2-mm-thick Epon coating in a 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 and then sliced in an ultra microtome. Ultrasections were contrasted with uranyl acetate and lead citrate. TEM images were generated using a Jeol 2100 instrument.

RESULTS

This study was devoted to investigating the potential presence of intracellular contamination in commercial human liver cells, HepG2 and C3A cell lines. Primary hepatocytes express a typical cubic cell shape and often contain two nuclei, while HepG2 cells have an epithelial-like morphology and contain one nucleus. The C3A line was derived from a sub-clone of HepG2, but more closely resembles primary hepatocytes.

If mammalian cells cultured for the first 36 hours under defined and approved conditions after cryo storage, following procurement from an external source, show intracellular, but not extracellular evidence of foreign particle contamination, this is a potentially novel finding.

The absence of extracellular contamination was confirmed by cell lyses and supernatant culture on different agar plates (blood, brain and heart infusion, MacConky agar, Mueller Hinton agar, nutrient agar), all of which showed no visible bacterial growth.

The photomicrograph in Plate I clearly shows the presence of numerous particles that resemble bacteria inside the cells. Most of the bacteria observed were enclosed by endocytic vacuoles. In addition, some bacteria were free in the cytoplasm, perhaps as a result of escape from endocytic vacuoles by bacterium-induced lyses of the vacuole membrane, (Jerome Boudeau 1999). Application of traditional gram –ve and +ve staining did not however generate accepted outcomes and the Plate I type of imaging did not support normal relatively intense staining. The extended culture time, post confluence with modest final pH decline facilitated and accelerated cytoplasmic bacterial growth and population scale. Figure 2 represents a similar intracellular domain with DAPI stain, confirming the scale of DNA containing particles – their magnitude is considerably more than indigenous mitochondria. Size of Micrococcus bacteria was ranging between 0.5mm – 1 mm, both TEM and LM images supported the presents of Micrococcus,

diplococcic and tetracoccus bacteria, which eliminate the possibility of mitochondrial resemblance.

TEM was performed on cell monolayers to contribute to the identification of any intracellular microorganisms. Bacteria like particles were observed adhered closely to C3A cells in Fig. 3 & 4. 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, (Jerome Boudeau 1999).

The DNA genome extracted from C3A cells and universal primers were used for the amplification and sequencing of the 16SrRNA fragment. Efficient extraction to secure sufficient contaminant DNA for identification and analysis required extended cell growth and combined gram +ve/-ve extraction processes. The first primer set PCR product gave multiple bands, while the second one gave only one band. The bands were excised, and cleaned up using a Qiagen Kit for cleaning agarose gel. Most fragments from the first primer set showed 100% similarity to the human genome, while one fragment of the 16SrRNA (size of ~550 bp) had no similarity to human DNA. Based on the phylogenetic tree (Figure1), this implied a relationship between the amplified fragment and selected representatives of an *Escherichia* strain bacteria. Comparison of test fragment 16S (~550 bp) data to known sequences of BLASTn (NCBI) database confirmed that the sample gene sequence had 98% sequence similarity with *Escherichia coli* partial 16S rRNA and 98% sequence similarity to uncultured bacterium clone SHZB491 16S rRNA. The sequence data indicative of intracellular prokaryotic presence was proportionately less than the visual results.

Second primer set PCR product about of ~ 1400 base pairs in size is shown in Table 2. The resulting sequence was checked for similarity to other known sequences using NCBI's BLAST and Ribosomal Database Project (RDP). The sequence shared 99% similarity with 16S rDNA gene sequence of an uncultured organism Clone EIUO124-T3104 and 99% Similarity to *Escherichia fergusonii* strain KRT1 16D ribosomal RNA gene partial equene.

The sequence data therefore confirms that the isolate is a member of the bacteria genus. The similarity rank program classifier (ECOLOGY 1999-2011) accessible in a ribosomal database project (Wang. 2007) classified the sample fragment as a novel genome species of bacteria genus

with a confidence threshold of 98%. To support this novelty and provide a basis for future and collaborative analysis, this 16S sequence data was submitted to the EMBL for accession number approval. An accession number is a unique identifier of sequence data to support tracking of emergent versions over time. However, allocations of accession numbers do not intrinsically imply a unique sequence. The GenBank accession numbers are: HE994466 isolate contaminant A and HE994467 isolate contaminant B.

