Peer reviewed version

Link to published version (if available):
10.1093/jac/dkx454

Link to publication record in Explore Bristol Research
PDF-document

University of Bristol - Explore Bristol Research
General rights

1   **Phenotypic antimicrobial susceptibility testing of *Chlamydia trachomatis* isolates from**

2   **patients with persistent and successfully treated infections**

3   Rachel PITT[1,2]*, Sarah ALEXANDER[2], Catherine ISON[2], Patrick HORNER[3], Emma HATHORN[4], Penny

4   GOOLD[4], Neil WOODFORD[1] and Michelle COLE[1,2]

5   [1] Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, National

6   Infection Service, Public Health England, London, U.K., [2]formerly of The Sexually Transmitted

7   Bacteria Reference Unit, Public Health England, London, U.K., [3]Population Health Sciences, Bristol

8   Medical School, University of Bristol, UK. [4]Whittal Street Clinic, University Hospitals Birmingham

9   NHS Foundation Trust, Birmingham, U.K.

10

11   *Corresponding Author Address: Antimicrobial Resistance in Sexually Transmitted Infections

12   (AMRSTI), Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit,

13   National Infection Service, Public Health England, Colindale, NW9 5EQ

14   Rachel.pitt@phe.gov.uk

15   Tel: 020 8327 7339

16

17   **Running title: Susceptibility testing of *Chlamydia trachomatis***

18

19

20

21

22   **Synopsis**

23   Objectives: Antimicrobial susceptibility data for *Chlamydia trachomatis* are lacking.

24   Methodologies for susceptibility testing in *C. trachomatis* are not well-defined, standardised

25   or performed routinely owing to its intracellular growth requirements. We sought to

26   develop an assay for the *in vitro* susceptibility testing of *C. trachomatis* isolates from two

27   patient cohorts with different clinical outcomes.

28   Methods: Twenty-four clinical isolates (11 from persistently infected and 13 from

29   successfully treated patients) were overlaid with media containing two-fold serial dilutions

30   of azithromycin or doxycycline. After incubation, aliquots were removed from the stock

31   inoculum (SI) and each antimicrobial concentration for total RNA extraction, complementary

32   DNA generation and real-time PCR. The MIC was defined as the lowest antimicrobial

33   concentration where a 95% reduction in transcription was evident in comparison with the SI

34   for each isolate.

35   Results: MICs of azithromycin were comparable for isolates from the two patient groups

36   (82% ≤0.25 mg/L persistently infected and 100% ≤0.25 mg/L successfully treated patients).

37   Doxycycline MICs were at least two-fold lower for isolates from the successfully treated

38   patients (53.9% ≤0.064 mg/L) than for the persistently infected patients (100% ≥0.125 mg/L)

39   (*p*=0.006, Fisher's exact test). Overall, 96% of isolates gave reproducible MICs when re-

40   tested.

41   Conclusions: A reproducible assay was developed for antimicrobial susceptibility testing of

42   *C. trachomatis*. MICs of azithromycin were generally comparable for the two different

43   patient groups. MICs of doxycycline were significantly higher in the persistently infected

44   patients. However, interpretation of elevated MICs in *C. trachomatis* is extremely

45   challenging in the absence of breakpoints, or wild-type and treatment failure MIC

46   distribution data.

