1 **Chlamydial clinical isolates show subtle differences in persistence phenotypes and growth**

2 ***in vitro***

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Abstract

Urogenital *Chlamydia trachomatis* infection is the most common sexually transmitted bacterial infection throughout the world. While progress has been made to better understand how type strains develop and respond to environmental stress *in vitro*, very few studies have examined how clinical isolates behave under similar conditions. Here, we examined the development and persistence phenotypes of several clinical isolates, to determine how similar they are to each other, and the type strain *C. trachomatis* D/UW-3/Cx. The type strain was shown to produce infectious progeny at a higher magnitude than each of the clinical isolates, in each of the six tested cell lines. All chlamydial strains produced the highest number of infectious progeny at 44 h PI in the McCoy B murine fibroblast cell line, yet showed higher levels of infectivity in the MCF-7 human epithelial cell line. The clinical isolates were shown to be more susceptible than the type strain to the effects of penicillin and iron deprivation persistence models in the MCF-7 cell line. While subtle differences between clinical isolates were observed throughout the experiments conducted, no significant differences were identified. This study reinforces the importance of examining clinical isolates when trying to relate *in vitro* data to clinical outcomes, as well as the importance of considering the adaptations many type strains have to being cultured *in vitro*.

Introduction

Chlamydia trachomatis is an obligate intracellular pathogen and is the most common bacterial sexually transmitted infection (STI) worldwide. There are more than 83,000 *Chlamydia* infections recorded in Australia each year (1). The pathology and sequelae associated with chlamydial diseases are thought to be associated with the infected individual's inflammatory response and potentially influenced by a number of important host factors (2). In women, chlamydial disease ranges from mild cases of cervicitis, endometritis and salpingitis, to more serious cases of pelvic inflammatory disease (PID), tubal infertility, and life-threatening ectopic pregnancy (3-5).

Cases of urogenital *Chlamydia* are treated with either azithromycin or doxycycline. While evidence suggests doxycycline (usually 7 day regimen) is slightly more effective at clearing infection, 1g single-dose azithromycin is often prescribed for its simple one-off treatment dose (6, 7). Azithromycin is a broad-spectrum macrolide antibiotic with a relatively long half-life (40 - 68 h), high lipid solubility, and accumulates within macrophages migrating to the site of infection (8, 9). Despite its high efficacy, instances do occur in which monitored women remain infected after treatment. The reasons for this remain unclear, but potential explanations include; reinfection from an untreated partner, chlamydial gastrointestinal colonization and auto-inoculation of the cervical site or treatment failure (10, 11). Direct macrolide resistance is generally not considered a probable cause due to the antibiotic's mechanism of action, and the scarcity of clinical isolates with validated genotypic or phenotypic resistance to azithromycin (12-16). Furthermore, mutants created *in vitro* with single nucleotide polymorphisms (SNPs) in their 23S rRNA and L4 protein-encoding genes show poor biological fitness and viability (17-20), further supporting their emergence in the population is likely to be rare.

62   When combined, *in vitro* and clinical evidence suggests that *C. trachomatis* rarely survives

63   treatment, which likely reflect both the pathogen and its intracellular niche. One candidate

64   mechanism is chlamydial persistence, which is believed to be a positive and beneficial adaptation

65   unique to the genus *Chlamydia* (21, 22). The persistence phenotype is characterised by several

66   reversible morphological, transcriptional and metabolic changes (23-27). The collective profiles

67   of these changes vary depending on the inducer of persistence; however, the morphologically-

68   aberrant chlamydial cells observed during persistence, altered inclusion sizes and the reversible

69   loss of both cultivability and replicative capacity are the universal hallmarks of chlamydial

70   persistence (reviewed (28)).

71   Several environmental conditions and exogenous stimuli have been demonstrated to induce

72   chlamydial persistence *in vitro* (29). Penicillin and IFN-γ are two of the most extensively studied

73   of these stimuli, and have both been used to characterise different aspects of the persistence

74   phenotype, as reviewed by Wyrick (30). Use of the iron-chelating agent deferoxamine mesylate

75   has shown that iron restriction not only induces persistence in *C. trachomatis*, but also alters the

76   pathogen's signaling pathways that modulate host-cell apoptosis (31, 32). Interestingly, IFN-γ

77   also decreases cellular levels of iron in infected cells by downregulating their transferrin receptor

78   expression (33).

79   Previous investigations into chlamydial persistence have established that type strains have

80   variations in their degree of responsiveness to certain stimuli, and have altered susceptibilities to

81   antibiotics while in the persistent state (34-37). In the present study, we examined the *in vitro*

82   phenotypes of selected clinical isolates of *C. trachomatis* isolated during the Australian

83   *Chlamydia* Treatment Study (ACTS)(11). Their relative abilities to infect and develop in

84   different cell lines were measured, as were their responses to two widely used and

85   physiologically relevant models of *in vitro* chlamydial persistence. Their susceptibility to

86   azithromycin during persistence was also investigated, to better understand whether persistence

87   has implications for treatment with frontline antibiotics when analysed on recent clinical isolates.

88

89   **Methods**

90   **Cell culture and cultivation of *Chlamydia***

91   McCoy B, MCF-7, CACO-2, HeLa, SiHa, and ARPE-19 cell lines (details in **Table 1**) were

92   cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % vol vol^{-1} fetal

93   calf serum (FCS; Sigma), 4 mM analyl-glutamine (Sigma), 100 µg ml^{-1} streptomycin (Life

94   Technologies) and 50 µg ml^{-1} gentamicin (Life Technologies). A range of cell types available to

95   the study were selected to profile any possible differences in the clinical isolates. All cell lines

96   were incubated at 37 ˚C in a humid environment containing 5% CO_2. All cell lines were

97   regularly confirmed to be free of *Mycoplasma* contamination using either an in-house PCR, or

98   the Mycoalert™ Plus Mycoplasma Detection Kit assay (Lonza). All six clinical isolates used

99   were obtained from the Australian *Chlamydia* Treatment Study (ACTS). This was a cohort study

100   of women diagnosed with and treated for genital *Chlamydia* (11) to examine factors associated

101   with repeat chlamydia infection. In one case, two isolates from one participant after a repeat

102   positive event were included in the study in the event that they prove to have some detectable

103   difference in persistence phenotypes to analysed here. Isolates were collected from infected

104   women using swabs stored in a 2 ml cryovial tube containing a sucrose-phosphate glutamate

105   (SPG) buffer, at -80˚C, and couriered on dry ice. A unique code has been generated to identify

106   each isolate purely for the purposes of this paper.

107

108   Isolates were cultured from the original cervical swabs from women using a series of culture

109   steps to attain sufficient culture for these experiments (6 to12 passages depending on the isolate).