Discussion

In addition to importation of contaminants, intracellular variation in some indigenous particulates, can sometimes be visibly misinterpreted as bacteria. The benefit being, that while cell behavior may change, it is not deleterious. Cytoplasmic particles of intracellular or contaminant nature, may exhibit some mobility, this may be due to capillary action, Brownian motion, physical association with ER or organelles or actual indigenous motility. Intracellular bacterial mobility, not dependent on Brownian motion, implies E consumption with an impact on the 'host' cell. Intracellular components include, golgi apparatus, macrophages, lipids, and glycogen particles.

In most cell types, lipid droplets are usually less than 1 μm in size, although in hepatic steatosis, they may reach 10 μm - next to the endoplasmic reticulum, mitochondria, and peroxisomes, with some restricted mobility,(Tobias C. Walthera and Robert V. Farese 2009),(Reue 2011).

The liver is a central organ for lipid metabolism and in hepatocytes, lack of TGH (triacylglycerol hydrolase) expression alters lipid droplet morphology and dynamics. Increased (phosphatidylcholine) PC synthesis observed in TGH-deficient hepatocytes may result in smaller LDs, (Wang 2010).

Lipid droplets are indeed, organelles expressed in virtually all cells from bacteria to mammals and in addition to lipid synthesis, are involved in catabolism/E release, and trafficking, (Yang 2013). All microscopy and sequence data generated in this study did not support the initial belief that detected mobile particles could be indigenous.

For bacteria to be permanent occupants of eukaryotic cells, they must generate energy but impose negligible impact on the host. Foreign organisms occupying cells of another without pathological consequences are considered an endosymbiont. It is obviously generally accepted that the organelles, mitochondria in all eukaryotes and chloroplasts in photosynthetic plant originated from prokaryote occupants. Endosymbionts as part of their host adaptation lose many essential genes but maintain a core genome to provide some useful functions to their hosts, which in turn provide the bacteria with physical protection and essential nutrients, (Kumar S 2011). A genome

constructed to encode 387 protein-coding and 43 structural RNA genes could sustain a viable synthetic cell, which has effectively been supported by the JCVI, (Glass JI 2006).

In extreme forms of symbiosis, the host may benefit enormously from the bacterial interface, such as, *Olavius algarvensis*, (Woyke 2006). Available data assumes that the majority of symbionts interface with non-mammalian species, but there is now an acceptance that from a health and clinical perspective, symbiosis and human engagement does require more research, particularly with regard to potential immune deficiency, (Margulis 2006). All eukaryotic organisms, including humans, obviously host a significant proportion of bacteria, particularly in the intestine, but at *individual in vitro* cell level, the potential for stable, mutual, not readily detectable cohesion is remote.

Until recently, there was a selected belief in the existence of nanobacteria, which were considered to access cells from approved serum, to become relatively low density stable occupants and to partially manifest via impact on vacuolisation (Galvez J 1997). It is now accepted that these perceived nano particles are in fact mineral nanoparticles although the scale of such nano particle presence may still influence aspects of cell function and imagery (Pan Y 2009).

This research was motivated by a selective identification of what appeared to be particle contaminants of new hepatocyte cultures, with no replicable evidence of traditional external bacterial presence and transfer. It has confirmed the presence of nuclear materials in the cytoplasm of cultured cells using DAPI nucleic acid staining, (Fig. 2). Giemsa staining (Fig.1) did show many spore-like structures in the cytoplasm mainly sized 0.5-1 microns.

Intracellular bacterial numbers in host cell line are normally very low, immobile and with detectable effect on the host. Prolonged and accelerated culture that enhanced bacterial population number and proportion of cells affected, resulted in greater visible detection and evidence of intracellular bacterial mobility.

It must be emphasised that all cell lines subject to this analysis were deemed contaminant free by all approved test protocols performed by the providers and replicated in our own laboratory. Furthermore, these cells demonstrated normal cell proliferation, turnover and adherence. As previously indicated, the C3A line is a patented, highly selected subclone of Hep G2 that retains many of the properties of primary human hepatocytes. They exhibit strong contact inhibition at confluency, high expression of albumin (generally 25µg/mg total cell protein/24 hrs) and high albumin/alpha-fetoprotein at confluency, (usually 25µg/mg total cell protein/24 hrs), (Jun-Qiang Zhang 2010). As the cells become confluent, there is a marked reduction in AFP secretion and an increase in albumin secretion and they also

show nitrogen metabolizing activity comparable to perfused rat livers. The secretion of hepatic proteins in considerable amounts is mediated by microvilli, (Henics T 1999). It is accepted that mammalian cytosol cannot readily support bacterial replication and sustain prokaryote presence in a non-reactive way. Only few bacteria species, facultative intracellular pathogens, have been found to efficiently replicate in cytoplasm after microinjection, eg *Shigella* spp., the related entero-invasive *E. coli* strains, and *L. monocytogenes*, (Falkow 1992; M. Pilar Francino 2006). Evidence for the former species is in agreement with data generated in this study and supports the belief that effective adaptation of bacterial metabolism to the host cell environment is critical for successful replication in a foreign cell environment.