47

48

49

50

51

52

53

54

55

56

57

58

59

60

61

Introduction

Chlamydia trachomatis is the most prevalent bacterial sexually transmitted infection worldwide with 202,546 diagnoses in England in 2016. [1] Current first-line recommended treatment regimens for uncomplicated infection are 1 g stat azithromycin, or 100 mg doxycycline twice a day for seven days. [2] Whilst the efficacy of these treatments is considered to be extremely high, [3, 4] treatment failure with 1 g stat azithromycin has been demonstrated in *C. trachomatis*-positive men with non-gonococcal urethritis and rectal chlamydia and in women not at risk of re-infection. [5-7] Further reports of treatment failure have been described in patients where the risk of re-infection is low. [8-15] There are a number of possible reasons why patients may remain positive for chlamydia after treatment: non-adherence to the treatment regimen; re-infection from a new or untreated partner; inadequate exposure to the antimicrobial as a result of host pharmacokinetics or short duration of treatment, [16] and heterotypic or homotypic antimicrobial resistance. Heterotypic resistance, also known as phenotypic switching, occurs when a heterogeneous population of both resistant and susceptible organisms replicate from a single predecessor. [7] It is not genetically inherited but is a result of adaptations by the bacteria to make them less susceptible to the antimicrobial e.g. induction of slow growing, non-replicative or persistent forms in the presence of antibiotic, which revert back to replicating forms once the antibiotic pressure has been removed resulting in a relapse in infection. Homotypic antimicrobial resistance is, by contrast, genetically inherited.

At high bacterial loads, e.g. as found in patients with symptoms of urethritis, [17] *C. trachomatis* has been shown to exhibit heterotypic resistance. [18, 19] Confirmed phenotypic decreased susceptibility to antimicrobials of clinical significance has been reported rarely in

85   *C. trachomatis.* [9, 11, 20, 21] Stable genotypic resistance to antimicrobials in clinical practice has

86   yet to be documented in human urogenital *C. trachomatis* infection. [16] However very little is

87   known about the susceptibility profiles of circulating strains because antimicrobial

88   susceptibility assays are not routinely performed and the methodology is neither

89   standardised nor well-defined. [7, 18, 22]

90   We describe the development of a robust antimicrobial susceptibility testing methodology

91   (adapted from Storm *et al* [23]), and report susceptibility data for azithromycin and

92   doxycycline for a cohort of *C. trachomatis* isolates from patients who were persistently

93   infected with *C. trachomatis*. Susceptibility data are also presented for *C. trachomatis*

94   isolates from a group of control patients who were *C. trachomatis*-positive at initial

95   presentation and were then confirmed to have been treated successfully.

96   **Methods**

97   *Patient recruitment*

98   As reported previously [24] patients with persistent *C. trachomatis* infections were recruited

99   from sexual health clinics across England and Wales. Patients were deemed to have a

100   persistent infection if they had tested positive at least twice by a *C. trachomatis*-specific

101   assay (e.g. a nucleic acid amplification test, NAAT), had fully adhered to the prescribed

102   treatment regimens in line with current guidelines [2] (including any abstinence periods) and

103   were assessed to be at low risk of re-infection. Risk of re-infection was categorised using

104   self-declared sexual contact behaviour in the time since initial diagnosis, reported via a

105   clinician-completed questionnaire. Patients were considered at low risk of re-infection

106   following treatment if they had: a) no sexual contact, b) protected sexual contact only, or c)

107   unprotected sexual contact with a partner who had not tested positive or who had tested

108   positive, but had been treated. These groups were designated categories 1, 2 and 3

109   respectively. Clinical data collected for some of the patients in this report were reported

110   previously. [24]

111   Control isolates were collected from patients who had been treated for *C. trachomatis*

112   infection in line with current UK guidelines [2] and had a negative test-of-cure by NAAT at

113   least 30 days later.

114   ***Ethical approval***

115   Patients with persistent infections were referred as part of an enhanced surveillance

116   programme and therefore ethical approval was not sought or required.   Public Health

117   England has permission to handle these data under the Health Service (Control of Patient

118   Information) regulation 2002, overseen by the Confidentiality Advisory Group.

119   Control patients were recruited through a sexual health clinic, ethics reference number

120   13/WM/0088.