110   Stocks were stored and propagated from sucrose phosphate glutamate (SPG) buffer which

111   consisted of 5 mM glutamic acid, 10 mM sodium phosphate, 250 mM sucrose balanced to pH

112   7.4. The five clinical isolates used in this study are shown in **Table 2**.

113

Table 1. Mammalian cell lines used in this study

Name	Cell Type	Origin	ATCC® Code (™)
McCoy B	Fibroblast	Fibroblast from *Mus musculus*	CRL-1696
MCF-7	Epithelial	Human mammary epithelium	HTB-22
CACO-2	Epithelial	Human colorectal adenocarcinoma	HTB-37
HeLa	Epithelial	Human cervical carcinoma	CRM-CCL-2
SiHa	Epithelial	Human cervical squamous cell carcinoma	HTB-35
ARPE-19	Epithelial	Human adult retinal pigmented epithelium	CRL-2302

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Table 2. Identification and details of the clinical isolates used in this study.

ACTS code (code)	*ompA* genotype	Clinical outcome
600 (1)	K	Repeat infection, first detection
600 (13)	K	Repeat infection, second detection

620 (1)	D	No repeat infection
628 (1)	E	No repeat infection
649 (1)	K	No repeat infection, genotypically close to 600 (1)

Infectivity and determination of viable progeny

McCoy B, MCF-7, CACO-2, HeLa, SiHa and ARPE-19 cells were cultured in 96-well plates, in triplicates for each condition and experimental analysis conducted. Cells were infected with each of the five clinical isolates and type strain D/UW-3/Cx at a multiplicity of infection (MOI) of 0.5. Cultures were immediately centrifuged at $500 \times g$ and $37°$ C for 30 minutes, then incubated under standard conditions ($37°$ C, 5% CO_2). At 4 hours post infection (h PI), the infectious media in each well was replaced with complete DMEM supplemented with 1 µg ml^{-1} cycloheximide before further incubation. To determine the infectivity of the isolates in each cell line cultures were fixed with 100% methanol at 30 h PI for evaluation by immunofluorescence and microscopy. Infectious progeny counts (inclusion forming units) of each isolate was determined from cultures harvested at 44 and 54-hour time points of infection. Inclusion forming units were determined by serially dilutions of stocks from each of the harvested cultures onto fresh cultures of McCoy B cells, which were fixed at 30 h PI for evaluation by immunocytochemistry.

ompA **genotyping**

DNA was extracted from the original swab samples collected (as per the ACTS study protocol) were swirled in 500 µl of PBS solution. A 200 µl aliquot of the swab/PBS homogenate was extracted using the MagNA Pure 96 (Roche Applied Science, Germany) automated system, according to the manufacturer's instructions and utilizing the MagNA Pure 96 DNA and Viral NA Small Volume Kit, and eluted in 100µl in MagNA Pure 96 elution buffer(11).

134   All *C. trachomatis* genotype determinations utilized a 5µl aliquot of PBS swab homogenate

135   elution, utilizing a series of qPCR amplification assays targeting the *ompA* gene of *C. trachomatis*

136   as described previously (38).

137

138   **Penicillin and iron deprivation persistence models**

139   Persistence models were conducted in MCF-7 cells. 2.5 x 10^4 MCF-7 cell monolayers cultured

140   for 24 h were infected at multiplicity of infection (MOI) 0.8 (slighly higher than the previous

141   cultures due to expected loss of some organisms in the persistence model). Cultures were

142   immediately centrifuged at $500 \times g$ and 37° C for 30 minutes, then incubated under standard

143   conditions (37° C, 5% CO_2, 95% air). At 4 h post infection (h PI), the infectious media in each

144   well was replaced with complete DMEM supplemented with 1 µg ml^{-1} cycloheximide as well as

145   0, 0.02, 0.05, or 1.0 U ml^{-1} benzylpenicillin (Pen G). 2,2'-Bipyridyl (Bpdl) was used in

146   accordance with the methods previously outlined by Thomson and Carabeo (doses of –400µM)

147   (39). Cultures were incubated until 44 h PI, where they were harvested stocked in SPG for

148   analysis of inclusion forming units, or washed and media replaced with fresh media without the

149   penicillin or supplemented with $FeCl_3$ until 96 or 110 h PI of culture and stocked in SPG. The

150   infectious yield of all cultures at each of the time points was determined by infecting serial

151   dilutions of stored stocks into monolayers of McCoy B cells using the standard protocol and

152   cultures were fixed with methanol at approximately 30 h PI, before immunocytochemistry and

153   visualisation by fluorescence microscopy. Cultures for infectivity yields were routinely

154   conducted in 96 or 48 well plates, replicate cultures were conducted in 24 well plates on top of

155   1.5mm coverslips for the cases where fixing, immunocytochemistry and imaging by microscopy

156   to eludicate phenotype was part of the experiment. Azithromycin treatment was conducted to

157   determine each isolates MICs using the methodology previously outlined (40), each isolates MIC

158   dose was then used to treat the isolate during and in the absence of the persistence inducing

159   conditions to evaluate if the isolate's capacity to survive the antibiotic treatment at the MIC

160   changed by being in persistence.

161   **Immunofluorescence microscopy**

162   Cultures were fixed with methanol, permeabilized with triton X-100 (0.5%) in Dulbecco's

163   phosphate-buffered saline (dPBS) and blocked with 1% bovine serum albumin (BSA) in dPBS

164   overnight. Once blocked, cultures were then further incubated with dPBS containing 4',6-

165   diamino-2-phenyindole (DAPI; 1/40,000) and rabbit sera (1/500) containing antibodies raised

166   against the *C. trachomatis* high-temperature requirement A (HtrA) protein for 1 h at room

167   temperature (antibody and protocol previously described (41, 42)). Following this, cultures were

168   washed four times with dPBS containing 0.2% tween 20, and then incubated with dPBS

169   containing an Alexa Fluor 488-conjugated goat anti-rabbit antibody (1/600; Thermo Fisher

170   Scientific) for 45 minutes at room temperature. The stained cultures were then washed five times

171   more with the dPBS tween solution. Cultures on 13 mm (No. 1.5) coverslips were stained and

172   labelled using the same process, with the addition of a mouse anti-α-tubulin (1/500; Thermo

173   Fisher Scientific) in the first antibody incubation, and an Alexa Fluor 546-conjugated goat anti-

174   mouse antibody (1/600; Thermo Fisher Scientific) in the second incubation. Stained coverslips

175   were mounted to clear glass slides using *n*-propyl gallate (NPG) and sealed using clear varnish

176   around the edge of the coverslip. Stained and labelled plate cultures were visualized and imaged

177   using the Nikon Eclipse Ti-S fluorescent microscope or the GE InCell 3000 high-throughput

178   fluorescent microscope. Coverslips were visualized and imaged using the Nikon Eclipse Ti-E

179   confocal microscope.