The entire sequence of the 16S rRNA is approximately, 1500 bp and a 0.5 – 1% differential would be expected to embrace a new taxon, which is again supportive of these findings, that the bacterial occupant in these cells is a new species, (Raoult 2005; Kumar S 2011). However, it is accepted that further confirmatory work must be performed to ensure that no new and subtle basis for external contamination occurred or the visual data complies more effectively with non-contaminant modified intracellular particles and the novel sequence data represents gene transfer or post experiment moderation. For example DMSO residue in media can increase hepatocyte albumin production and resultant cytoplasmic morphology and particle presence, (Isom HC 1985), and exposure to selected xenobiotics may increase cell vacuolisation with potential subsequent apoptosis or autophagy and enhanced and partially mobile vacuoles can be misinterpreted as cytosolic occupants, (Jun-Qiang Zhang 2010). However, the microscopy imaging did not support these possible processes.

Bacterial species have at least one copy of the 16S rRNA gene containing highly conserved regions together with hyper variable regions. The use of 16S rRNA gene sequences to identify new strains bacteria is gaining momentum in recent years (Vimlesh Yadav 2009). This work currently supports the use of 16S rRNA gene sequence to characterize a bacterial isolate from mammalian cell lines. The further work will confirm the intracellular sustainability of this bacterial strain and its minimal negative impact on the host cell. It is accepted that even more immediate time work will be conducted to confirm that these prokaryotes are indigenous in the hepatocytes and are having no significant impact on host behaviour or expression.

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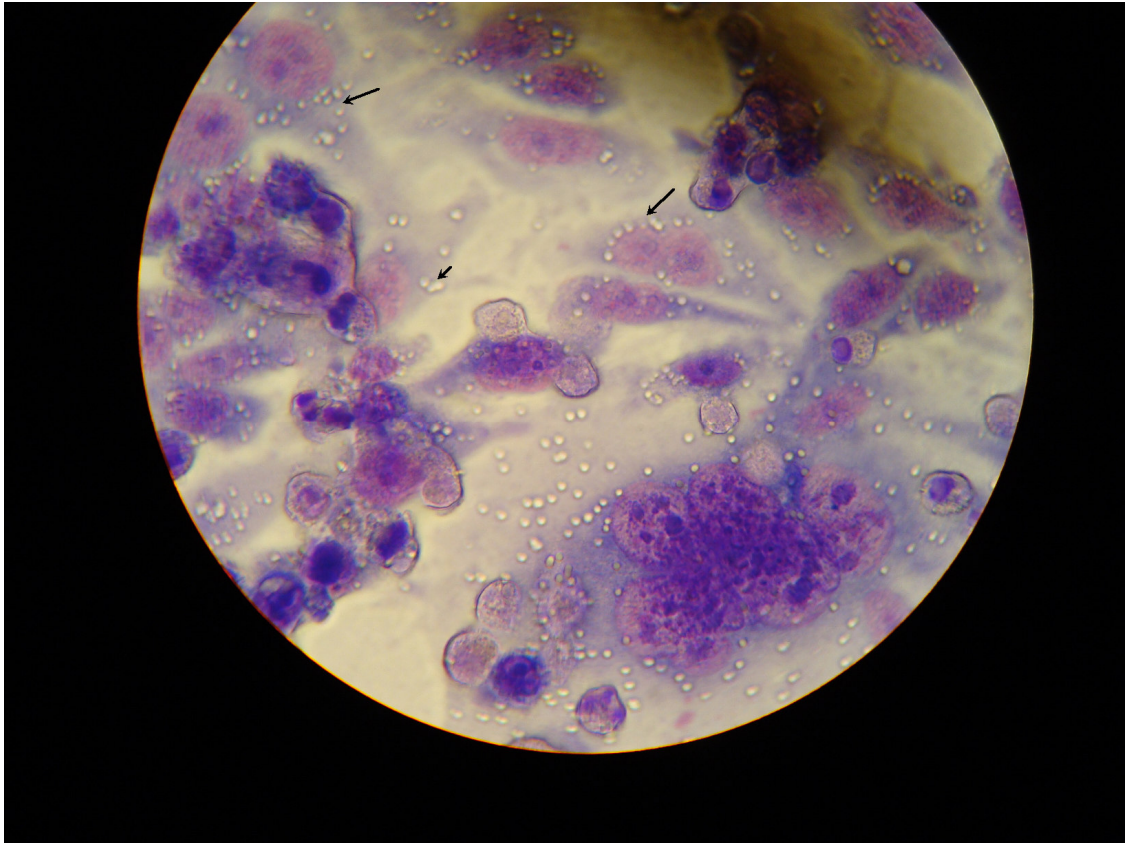


