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TITLE 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

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38 mycoplasmas accounting for 19%, followed by mixed infection (8%), fungi (8%) and bacteria (4%),
39 (Mirjalili A 2005). There are four main sources of contamination, cells themselves, associated labware,
40 cell media, and air and laboratory environment. All cell culture laboratories must implement effective
41 practices to ensure these four domains are compliant with necessary quality practice, (Ryan 2005).
42 Media incorporating antibiotics are efficient against many bacterial species, but they cannot protect
43 cultures against all bacterial contaminants, especially mycobacteria or corynebacteria, (Lelong-Rebel IH
44 2009). Some bacteria and other pathogenic microbes bind to the host cell surface and then become
45 internalized via microbial invasion. Intracellular bacteria can resist treatment by aminoglycoside
46 antibiotics, whereas bacteria bound to the outside of mammalian cells are rapidly killed. Cryostorage may
47 also be a mechanism for contamination transfer, particularly, viral, (Mirabet V 2012).
48 Routine successful cell culture is highly dependent upon adherence to very secure and aseptic practices.
49 Pen-strep and other antibiotics should not routinely be incorporated in media, because they will risk
50 increased bacterial resistance, enhance mycoplasma inclusion and selected intracellular reactions such as
51 protein and ATP synthesis, (Freshney 2000; Coecke S 2005).
52

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

57 There have been many studies analysing the use of molecular assays for the detection of positive bacterial
58 contamination (Marlowe 2003). PCR screening assays provide fast, dependable and cost-effective
59 methods for quality assessment, ultimately resulting in faster product release and product optimization –
60 detection can be based on generic primer sequences to maximize detection of difference bacterial strains,
61 (Jimenez 2001; Lleo 2005). The highly conserved bacterial ribosomal DNA sequence has been employed
62 in PCR-based assays to determine sterility of pharmaceutical samples, (Jimenez 2007). Nucleic acid
63 amplification has been described as a significant improvement in technology for microbial research
64 laboratories and microbial diagnostic industries, due to sensitivity and capacity to be automated, (Nocker
65 2008).

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

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70 While many undesirable organisms may consume nutrients from cells lines in cultures, they may also
71 exploit cells themselves. Predatory bacteria have been shown to feed on other bacteria (KL. 2007;
72 Blazkova H 2009), particularly in a limited nutrient environment, (Nandy SK 2006). Experimental results
73 may also be altered due to unwanted activation of cells. Different cellular functions, including those
74 triggered by tool-like receptors, can be activated by variety of bacterial components, (Blazkova H 2009;
75 Testro AG 2009; Zenk SF 2009).

76 Highly biologically reactive molecules have major influences *in vivo* on humoural and cellular systems. Endotoxin
77 residues affect the growth or performance of *in vitro* cultures and are a significant source of experimental variability,
78 (Case Gould 1984; Ryan 2005).
79 Frequency and absence of formal contaminant detection is reflected in several online science blogs that
80 describe cell culture contaminants that resemble black specks in the cell culture
81 (http://www.youtube.com/watch?v=lo96fcXfcVs ,http://www.scientistsolutions.com/t11616-
82 black+dots+in+cell+culture.html,
83 http://www.researchgate.net/post/Human_cancer_cell_line_culture_contamination).The researchers on
84 these blogs describe black dots that are mobile and resemble rods and dots in more detail, implying *bacilli*
85 and *cocci*. More recently, intracellular *Achromobacter* have been shown to represent a cell culture
86 problem due to scale, some cell tolerance and antibiotic resistance, but which are confirmatory detectable
87 by 16S rDNA analysis, (Jennifer Sue Gary 2010). The latter paper is expressing intracellular mobile
88 'black dots or rods', is in agreement with numerous cell culture on line questions and blogs, which tend to
89 lack appropriate technical descriptions and analysis. They also tend to fail to indicate whether the
90 contaminants were intrinsic in the cells on receipt or were a consequence of handling error.
91 This study took cognisance of prior findings, but was stimulated by a period of apparent
92 intracellular contaminant presence in selected hepatocyte cell lines, detected within 24 hrs of
93 culture following cryo storage, that was compliant with approved quality procedures. The full
94 evidential origin of this contamination has yet to be confirmed, whether a unique and rare
95 mammalian endosymbiont, a new mode of external transfer, or sub-optimal sera, but current
96 sequence evidence at least implies a novel bacterial strain.

97

98 ***Materials and Methods***

99 ***In vitro* culture**

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

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104 C3A cells were cultured in Minimum Essential Medium Eagle (MEM) with 10% FCS, 2 mM L-
105 glutamine, 1 mM sodium pyruvate, and 1% non-essential amino acids, at 37 °C and 5% CO_2. All
106 experiments were conducted using cells between passage 7 and 25. 100 U/ ml penicillin/
107 streptomycin could be added transiently to media for new set-up cultures or when a
108 contamination risk was perceived all media constituents were sourced from Sigma Aldrich
109 Ireland.