180   **Data analysis and graphing**

181   Raw data was compiled using Microsoft Excel 2010 before being transferred into GraphPad

182   Prism version 8.0.0 for Windows for statistical analysis and graphing with each value and any

183   applicable statistical testing described in the respective figure legends.

184

185   # Results

186   **Clinical isolates differed in infectivity and growth more profoundly than the differences**

187   **caused by the cell lines**

188   Six cell lines were selected, to compare how susceptible they were to infection by four of the

189   clinical isolates, and type strain D/UW-3/Cx (**Fig 1.**). The human mammary epithelial cell line

190   (MCF-7) was found to result in the highest cell infection levels after exposure to the chlamydial

191   strains for each of the four clinical isolates, with 35-40% of all counted cells becoming infected.

192   Both the retinal cell line (ARPE-19) and cervical carcinoma cell line (SiHa) were observed to

193   have the lowest percentage of infectivity (5-10%) for all four clinical isolates, while the type

194   strain was observed to still have produced inclusions in 25-30% in both these lines. The proposed

195   0.5 infectivity or MOI was calculated from an ifu/ml yield in McCoy B cells. While the type

196   strain showed a similar level of infectivity across the McCoy B, MCF-7, HeLa and SiHa lines, it

197   did show slightly lower levels in CACO-2 and ARPE-19. The same six cell lines used for the

198   infectivity assay were infected and harvested at two timepoints, to determine the number of

199   infectious progeny that had been produced during the developmental period. All four isolates

200   produced the most infectious progeny (IFU ml^{-1}) in the McCoy B cell line (**Fig. 1**). For example,

201   clinical isolate 600(1) grown in McCoy B cells yielded 1.2×10^6 at 44 h PI, while in HeLa cells, it

202   yielded 3.7×10^4 IFU ml^{-1}. Similarly, the type strain produced higher levels of progeny in the

203   McCoy B cells (9.8×10^6), in CACO-2 (9.4×10^6), and HeLa cells (7.1×10^6), compared to MCF-7

204   (1.1×10^6), SiHa (1.9×10^6) cells, and ~100-fold fewer in ARPE-19 cells (5.9×10^4). The ARPE-19

205   cell line was also shown to produce the least number of infectious progeny for clinical isolates,

206   with all four showing no detectable progeny. Less variation in the infectivity and infectious

207   progeny production from each of the clinical isolates was observed in the SiHa cell lines

208   compared to the other cell lines (**Fig. 1**).

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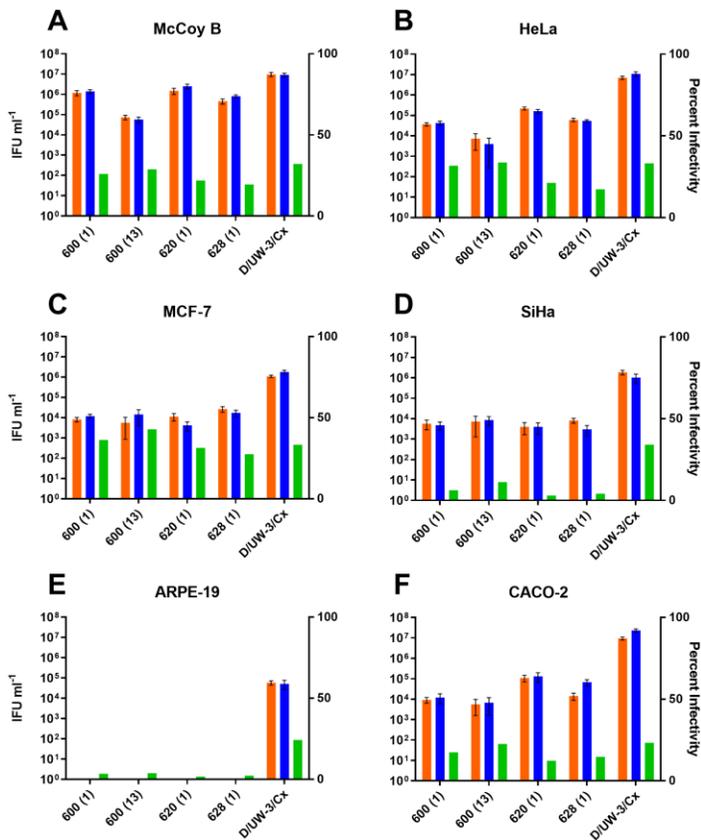
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212   **Figure 1. Infectivity and infectious progeny yield in different cell types for the clinical**

213   **isolates.** The figure shows the inclusion forming units yielded (left y axis) from each isolate

214   (orange indicates yields at 44 h PI and blue indicates yields at 54 h PI) and the % infectivity at 30

215   h PI (right y axis, green bar). Each cell line analysed includes A: McCoy B, B: Hela, C: MCF-7,

216   D: SiHa, E: APRE-19, and F: CACO2. The bars for IFU ml^{-1} include a total of n=27

217   representative images to determine the mean of a minimum of n=3 separate experimental

218   replicates, shown with standard deviation. The bars for % infectivity are representative of n= 9

219   experimental replicates. The data is shown from a single experiment, and is consistent with other

220   attempts at the experiment.

221

222   **Clinical isolates show similar susceptibilities to conditions of iron deprivation at lower**

223   **doses than type strain D/UW-3/Cx**

224   In order to more closely examine if persistence phenotypes were impacted by *ompA* genotypes,

225   and the known outcomes in participants in the ACTS trial an additional isolate was included in

226   these persistence experiments to have a close genotype to the isolate from a participant that

227   experienced repeat infection. Five clinical isolates and the type strain were cultivated in MCF-7

228   cells, treated with 100, 200 (D/UW-3/Cx only) and 400 µM bipyridal (Bpdl) ± 100 µM iron as

229   FeCl$_3$, with infectious progeny determined at 96 h PI. As seen in **Fig. 2A**, the type strain was

230   able to recover from up to 100 µM of Bpdl (7.1x10^4 IFU ml^{-1} before recovery) with the

231   supplementation of FeCl$_3$ into culture (3.1x10^8 IFU ml^{-1} after recovery). The five clinical isolates

232   showed lower recovery, even from at lowest dose of Bpdl, as seen in **Fig. 2**. For example (**Fig.**

233   **2C**), at the 100 µM dose of Bpdl with and without recovery, 600(13) yielded 3.1x10^3 and 7.0x10^2

234   IFU ml^{-1} (near the limit of detection for this assay) respectively. All five isolates produced

235   infectious progeny in a manner that was dose-dependent, with slightly lower levels of infectious

236   progeny present in cultures treated with 400 µM than those treated with 100 µM.