Figure 1: The arrow in the Light microscope image showing 'transparent' round shape bodies observed adjacent to lysed Giemsa stained cells while the nucleus and cytoplasm are stained with purple and pink colour (x200). Image restricted by condenser to maximise core illumination.

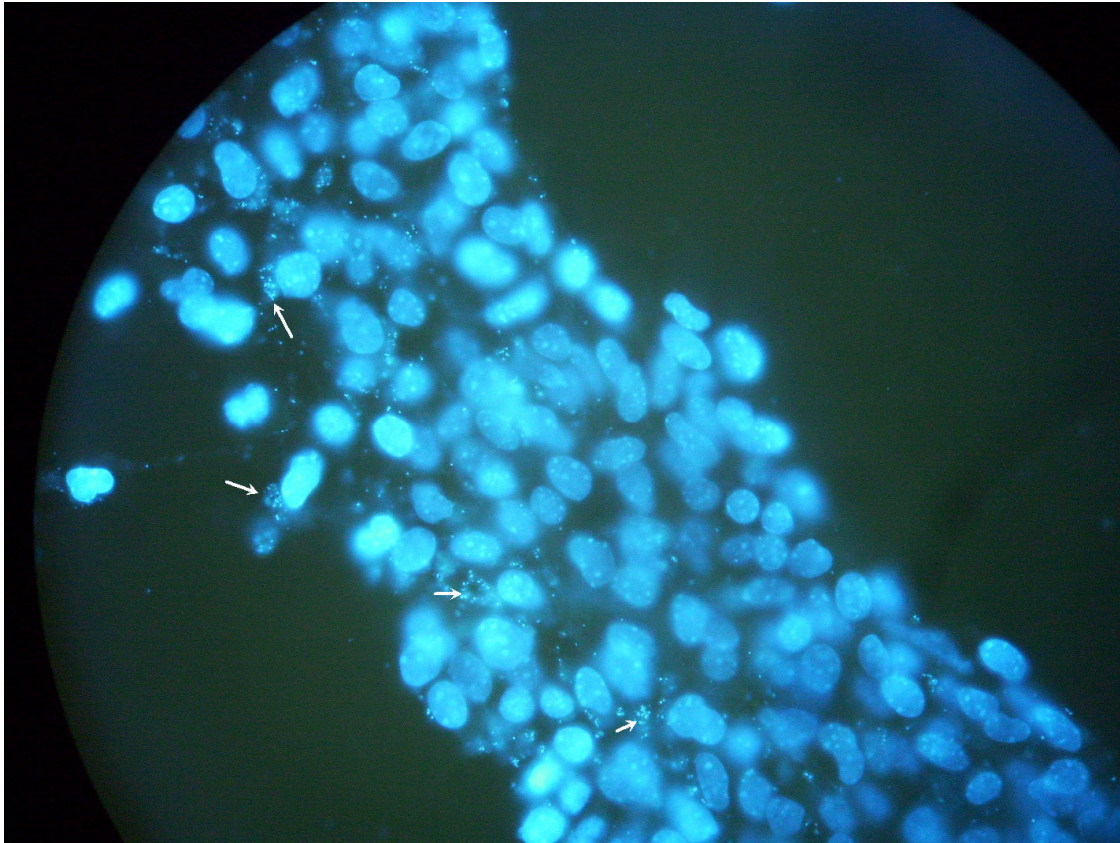
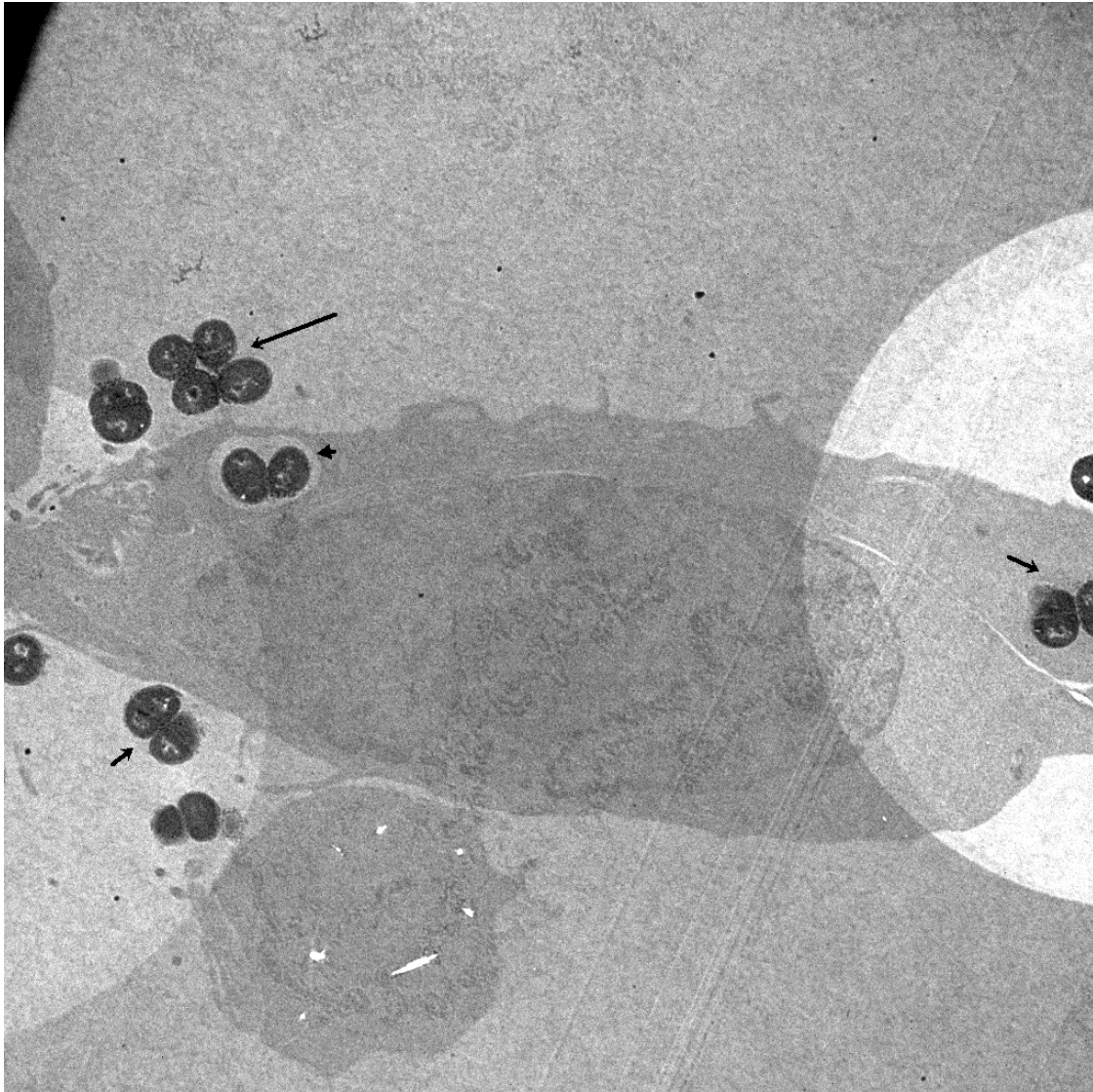


Figure 2: Fixed cells on cover slips and stained with counterstain DAPI. Arrow point to the Spots of nuclear materials observed around the nucleus of cells in the image (x100).



36980.tif

Print Mag: 2420x @ 51 mm

10:31:40 a 03/05/12

TEM Mode: Imaging

2 microns

HV=100.0kV

Direct Mag: 1500x

CMA

Figure 3: TEM micrographs of C3A cells infected with unknown bacteria (~ 1 μm dia). Cross section of the cells monolayer showing numerous intracellular bacteria. Micrograph showing membrane 'ruffling' upon contact with bacteria.