110 **Cell Lysate preparation**

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

116 **DNA Extraction**

117 To ensure the isolation of the microbe genome and process of freeze-thaw using liquid nitrogen
118 and 60 C bath to lyse the spore-like microbe, and a Sigma Aldrich kit for genome extraction (Gen
119 Elute Bacterial Genomic DNA Kit), was applied. Centrifuged pellets were suspended in a 500 µl of
120 TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The DNA concentration was determined by A260/280
121 nm absorbance spectroscopy of 2 µl samples using a *Picodrop* spectrophotometer.

122 **DNA amplification**

123 59 ng of DNA was added to 50 µl of PCR reaction mixture using a Bioline Kit. The primers of 16S genes
124 are listed in Table 1. The reaction was run using the following cycling parameters: 95 ºC for 4 min, 30
125 cycles of 30 sec at 95 ºC, 30 sec at 55 ºC and 45 sec at 72 ºC , with final elongation step of 10 min at 72 ºC
126 before a 4 ºC hold. (RoboCycler® Gradient 96, Stratagene). 10 µl of the reaction mixture was separated
127 on 1% TEA agarose with ethidium bromide at 8V/cm and the reaction product was visualized and scanned
128 by a Gel doc/UV trans-illuminator (Syngene). The PCR product was purified with a Qiagen gel extraction
129 kit. Several quality control steps, including negative control for masteries used in preparing cell culture
130 and positive control such as Bacterial genome, where included which conducting this experiments.

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

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136 **Differential Giemsa staining**

137 Cells were seeded at $5x10^3$ per 50 µl on cover slips and cultured for 72- 96 h at 37 ºC without changing
138 medium. The cells were then washed with phosphate-buffered saline (PBS), and fixed with 3:1
139 methanol/acetic acid for 10 min at room temperature. Cells were immersed in a Giemsa solution (10%
140 v/v) for 15 min at room temperature. Staining was followed by rinsing the cover-slips for two to five
141 minutes in phosphate buffer, air-dried, and mounted on microscope slides in DPX (1:1 glycerol: PBS) and
142 examined under an oil-immersion objective at overall 1000x magnification (Leica SP5 & Leica Diaplan).

143 **TEM**

144 Cross sections of C3A cells were prepared as follows. The cells were fixed with 3% glutaraldehyde in 0.2
145 M cacodylate buffer (pH 7.4) at 4°C for 2 h and post fixed in 1% OsO_4 in cacodylate buffer at 4°C for 1 h.
146 After dehydration in a graded series of ethanol concentrations, the cultures were embedded in a 2-mm-
147 thick Epon coating in a tissue culture well and polymerized for 3 days at 60°C. Suitable areas were
148 reoriented either parallel or perpendicular to the cell layer surface on Epon blocks with an Epon mixture
149 and then sliced in an ultra microtome. Ultrasections were contrasted with uranyl acetate and lead citrate.
150 TEM images were generated using a Jeol 2100 instrument.
151

152 **RESULTS**

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

158
159 If mammalian cells cultured for the first 36 hours under defined and approved conditions after cryo
160 storage, following procurement from an external source, show intracellular, but not extracellular evidence
161 of foreign particle contamination, this is a potentially novel finding.

162 The absence of extracellular contamination was confirmed by cell lyses and supernatant culture on
163 different agar plates (blood, brain and heart infusion, MacConky agar, Mueller Hinton agar, nutrient
164 agar), all of which showed no visible bacterial growth.
165
166 The photomicrograph in Plate I clearly shows the presence of numerous particles that resemble
167 bacteria inside the cells. Most of the bacteria observed were enclosed by endocytic vacuoles. In
168 addition, some bacteria were free in the cytoplasm, perhaps as a result of escape from endocytic

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169 vacuoles by bacterium-induced lyses of the vacuole membrane, (Jerome Boudeau 1999).
170 Application of traditional gram –ve and +ve staining did not however generate accepted
171 outcomes and the Plate I type of imaging did not support normal relatively intense staining. The
172 extended culture time, post confluence with modest final pH decline facilitated and accelerated
173 cytoplasmic bacterial growth and population scale. Figure 2 represents a similar intracellular
174 domain with DAPI stain, confirming the scale of DNA containing particles – their magnitude is
175 considerably more than indigenous mitochondria. Size of Micrococcus bacteria was ranging
176 between 0.5mm – 1 mm, both TEM and LM images supported the presents of Micrococcus,
177 diplococcic and tetracoccus bacteria, which eliminate the possibility of mitochondrial
178 resemblance.