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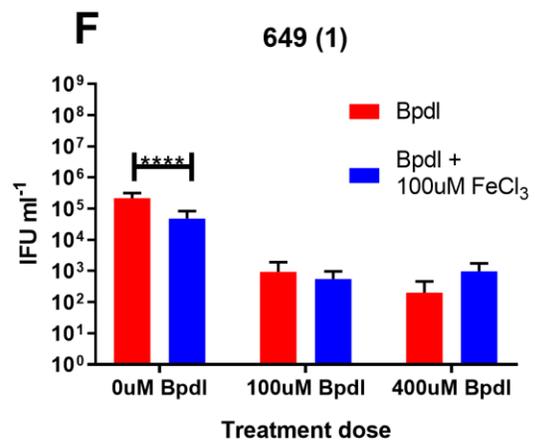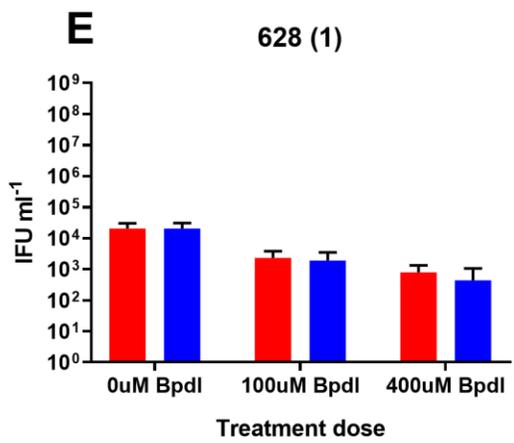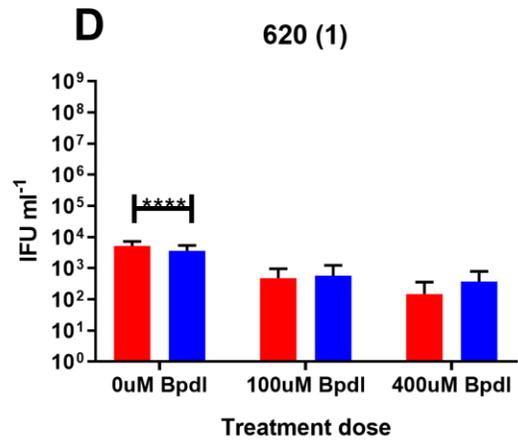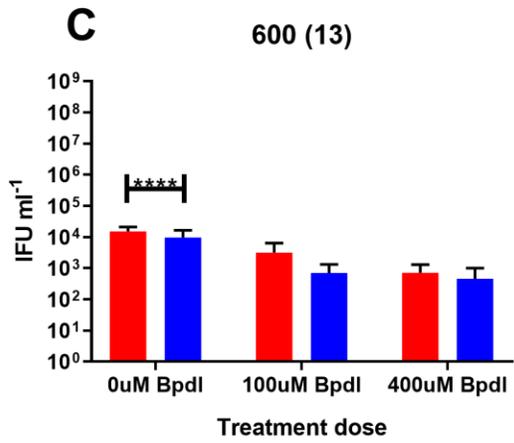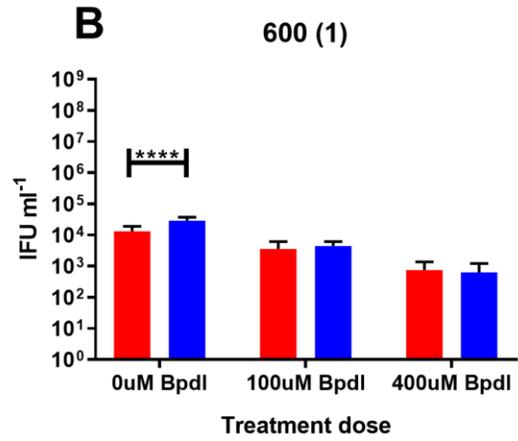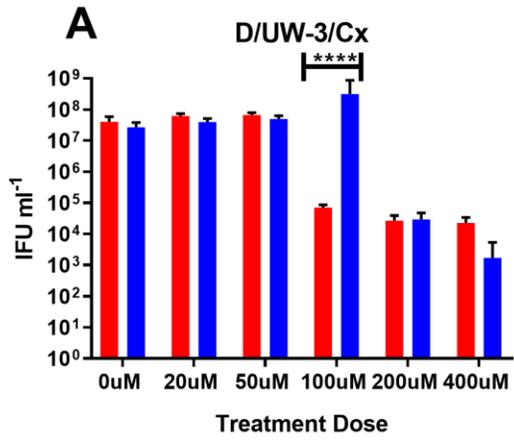
240   **Figure 2. Iron deprivation related persistence and recovery of the clinical isolates.** The

241   graphs show the infectious progeny produced by type strain D/UW-3/Cx (A) and five clinical

242   isolates (B: 600(1); C: 600(13); D: 1-020(1); E: 628(1); F: 649 (1)) in MCF-7 cells treated with

243   Bpdl $\pm$ 100 µM iron as $FeCl_3$ for recovery. Each bar represents the mean of three experimental

244   replicates analysed using n=10 separate fields of view from n=3 experimental replicates with

245   error bars showing the standard error of the mean (SEM). Data was analysed by way of a 3-way

246   ANOVA using GraphPad Prism version 8.0.0 for Windows. Significant differences are indicated

247   by **** p<0.0001. The data is shown from a single experiment, and is consistent with other

248   attempts at the experiment.

249

250   **Treatment with azithromycin during iron deprivation-induced persistence decreases the**

251   **number of recoverable infectious progeny among clinical isolates**

252   Each of the five clinical isolates and the type strain were cultured in MCF-7 cells treated with

253   Bpdl, subsequently treated with azithromycin (Az), and then recovered with supplementation of

254   iron. The azithromycin dose used was the MIC that had been determined for each isolate. The

255   MICs were as follows: 600 (1): 0.032 µg/ml, 600 (13): 0.125 µg/ml, 620: 0.032 µg/ml, 628:

256   0.064 µg/ml; 649: 0.064 µg/ml, and D/UW-3/Cx: 0.064 µg/ml. All strains showed a significant

257   decrease in infectious progeny at 44 h PI when treated with azithromycin, Bpdl, or both (**Fig. 3,**

258   p<0001). Compared to the untreated control at 44 h PI (5.4×10^5 IFU ml^1), D/UW-3/Cx showed

259   an impaired ability to recover from the effects of Bpdl when also treated with azithromycin

260   (2.9×10^4 IFU ml^{-1}), even with iron supplementation (5.0×10^2 IFU ml^{-1}). Compared to their

261   respective untreated controls at 44 h PI (**Fig. 3**), all five clinical isolates were found to have only

262   slight differences in their resulting infectious yield when treated with the combinations of

263   azithromycin, Bpdl, and FeCl$_3$. For example, the untreated control 600(13) culture produced

264   2.2×10^4 compared to only 1.5×10^3 IFU ml^{-1} after treatment and recovery. Analysis of the cultures

265   by confocal microscopy showed morphologies consistent with persistence, as inclusions visible

266   were consistent with persistence after treatment with penicillin; and regular development after

267   allowing for recovery from the drug (**Fig. 4,** representative images from some isolates).