36995.tif

Print Mag: 4040x @ 51 mm

11:00:50 a 03/05/12

TEM Mode: Imaging

2 microns

HV=100.0kV

Direct Mag: 2500x

CMA

Figure 4: TEM micrographs of C3A cells infected with unknown bacteria (~ 1 μm dia). Bacteria are engulfed by elongated microvilli from infected epithelial cells. High magnification showing partially lysed vacuole membrane containing bacteria, indicating the ability of bacteria to escape from the endocytic vacuoles.

Table 1: List of primer sequences used for the analysis

Primer	Forward primer	Reverse primer
General	5' TGAGCTCAAGCTTCAGCMGTCCGCGGTAATWC-3'	5'-TTTTGGATCCTCTAGAACGGGCGGTGTGTRC-3
U16S-staph	5' GGAATTCAAAGGAATTGACGGG-3'	5' CGGGATCCCAGGCCCGGAACG-3'
Universal	27f -AGAGTTGATCVTGGCTCAG	1492r-AACCTTGTACGATT

Table 2: List of result sequences which have an EMBO data Base Reference number

Accession#: HE994466	ccaagtcaa gactgatgtg ctatgggtgt ggatacgtat attcactcc ttacaatct	60
	attacttga cccgactgc ttgagccaca ataaaaatct tctatgaagg ccctatgtg	120
	ggcttaagta tataccgcc tatgtacca tctatggctc tactaatatg atctccagtc	180
	cgaaggatt taaatcagag gataacacgg aagataatac tatatgggga ttattgctca	240
	acggggctga gcctgtagca cccatgacgc gtgaatcaat tatgacttcg ggttgtaaag	300
	tactttcagc ggggaggaag ggagtaaagt taataccttt gctcattgac gttaccgca	360
	gaagaagcac cggtaactc cgtgccagca gccgcgtaa tacggaaggt gcaagcgtta	420
	atcgaatta ctggcgtaa agcgcacgca ggcggtttgt taagtcagat gtgaaatccc	480
	cgggtcaac ctgggaactg catctgatac tggcaagctt gagtctcgtg gaggggggta	540
	gaattccagg tftagcgtg aaatgcgtag agatctggag gaataccggt ggcgaagcgc	600
	gccccctgga cgaagactga cgctcagggt cgaagcgtg gggagcaaac aggattagat	660
	accctggtag tccacccctg aaacgatgac gacttgagg ttgtgccctt gagcgtggc	720
	ttccggagct aacgcgttaa gtcgaccgcc tggggagtac ggcgcaagg ttaaaactca	780
	atgaattgac gggggccgc acaagcgtg gagcatgtgg ttaattcga tgcaacgcga	840
	agaaccttac ctggtttga catccacaga ctttccaga gatggaattg gtccttcgg	900
	gaaactgta gacaggtgct gcatggctgt cgtcaagctc gtgtgtgaa atgttggtt	960
	aagtcccgca acgagcga ccccttatcc ttgttgcca gcggtccggc cggggaactc	1020
	aaggagactg ccaggtgata aactggaggg aaggtggggg atgacgtcaa gtcacatgg	1080
	gcccctacga ccaggccta actcactgac tacaatggcg catacaaga agaagcgacc	1140
	ttcgcgagag ccagcggact tcataagtgg cggctgtagt ccggattggg agtctgcaac	1200
	tcgacctca tfgaagctc gaatccgcta gtattcgtgg aatccagaat gccacggtg	1260

	aattacgttc acgggtcctt gtaactaccc gtccccgtca actcatggga agttgaaatt 1320
	gccaaagaaa gctacgtgag tctttatgcc ttfgcgacgg tcttagcacg tttgtgggta 1380
	atccatgag tactctggat g 1401
Accession#: HE994467	gttaatact ttgctcattg acgttacccg cagaagaagc accgctaac tcegtccag 60
	cagccgcggt aatacggagg gtgcaagcgt ttaatcggaa ttactgggcg taaagcgcac 120
	gcagcgggtt tgtaagtca gatgtaaat cccccggctc aactgggaa ctgcatctga 180
	tactggcaag cttgagtctc gtagaggggg gtagaattcc aggtgtagcg tgaaatgcg 240
	tagagatctg gaggataacc ggtggcgaag gcggcccctt ggacgaagac tgacgtcag 300
	gtgcgaaagc gtggggagca aacaggatta gataccctgg tagtccacgc cgtaaacgat 360
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	ggcgatata aagagaagcg acctcgcgag agcaagcggga cctcataaag tgcgtcgtag 840
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	gaatgccacg gtgaatacgt tcccggacct tg 932