179 TEM was performed on cell monolayers to contribute to the identification of any intracellular
180 microorganisms. Bacteria like particles were observed adhered closely to C3A cells in Fig.3 & 4.
181 The adhered bacteria strikingly induced the elongation of microvilli from the cell surface. At the
182 site of close contact between the bacteria and the epithelial cell, the elongated microvilli
183 surrounded the adherent bacteria. In addition, dense area of staining, possibly related to an
184 accumulation of cytoskeleton components were observed beneath the sites of intimate contact,
185 (Jerome Boudeau 1999).

186
187 The DNA genome extracted from C3A cells and universal primers were used for the amplification and
188 sequencing of the 16SrRNA fragment. Efficient extraction to secure sufficient contaminant DNA for
189 identification and analysis required extended cell growth and combined gram +ve/-ve extraction
190 processes. The first primer set PCR product gave multiple bands, while the second one gave only one
191 band. The bands were exited, and cleaned up using a Qiagen Kit for cleaning agarose gel. Most fragments
192 from the first primer set showed 100% similarity to the human genome, while one fragment of the
193 16SrRNA (size of ~550 bp) had no similarity to human DNA. Based on the phylogenetic tree (Figure1),
194 this implied a relationship between the amplified fragment and selected representatives of an *Escherichia*
195 strain bacteria. Comparison of test fragment 16S (~550 bp) data to known sequences of BLASTn (NCBI)
196 database confirmed that the sample gene sequence had 98% sequence similarity with *Escherichia* coli
197 partial 16S rRNA and 98% sequence similarity to uncultured bacterium clone SHZB491 16S rRNA. The
198 sequence data indicative of intracellular prokaryotic presence was proportionately less than the visual
199 results.
200 Second primer set PCR product about of ~ 1400 base pairs in size is shown in Table 2. The resulting
201 sequence was checked for similarity to other known sequences using NCNI's BLAST and Ribosomal

202 Database Project (RDP). The sequence shared 99% similarity with 16S rDNA gene sequence of an
203 uncultured organism Clone ElUO124-T3104 and 99% Similarity to Escherichia fergusinii strain KRT1
204 16D ribosomal RNA gene partial equene.

205
206 The sequence data therefore confirms that the isolate is a member of the bacteria genus. The similarity
207 rank program classifier (ECOLOGY 1999-2011) accessible in a ribosomal database project (Wang. 2007)
208 classified the sample fragment as a novel genome species of bacteria genus with a confidence threshold of
209 98%. To support this novelty and provide a basis for future and collaborative analysis, this 16S sequence
210 data was submitted to the EMBL for accession number approval. An accession number is a unique
211 identifier of sequence data to support tracking of emergent versions over time. However, allocations of
212 accession numbers do not intrinsically imply a unique sequence. The GenBank accession numbers are:
213 HE994466 isolate contaminant A and HE994467 isolate contaminant B.

214 ***Discussion***

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

222 In most cell types, lipid droplets are usually less than 1 µm in size, although in hepatic steatosis, they may
223 reach 10 µm - next to the endoplasmic reticulum, mitochondria, and peroxisomes, with some restricted
224 mobility,(Tobias C. Walthera and Robert V. Farese 2009),(Reue 2011).
225 The liver is a central organ for lipid metabolism and in hepatocytes, lack of TGH (triacylglycerol
226 hydrolase) expression alters lipid droplet morphology and dynamics. Increased (phophatidylcholine) PC
227 synthesis observed in TGH-deficient hepatocytes may result in smaller LDs, (Wang 2010).
228 Lipid droplets are indeed, organelles expressed in virtually all cells from bacteria to mammals and in
229 addition to lipid synthesis, are involved in catabolism/E release, and trafficking, (Yang 2013).
230 All microscopy and sequence data generated in this study did not support the initial belief that detected
231 mobile particles could be indigenous.
232 For bacteria to be permanent occupants of eukaryotic cells, they must generate energy but impose
233 negligible impact on the host. Foreign organisms occupying cells of another without pathological
234 consequences are considered an endosymbiont. It is obviously generally accepted that the organelles,
235 mitochondria in all eukaryotes and chloroplasts in photosynthetic plant originated from prokaryote
236 occupants. Endosymbionts as part of their host adaptation lose many essential genes but maintain a core