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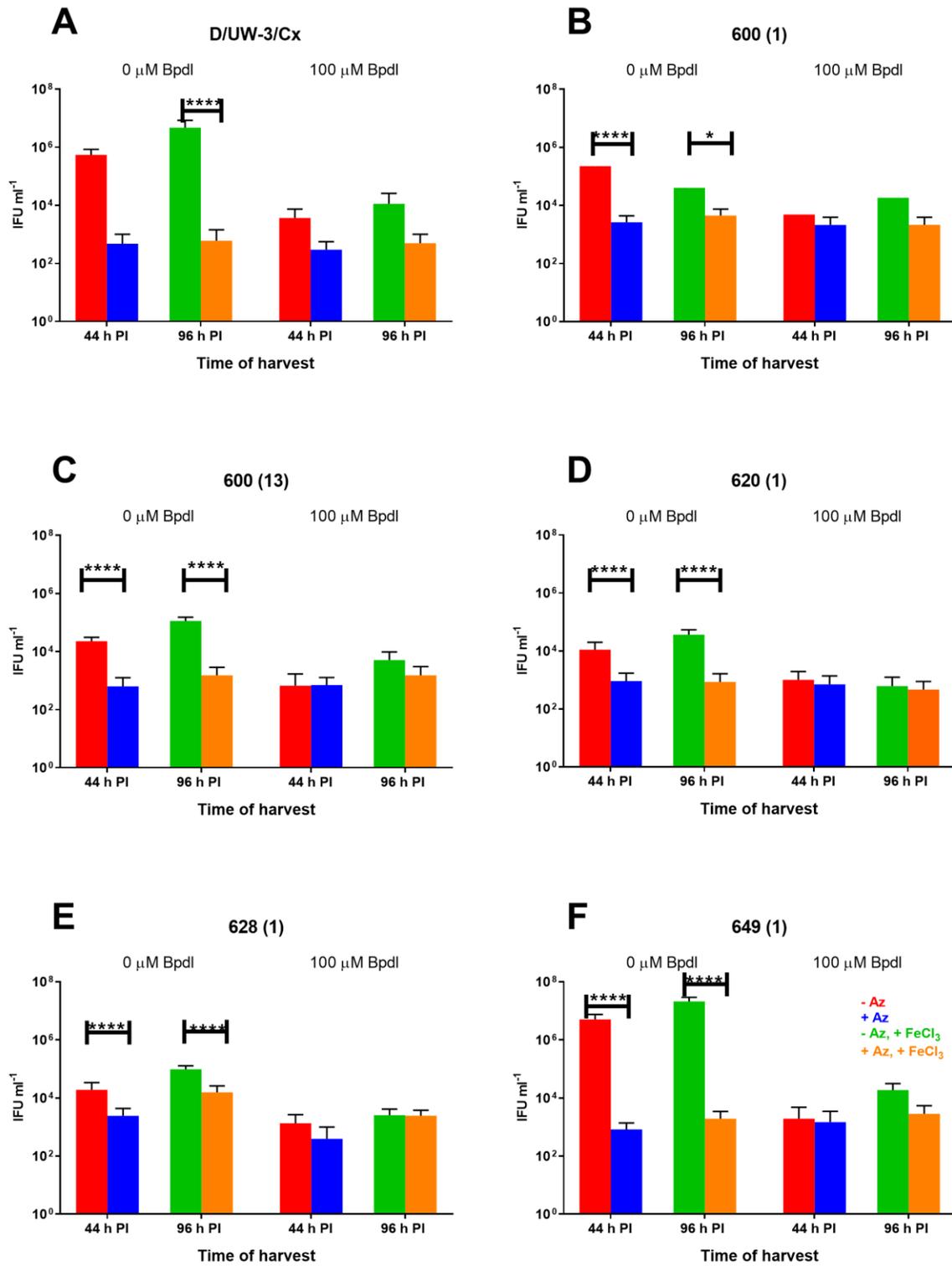
272 **Figure 3. Iron deprivation induced persistence and recovery of the chlamydial isolates.**

273 Infectious progeny (inclusion forming units, y axis) produced by the type strain D/UW-3/Cx (A)

274 and five clinical isolates (B: 600(1); C: 600(13); D: 620 (1); E: 628(1); F: 649(1)) in MCF-7 cells

275 treated with Bpdl, Az, ± 100 µM iron as $FeCl_3$ for recovery. Azithromycin dose was the MIC for

276 the strain; 600 (1): 0.032 µg/ml, 600 (13): 0.125 µg/ml, 620: 0.032 µg/ml, 628: 0.064 µg/ml;

277 649: 0.064 µg/ml, and D/UW-3/Cx: 0.064 µg/ml. Each bar represents the mean of three

278 experimental replicates analysed using n=10 separate fields of view from n=3 subsequent

279 infectivity (passage) wells with error bars showing the standard error of the mean (SEM). Data

280 was analysed by way of a 3-way ANOVA using GraphPad Prism version 8.0.0 for Windows.

281 Significant differences are indicated by **** $p < 0.0001$, and * $p = 0.0138$. The data is shown from

282 a single experiment, and is consistent with other attempts at the experiment.