121   **Culture methods**

122   ***Stock inoculum culture***

123   Clinical specimens (persistently infected group: 6 specimens from male patients [5 urethral

124   swabs and 1 rectal swab] and 5 specimens from female patients [4 cervical swabs and 1

125   urethral swab; successfully treated group: 2 specimens from male patients [1 urethral swab

126   and one swab from an unknown site and 11 specimens from female patients [3 cervical

127   swabs, 8 self-collected vaginal swabs were inoculated on to confluent McCoy cell mono-

128   layers in shell vials. Shell vials were centrifuged at 2300 x g for 1 h at 35°C and were then

129   incubated for 4 h at 35°C in 5% CO_2. The inoculum was then aspirated and the infected

130   mono-layer was overlaid with Dulbecco's Modified Eagles Medium (DMEM, Gibco, Hemel

131   Hempstead, U.K.) supplemented with 10% foetal bovine serum (Gibco), 200 mM L-

132   glutamine (Sigma, Gillingham, U.K.), 1 mg/L cycloheximide (Sigma), 100 mg/L gentamicin

133   (Gibco), 25 U/mL nystatin (Sigma) and 100 mg/L vancomycin (Sigma). Shell vials were

134   incubated for 48 h at 35°C in 5% CO_2 to produce a stock inoculum of each strain for

135   antibiotic susceptibility testing assays. Inclusion forming units (IFUs) were visualised after

136   staining with the MicroTrak® *Chlamydia trachomatis* culture confirmation test (Trinity

137   Biotech, Newmarket, U.K.).

138   ***Susceptibility assays***

139   MICs of azithromycin and doxycycline were determined as follows; confluent McCoy cell

140   monolayers in 48-well plates were overlaid with the stock inoculum of each strain (10^3-10^5

141   inclusion forming units per well), plates were centrifuged for 1 h at 1350 x g and 35°C and

142   were then incubated at 35°C, 5% CO_2 for 4 h to facilitate infection. Wells were aspirated

143   and overlaid with two-fold serial dilutions of antimicrobial (0.125-2 mg/L azithromycin or

144   0.064-1 mg/L doxycycline) in supplemented DMEM (as above). An antimicrobial-free

145   control was included for each strain to allow identification of assay failure. Due to the lack

146   of known azithromycin- or doxycycline- resistant control isolates of *C. trachomatis*, a

147   susceptible control *C. trachomatis* isolate (from a successfully treated patient, isolate 314)

148   was used in the azithromycin assays, and the tetracycline-resistant *C. suis* strain R19 [25] was

149   used in the doxycycline assays. Plates were then incubated for 48 h at 35°C in 5% CO_2.

150   **RNA extractions and cDNA generation**

151   The RNeasy Plus Mini Kit (QIAgen, Manchester, U.K.) was used as per the manufacturer's

152   instructions to extract total RNA from aliquots of culture media collected from each

153   antibiotic concentration and negative control at varying stages during the susceptibility

154   assay, namely the initial inoculum (P0) and after the 48 h incubation with antimicrobial (P1).

155   Complementary DNA (cDNA) was reverse transcribed from the total RNA (2 µL per reaction)

156   using the Quantitect reverse transcription kit (QIAgen) as per manufacturer's instructions.

157   This kit includes a step for removing contaminating genomic DNA negating the need for a

158   separate *DNase*I digestion.

159   **Real Time-PCR to detect transcription and interpretation of MIC endpoint**

160   cDNA was used as template for real time PCR (RT-PCR) on the RotorGene (QIAgen) platform

161   (primer and probe sequences in Table 1) to quantify the *C. trachomatis* transcripts and allow

162   assignment of an MIC of each antimicrobial for each strain. The method described by Storm

163   *et al* [23] was modified to facilitate use of an L2 internal control, prepared in-house, for

164   transcript quantification. In place of the *omp2* gene target a predicted virulence factor on

165   the *C. trachomatis* cryptic plasmid was used as the chlamydia specific target. [26] The McCoy

166   cell B-actin gene (inhibition control) and the *C. suis* R19 23S rRNA gene were detected

167   qualitatively only where appropriate. To increase assay sensitivity each target was run as a

168   separate reaction.