237 genome to provide some useful functions to their hosts, which in turn provide the bacteria with physical
238 protection and essential nutrients, (Kumar S 2011). A genome constructed to encode 387 protein-coding
239 and 43 structural RNA genes could sustain a viable synthetic cell, which has effectively been supported by
240 the JCVI, (Glass JI 2006).
241 In extreme forms of symbiosis, the host may benefits enormously from the bacterial interface, such as,
242 *Olavius algarvensis,*(Woyke 2006) . Available data assumes that the majority of symbionts interface with
243 non-mammalian species, but there is now an acceptance that from a health and clinical perspective,
244 symbiosis and human engagement does require more research, particularly with regard to potential
245 immune deficiency, (Margulis 2006). All eukaryotic organisms, including humans, obviously host a
246 significant proportion of bacteria, particularly in the intestine, but at *individual in vitro* cell level, the
247 potential for stable, mutual, not readily detectable cohesion is remote.
248
249
250 Until recently, there was a selected belief in the existence of nanobacteria, which were considered to
251 access cells from approved serum, to become relatively low density stable occupants and to partially
252 manifest via impact on vacuolisation (Galvez J 1997). It is now accepted that these perceived nano
253 particles are in fact mineral nanoparticles although the scale of such nano particle presence may still
254 influence aspects of cell function and imagery (Pan Y 2009).
255 This research was motivated by a selective identification of what appeared to be particle contaminants of
256 new hepatocyte cultures, with no replicable evidence of traditional external bacterial presence and transfer.
257 It has confirmed the presence of nuclear materials in the cytoplasm of cultured cells using DAPI nucleic
258 acid staining, (Fig. 2). Giemsa staining (Fig.1) did show many spore-like structures in the cytoplasm
259 mainly sized 0.5-1 microns.
260 Intracellular bacterial numbers in host cell line are normally very low, immobile and with detectable effect
261 on the host. Prolonged and accelerated culture that enhanced bacterial population number and proportion
262 of cells affected, resulted in greater visible detection and evidence of intracellular bacterial mobility.
263
264 It must be emphasised that all cell lines subject to this analysis were deemed contaminant free by all
265 approved test protocols performed by the providers and replicated in our own laboratory. Furthermore,
266 these cells demonstrated normal cell proliferation, turnover and adherence. As previously indicated, the C3A
267 line is a patented, highly selected subclone of Hep G2 that retains many of the properties of primary human
268 hepatocytes. They exhibit strong contact inhibition at confluency, high expression of albumin (generally 25µg/mg
269 total cell protein/24 hrs) and high albumin/alpha-fetoprotein at confluency, (usually 25µg/mg total cell protein/24
270 hrs), (Jun-Qiang Zhang 2010). As the cells become confluent, there is a marked reduction in AFP secretion and an
271 increase in albumin secretion and they also show nitrogen metabolizing activity comparable to perfused rat livers.
272 The secretion of hepatic proteins in considerable amounts is mediated by microvilli, (Henics T 1999). It is accepted
273 that mammalian cytosol cannot readily support bacterial replication and sustain prokaryote presence in a non-

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274 reactive way. Only few bacteria species, facultative intracellular pathogens, have been found to efficiently replicate
275 in cytoplasm after microinjection, eg *Shigella* spp., the related entero-invasive *E. coli* strains, and *L. monocytogenes*,
276 (Falkow 1992; M. Pilar Francino 2006). Evidence for the former species is in agreement with data generated in this
277 study and supports the belief that effective adaptation of bacterial metabolism to the host cell environment is critical
278 for successful replication in a foreign cell environment.

279

280 The entire sequence of the 16S rRNA is approximately, 1500 bp and a 0.5 – 1% differential would be
281 expected to embrace a new taxon, which is again supportive of these findings, that the bacterial occupant
282 in these cells is a new species, (Raoult 2005; Kumar S 2011). However, it is accepted that further
283 confirmatory work must be performed to ensure that no new and subtle basis for external contamination
284 occurred or the visual data complies more effectively with non-contaminant modified intracellular
285 particles and the novel sequence data represents gene transfer or post experiment moderation. For
286 example DMSO residue in media can increase hepatocyte albumin production and resultant cytoplasmic
287 morphology and particle presence, (Isom HC 1985), and exposure to selected xenobiotics may increase
288 cell vacuolisation with potential subsequent apoptosis or autophagy and enhanced and partially mobile
289 vacuoles can be misinterpreted as cytosolic occupants, (Jun-Qiang Zhang 2010). However, the
290 microscopy imaging did not support these possible processes.
291 Bacterial species have at least one copy of the 16S rRNA gene containing highly conserved regions
292 together with hyper variable regions. The use of 16S rRNA gene sequences to identify new strains bacteria
293 is gaining momentum in recent years (Vimlesh Yadav 2009). This work currently supports the use of 16S
294 rRNA gene sequence to characterize a bacterial isolate from mammalian cell lines. The further work will
295 confirm the intracellular sustainability of this bacterial strain and its minimal negative impact on the host
296 cell. It is accepted that even more immediate time work will be conducted to confirm that these
297 prokaryotes are indigenous in the hepatocytes and are having no significant impact on host behaviour or
298 expression.