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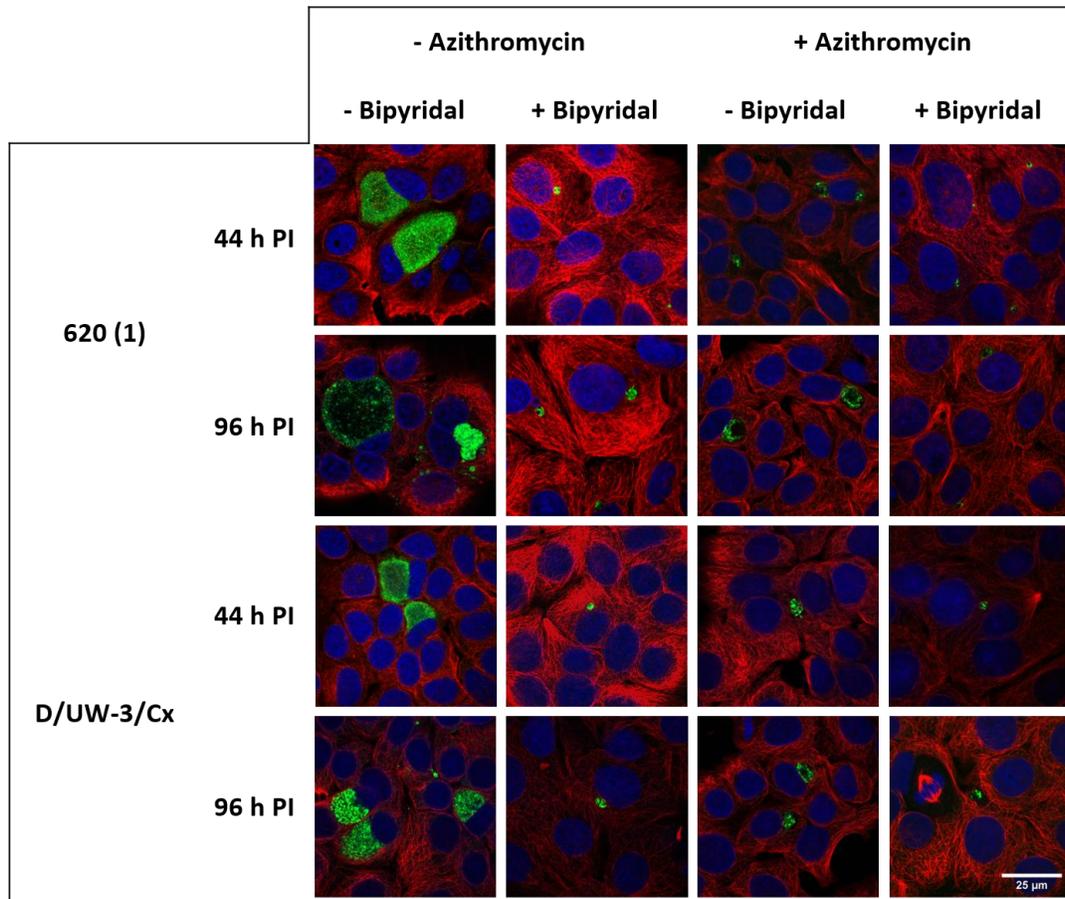
Figure 4. Confocal microscopy of *Chlamydia* cultures during iron-deprivation persistence.
MCF7 cells infected with 620 (1) (top two rows) or type strain D/UW-3/Cx (bottom two rows) were fixed and visualised using immunofluorescence. Active and persistent inclusions containing chlamydial HtrA appears as green, while the host alpha-tubulin appears as red and the host cell nucleus as blue. This images are representatives of observations for each experimental condition.

Clinical isolates entered persistence at lower doses of penicillin compared to the type strain

Several doses of penicillin were used to induce persistence in each strain, which was confirmed by measuring viability before and after recovery from the drug. The data shown in **Fig. 5** demonstrates that compared to the untreated controls at 44 h PI, the addition of 0.02 U ml^{-1}

296   penicillin slightly impacted the development of each clinical isolate, while the 0.05 U ml^{-1} and

297   1.0 U ml^{-1} doses had rendered them non-cultivable. Conversely, only the 1.0 U ml^{-1} dose caused

298   the type strain to enter persistence, with far less pronounced effects than the clinical isolates

299   prevalent at the lower doses. 600(1) and 600(13) produced 2.1×10^6 and 5.5×10^5 IFU ml^{-1} in their

300   respective untreated cultures, compared to 6.6×10^5 and 8.7×10^4 IFU ml^{-1} in the cultures treated

301   with the lowest dose of penicillin. The type strain exhibited only slightly impacted growth at

302   both the lower doses (5.0×10^7 IFU ml^{-1} untreated versus 4.1×10^6 and 9.4×10^4 at 0.02 and 0.05,

303   respectively), and only became non-cultivable at the 1.0 U ml^{-1} dose. Each of the five clinical

304   isolates and the type strain were able to produce detectable (after recovery) infectious progeny by

305   110 h PI, all to a similar level, even after treatment with 1 U ml^{-1} of penicillin. Confocal

306   microscopy examination of the morphology of the cultures confirmed the presence of visible

307   forms consistent with persistent or recovered morphology (**Fig. 6**). Specifically, at 44 and 110 h

308   PI and in the absence of penicillin, each of the five clinical isolates and the type strain showed

309   typical morphologies consistent with regular development. At the same time point, cultures

310   treated with penicillin showed significantly smaller inclusions with enlarged particles inside,

311   morphologies consistent with persistence. Imaging of the cultures at 110 h PI (66 hours after the

312   removal of penicillin from culture) showed inclusions typical of regular inclusions.

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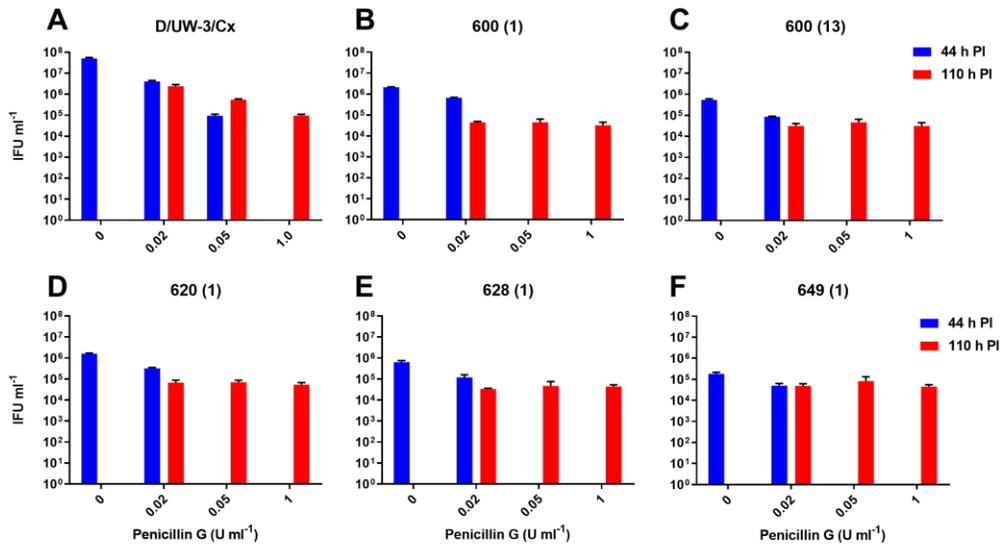
Figure 5. Enumeration of infectious progeny from penicillin persistence experiments.