299

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301

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309

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Table 1(on next page)

List of primers sequences used for the analysis

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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' GGAATTCAAAKGAATTGACGGG-3'	5' CGGGATCCCAGGCCCGGAACG-3'
Universal	27f –AGAGTTtGATCVTGGCTCAG	1492r-AACCTTGTTACGATT

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Table 2 (on next page)

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

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

Sequence	Accession
ccaagttcaa gactgatgtg ctatgggtgt ggatacgtat attccactcc tttacaatct 60 attacttgta cccgcactgc ttgagccaca ataaaaatct tctatgaagg ccctatgttg 120 ggcttaagta tatacccgcc tatgtgacca tctatggctc tactaatatg atctccagtc 180 cgaaaggatt taaatcagag gataacacgg aagataatac tatatgggga ttattgctca 240 acggggctga gcctgtagca cccatgacgc gtgaatcaat tatgacttcg ggttgtaaag 300 tactttcagc ggggaggaag ggagtaaagt taataccttt gctcattgac gttacccgca 360 gaagaagcac cggctaactc cgtgccagca gccgcggtaa tacggaaggt gcaagcgtta 420 atcggaatta ctgggcgtaa agcgcacgca ggcggtttgt taagtcagat gtgaaatccc 480 cgggctcaac ctgggaactg catctgatac tggcaagctt gagtctcgta gaggggggta 540 gaattccagg tgtagcggtg aaatgcgtag agatctggag gaataccggt ggcgaaggcg 600 gccccctgga cgaagactga cgctcaggtg cgaaagcgtg gggagcaaac aggattagat 660 accctggtag tccacgccgt aaacgatgtc gacttggagg ttgtgccctt gaggcgtggc 720 ttccggagct aacgcgttaa gtcgaccgcc tggggagtac ggccgcaagg ttaaaactca 780 atgaattgac gggggcccgc acaagcggtg gagcatgtgg tttaattcga tgcaacgcga 840 agaaccttac ctggtcttga catccacaga cttttccaga gatggaattg gtgccttcgg 900 gaaactgtga gacaggtgct gcatggctgt cgtcaagctc gtgttgtgaa atgttgggtt 960 aagtcccgca acgagcgcaa ccctttatcc tttgttgcca gcggtccggc cggggaactc 1020 aaggagactg ccaggtgata aactggaggg aaggtgggg atgacgtcaa gtcatcatgg 1080 gcccttacga ccagggccta actcacgtgc tacaatggcg catacaaaga agaagcgacc 1140 ttcgcgagag ccagcggact tcataagtgg cggtcgtagt ccggattggg agtctgcaac 1200 tcgacctcca ttgaagctcg gaatccgcta gtattcgtgg aatccagaat gccacggttg 1260 aattacgttc acgggtcctt gtaactaccc gtcccggtca actcatggga agttgaaatt 1320 gccaaagaaa gctacgtgag tctttatgcc tttgcgacgg tcttagcacg tttgtgggta 1380 attccatgag tactctggat g 1401	Accession#: HE

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gttaatacct ttgctcattg acgttacccg cagaagaagc accggctaac tccgtgccag 60	Accession#: HE
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Figure 1

Giemsa stained cells

Light microscope image showing ‘transparent’ round shape bodies (arrow) observed adjacent to lysed Giemsa stained cells, nucleus and cytoplasm are stained with purple and pink colour

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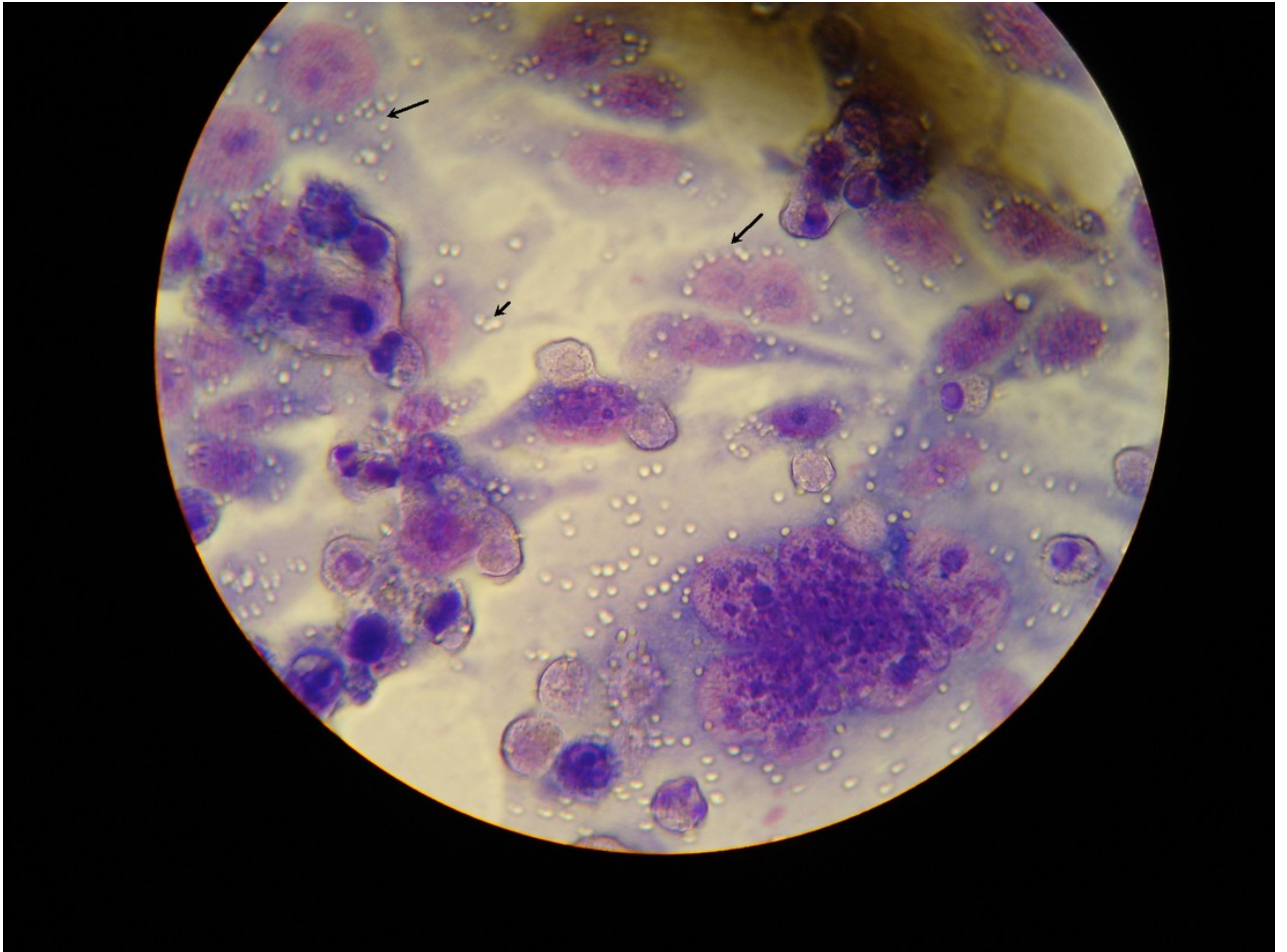

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Figure 2

DAPI Stain

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).