Inclusion forming units from culture harvested at 44 and 110 h PI are shown on the graph(s). The inclusion forming units per ml (y axis, log scale), and dose of Penicillin (x axis), and recoverable inclusion forming units per ml at 44 h PI (blue bars- during persistence), and 110 h PI (red bars - recovery). Data shown represents the mean value of n=3 separate experimental replicates wells with n=10 images analysed from each of n=3 wells, with error bars representative of the SEM. The data is shown from a single experiment and is consistent with other attempts at the experiment. This data was used to select the dose of penicillin G for the next experiments for each isolate and as such was not used for statistical analysis.

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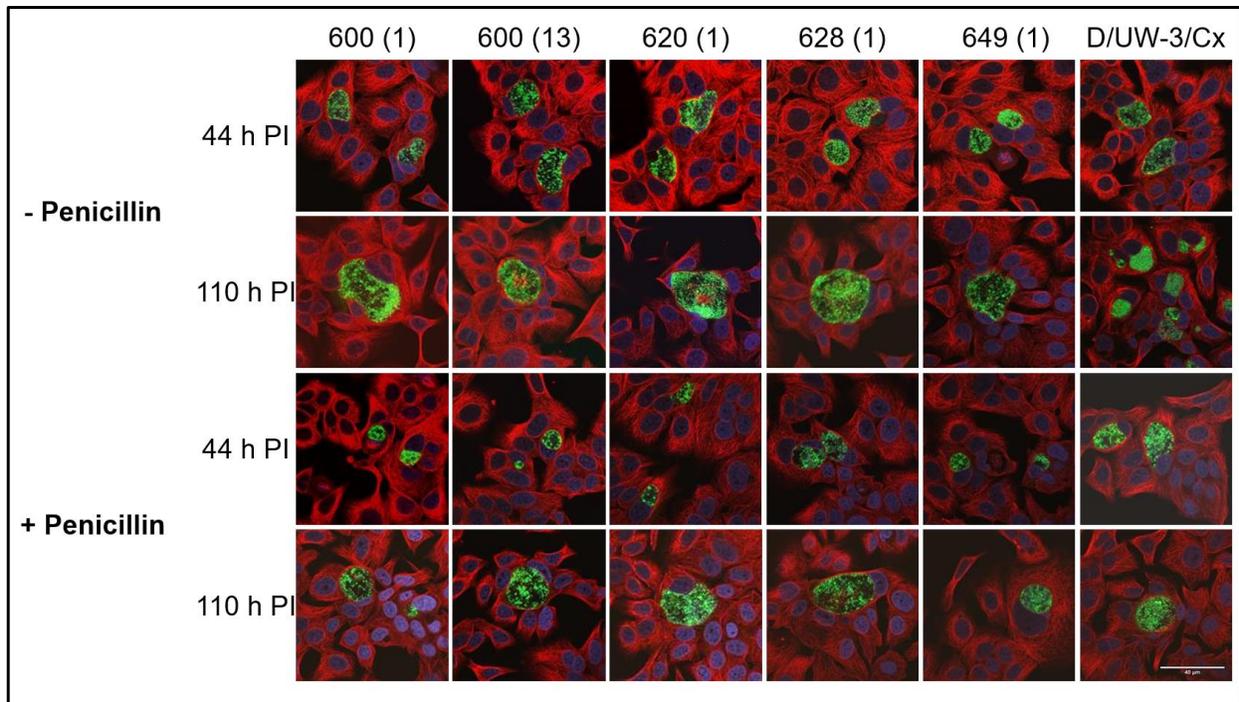
Figure 6. Confocal microscopy of the chlamydial cultures in presence and absence of

penicillin and post recovery. Active and persistent inclusions containing Chlamydial HtrA

appears as green, while the host alpha-tubulin appears as red and the host cell nucleus as blue.

This images are representatives of observations for each experimental condition.

Clinical isolates treated with azithromycin during penicillin persistence showed a dose-

dependent decrease in recoverable infectious progeny

To assess whether clinical isolates had altered susceptibilities to azithromycin during persistence,

three clinical isolates and D/UW-3/Cx were cultured in MCF-7 cells and treated with both

penicillin and azithromycin. Persistence was induced using 0.05 U ml^{-1} of penicillin for the three

clinical isolates and 1.0 U ml^{-1} for the type strain. At 44 h PI, **Fig 7** shows a complete loss of

infectious progeny for the penicillin-treated cultures, which was recoverable (albeit reduced) by

110 h PI. The infectious progeny of the four strains at 44 h PI was also observed to decrease by

344   up to 100-fold lower when treated with azithromycin alone. In the type strain, this effect was also

345   seen, whereby the recoverable infectious progeny decreased in the presence of azithromycin

346   only, with a complete loss of viability at 44 h PI when both azithromycin and penicillin were

347   added. Although no statistical differences were apparent for any isolates treated with

348   azithromycin during penicillin persistence. It appeared that the recovery of the clinical isolates

349   from persistence was further reduced when treated with azithromycin at the MIC, with two of the

350   isolates (600(13) and 649) appearing to be more impacted by the combination treatment.

351
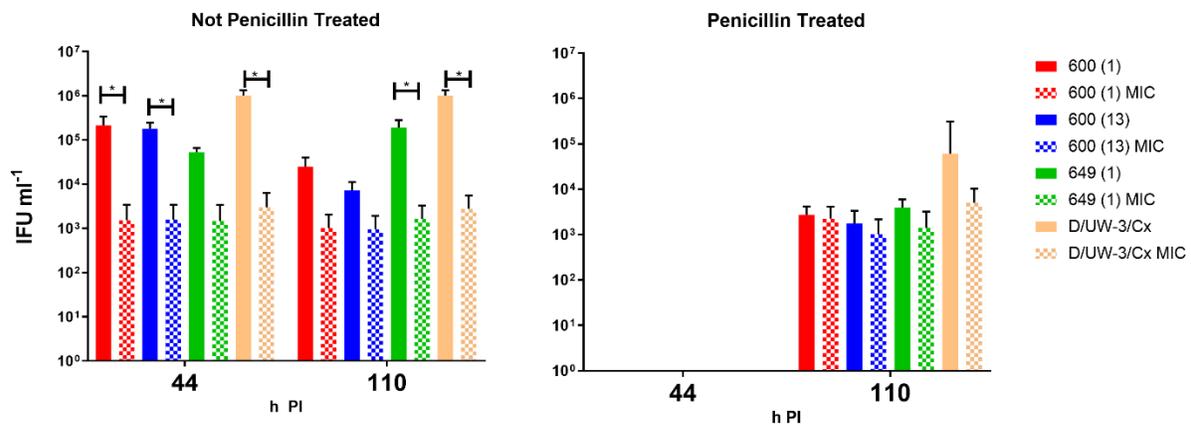
352

353   **Figure 7. Impact of azithromycin treatment during penicillin induced persistence.** The