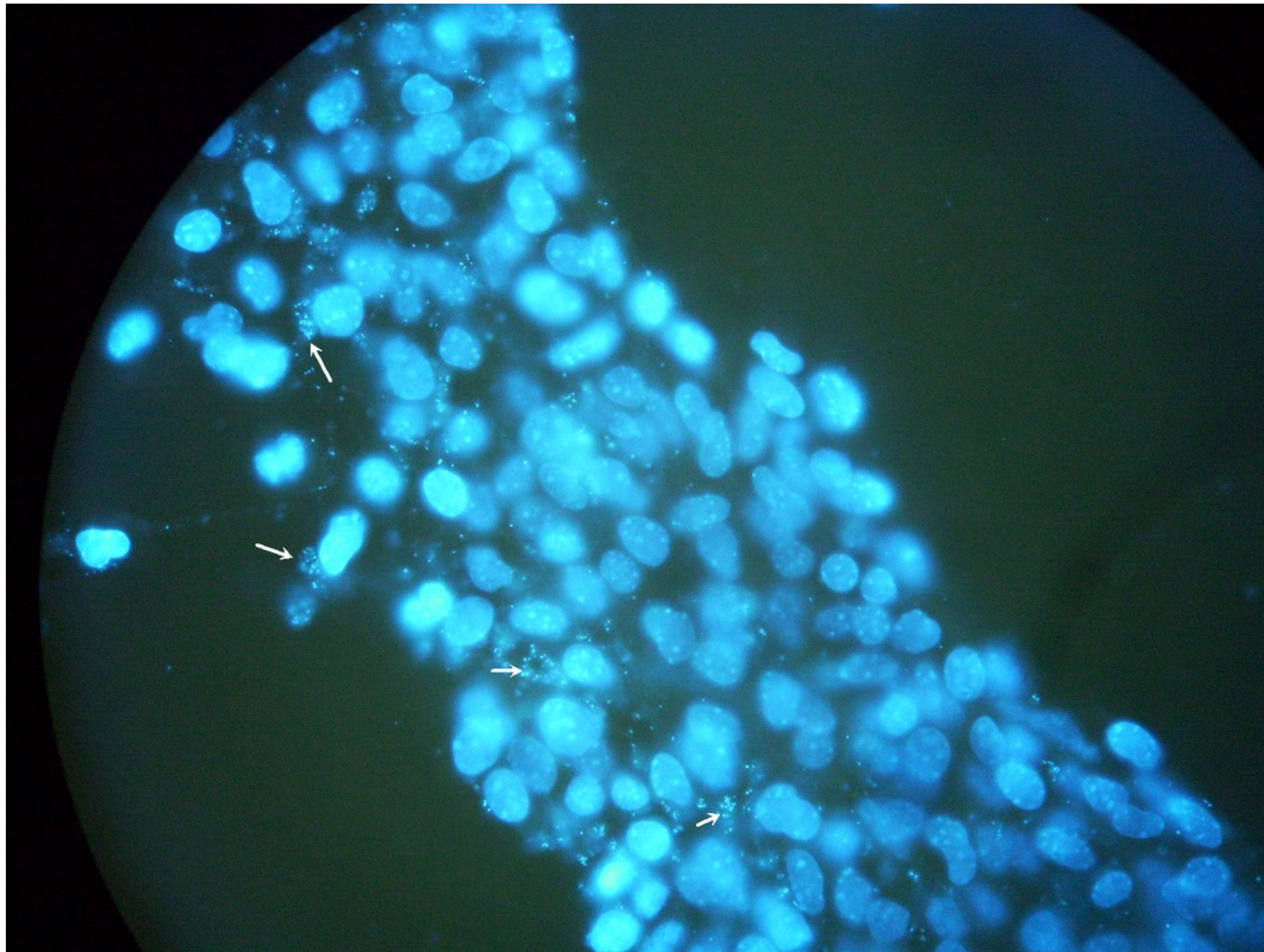

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Figure 3

TEM micrographs of C3A cells infected with unknown bacteria

Cross section of the cells monolayer showing numerous intracellular bacteria. Micrograph showing membrane 'ruffling' upon contact with bacteria. Bacteria are engulfed by elongated microvilli from infected epithelial cells.

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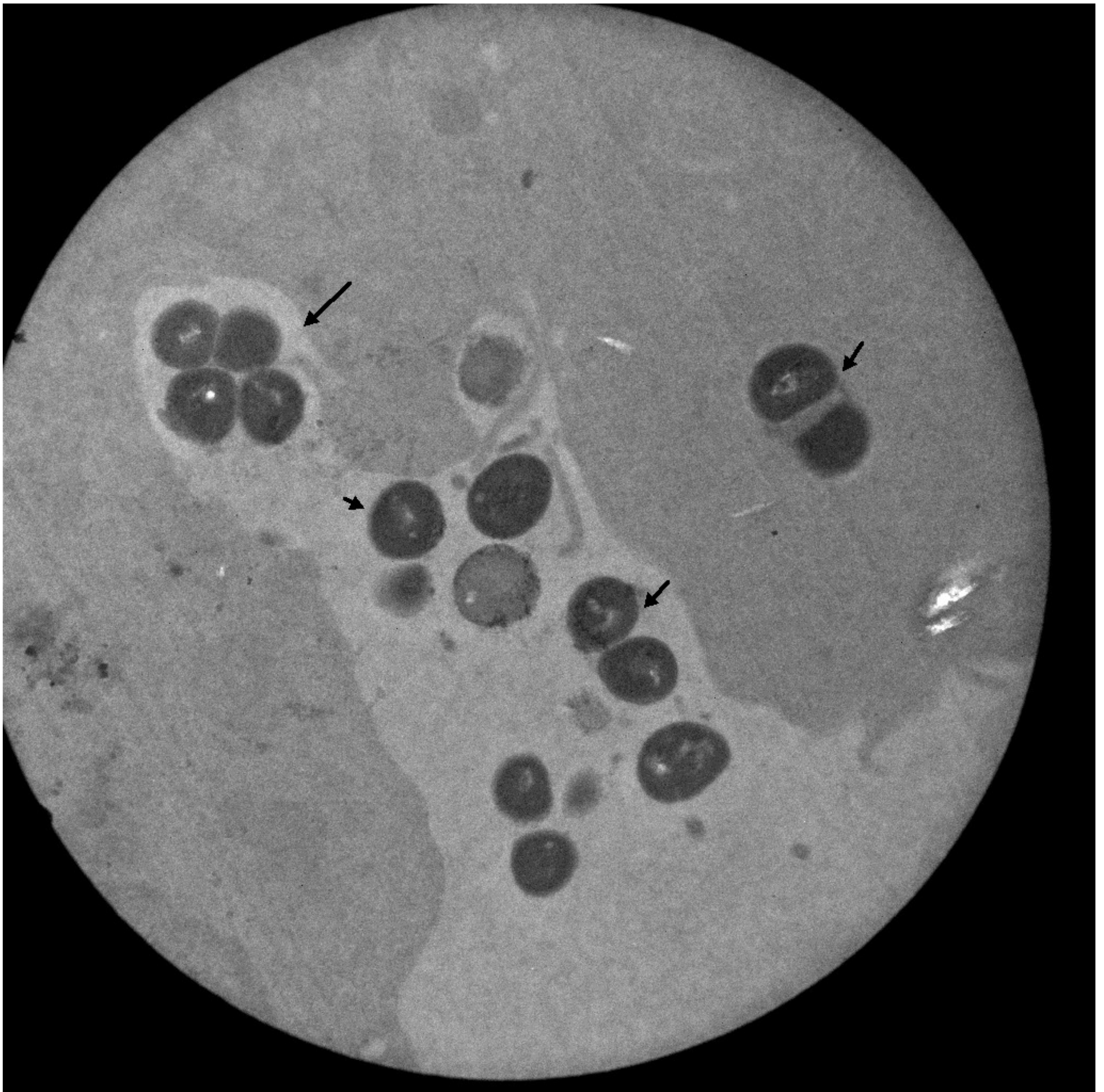

Figure 4

TEM micrographs of C3A cells infected with unknown bacteria (~ 1 m m 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. <?xml:namespace prefix = o ns = "urn:schemas-microsoft-com:office:office" />

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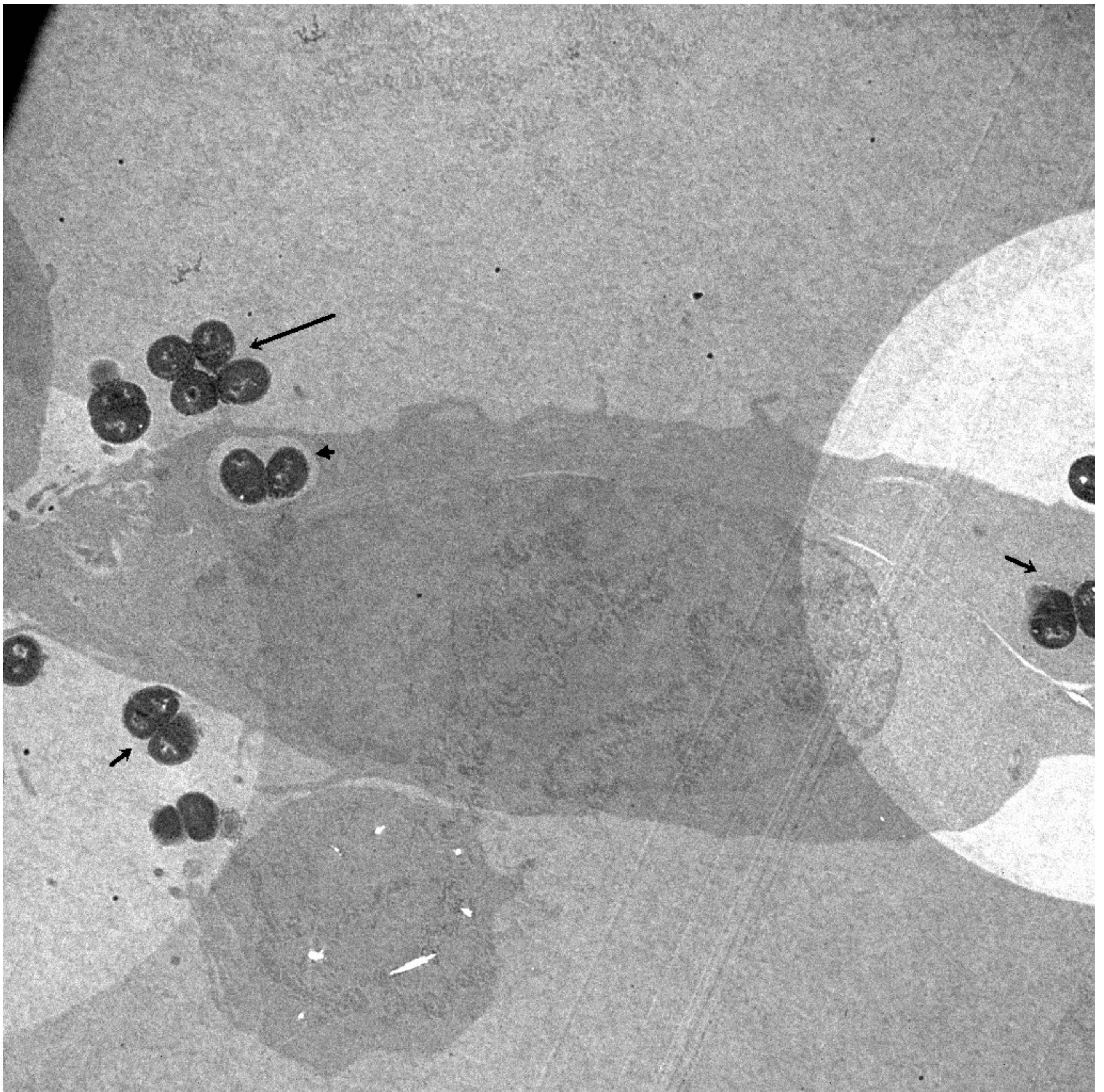

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