354   graphs show the IFU/ml of each clinical isolate and type strain D/UW-3/Cx in the presence of

355   azithromycin (at the minimum inhibitory concentration (MIC) for that strain), with and without

356   pretreatment and recovery from benzylpenicillin. Azithromycin dose; 600 (1): 0.032 μg/ml, 600

357   (13): 0.125 μg/ml, 620: 0.032 μg/ml, 628: 0.064 μg/ml; 649: 0.064 μg/ml, and D/UW-3/Cx:

358   0.064 μg/ml. The X axis shows each isolate grouped at both time points on the left- and right-

359   hand sides respectively and the Y axis shows infectivity of the cultures as IFU ml^{-1}. Data shown

360   represents the mean value of n=3 separate experimental replicate wells with an average of n=10

361   images analysed to input the result for each of n=3 wells, with error bars representative of the

362   SEM. The data is shown from a single experiment, although is consistent with other attempts at

363   the experiment. The p values for the significant different outcomes for the isolates are as follows

364   (left to right): 0.043, 0.099, 0.066, 0.027, 0.0066).

365

Discussion

367   The susceptibility and growth permissiveness of different cell lines to infection by *C.*

368   *trachomatis* enables examination of the host-pathogen relationship (43-46). Such studies

369   frequently find differences between strains in their ability to enter the host cell, and complete

370   their developmental cycle (47). The infectivity of the clinical isolates in the present study was

371   observed to be highest in the MCF-7 cell line, despite the long-standing practice throughout the

372   field of using McCoy or HeLa cells for cultivation and isolation of *C. trachomatis*, especially

373   from clinical samples (48-50). However, the infectious yields were highest in the McCoy B cell

374   line for each of the strains tested. The ARPE-19 cell line was observed to have a very low

375   susceptibility to infection by, and permissiveness of growth to all strains except D/UW-3/Cx, yet

376   was nonetheless able to be infected by each of the strains, even though it is not an epithelial cell

377   type. The inability to detect progeny from this cell line for the clinical isolates may reflect the

378   low yields being below detection of the assay, or that the clinical isolates in these cells are unable

379   to form infectious elementary bodies. Overall, these findings reinforce that there are phenotypic

380   differences between type strains and clinical isolates that likely reflects the adaptation of D/UW-

381   3/Cx to growth *in vitro* (51).

382   Although it has been shown that type strains of *C. trachomatis* have varying levels of

383   susceptibility to the *in vitro* effects of IFN-γ, fewer studies have examined how different strains

384   respond to penicillin or iron deprivation (52). In this study, we aimed to determine how clinical

385   isolates respond to such conditions. Both were selected on the basis that penicillin has been used

386   widely as a chlamydial persistence model so our findings can be interpreted in light of other

387   studies (37, 53-55); while iron deprivation is potentially a more clinically relevant model when

388   considering the physiology of the female reproductive tract (56-58). Penicillin is known to

389   induce persistence by interacting with chlamydial penicillin-binding proteins (PBPs), while host

390   cell-derived iron is essential for chlamydial development. Both points combined, raise the

391   possibility that genetic variation among infecting strains could result in differing thresholds at

392   which they divert from the regular developmental cycle into persistence.

393   To assess the susceptibility of each strain to penicillin, they were treated with a dose range in

394   MCF-7 cells. None of the clinical strains showed any notable difference in their levels of

395   susceptibility to penicillin persistence, with all entering a viable but non-cultivable state at 0.05

396   U ml^{-1} of the antibiotic. In contrast, the type strain D/UW-3/Cx remained cultivable up to the

397   maximum dose used, which was 1 U ml^{-1}. A recent study into the effects of beta-lactam

398   antibiotics on *C. trachomatis* showed that type strain E/UW-3/Cx entered persistence at 0.02 U

399   ml^{-1} of benzylpenicillin (37). Although using different host cells, this suggests there may be

400   different susceptibilities among type strains.

401

402   Similarly, there were no large observed differences in the responses of clinical isolates to the

403   effects of iron deprivation. In a recent review, Pokorzynski and colleagues postulated a complex

404   system by which *C. trachomatis* may be able to both passively and actively acquire ferrous and

405 ferric iron from within the host cytoplasm by modulating the hosts own iron trafficking pathways

406 (59). As with other instances of chlamydial persistence, it is possible that strain differences may

407 in turn result in slight differences in the proteins that conduct these functions.

408 The effects of azithromycin were tested on a selection of clinical isolates during both active and

409 persistent development. We observed no differences in the susceptibilities of active and

410 persistent infections, in any of the clinical strains tested. Previous findings by the Caldwell group

411 demonstrate that during IFN-γ-mediated persistence, a serovar D type strain of *C. trachomatis* is

412 significantly more susceptible to azithromycin (60). However, Wyrick and Knight have shown

413 that a serovar E type strain is less susceptible to the same antibiotic during penicillin-mediated

414 persistence (53). This reinforces previous findings that different inducers of persistence produce

415 different phenotypes (61), which likely reflects the physiological stress the inducer places upon

416 *C. trachomatis*.

417 Collectively these findings show that clinical isolates respond to the effects of penicillin, iron

418 deprivation, and azithromycin in a similar but more pronounced way than type strain D/UW-

419 3/Cx. This is important because these are recent clinical isolates, indicating that persistence may

420 occur more frequently and with lower thresholds *in vivo*. Here we demonstrated that clinical

421 isolates showed subtle variation in thresholds for persistence that may be more distinct and

422 impactful in the complex *in vivo* environment. These variations may translate into phenotypes *in*

423 *vivo* that could relate to heterogenic survival of antibiotic treatment, or different capacities to

424 survive in different tissue niches (e.g. rectal compared to urogenital) that could be relevant for

425 future investigation of chlamydial variation.

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429   **Conflict of Interest**

430   The authors declare no conflicts of interest.

437   **Author Statements**

438   The authors declare that there are no conflicts of interest.

439   MT: Investigation, Writing, original data preparation, and review and editing

440   SK: Investigation, Writing, original data preparation

441   AL: Investigation, Writing, original data preparation

442   LV: Investigation, Writing, original data preparation

443   SP: Investigation, Writing, original data preparation

444   JH: Supervision, Investigation, Writing, original data preparation, and review and editing

445   PT: Supervision, Investigation, Writing, original data preparation, and review and editing

446   WH: Conceptualisation, Supervision, Investigation, Writing, original data preparation, and

447   review and editing

448

449   **Ethical Approval**

450   Ethical approval for this study was granted by the Alfred Hospital Ethics Committee (HREC No.

451   223/12) and the Southern Eastern Sydney Local Health District Human Research Ethics

452   Committee (Southern Sector) (HREC No. 12/143).

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456   **References**

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