169   Twenty-five microliter reactions were prepared for each target in HotStarTaq master mix

170   (QIAgen). Primer and probe sequences can be found in Table 1. *C. trachomatis*-specific

171   target: 200 nM Ct-Forward primer, 320 nM Ct-Reverse primer, 200 nM Ct-Probe and 10 µL

172   cDNA. McCoy cell β-actin-specific target: 100 nM McCoy-Forward primer, 100 nM McCoy-

173   Reverse primer, 24 nM McCoy-Probe and 5 µL cDNA. *C. suis*-specific target: 200 nM R19-

174   Forward primer, 200 nM R19-Reverse primer, 200 nM R19-Probe and 5 µL cDNA. Reactions

175   were run on the RotorGene platform (QIAgen) using the following programme: initial

176   denaturation and Taq activation step of 95°C for 10 minutes followed by 50 cycles of 95°C

177   for 30 seconds, 60°C for 40 seconds (acquiring in the green [FAM, *C. trachomatis*-specific

178   PCR], yellow [JOE, McCoy cell-specific PCR] or red [Cy5, *C. suis*-specific PCR] channel) and

179   72°C for 40 seconds. A standard curve was generated using a previously quantified *C.*

180   *trachomatis* L2 cryptic plasmid positive control on each *C. trachomatis*-specific PCR run to

181   allow quantification of transcripts. As described by Storm *et al* [23] the MIC was assigned to

182   the lowest antimicrobial concentration where a ≥95% reduction in transcription was

183   observed after a passage in the presence of antimicrobial (P1) in comparison with the initial

184   inoculum (P0) for each strain. RT-PCR was used only for MIC assignment to negate

185   subjectivity of immunofluorescent staining interpretation.

186   **Statistical analysis**

187   Geometric means of the azithromycin and doxycycline MICs were calculated and linear

188   regression was used to analyse the relationship between the MICs and the different patient

189   groups. As absolute MICs were not available for a number of isolates (i.e. MICs were ≤ or ≥)

190   then MIC values a doubling dilution above or below the recorded MIC e.g. ≤0.064 mg/L was

191   analysed as 0.032 mg/L and ≥1 mg/L was analysed as 2 mg/L). Fisher's exact test was used

192   to compare azithromycin MICs ≤0.25 mg/L versus MICs >0.25 mg/L and doxycycline MICs

193   ≤0.064 mg/L versus MICs >0.064 mg/L in the persistently infected and successfully treated

194   patient groups respectively. Results for both tests were deemed significant if the *p* value

195   was ≤0.05.

196 **Results**

197 **Isolate retrieval**

198 Isolates were retrieved from eleven patients with persistent *C. trachomatis* infections that

199 met the inclusion criteria outlined previously (five in category 1, two in category 2 and four

200 in category 3). In addition, isolates were retrieved from thirteen control patients with linked

201 negative test-of-cure samples.

202 **MIC data**

203 Azithromycin MICs were ≤0.25 mg/L for 81.8% (9/11) of the isolates from patients with

204 persistent infections and for 100% (13/13) of isolates from the successfully treated control

205 patients (Table 2, Table 3). The azithromycin geometric mean MICs were 0.127 mg/L and

206 0.071 mg/L for isolates from the persistently positive group and the successfully treated

207 patient group, respectively. Azithromycin MICs for two isolates (18.2%) in the persistently

208 infected group were 2 mg/L and 0.5 mg/L (Table 2, Table 3). No difference (Fisher's exact

209 test, p = 0.3; linear regression p = 0.1) was observed between the azithromycin MICs for

210 isolates from the persistently infected patients compared with those for isolates from the

211 successfully treated patients. The MICs of doxycycline for the isolates from the successfully

212 treated patient group were significantly lower than MICs for isolates from the persistently

213 infected patient group (Fisher's exact test, p = 0.006); doxycycline MICs for 7/11 (63.6%)

214 isolates from patients with persistent infections were 0.125 mg/L, and for the remaining

215 four isolates (36.4%) were ≥1 mg/L. The doxycycline MICs for most (7/13, 53.9%) isolates in

216 the successfully treated group were ≤0.064 mg/L, at least two-fold lower than the lowest

217 MICs for isolates from the treatment failure group. The doxycycline MICs for the five

218   remaining isolates from the successfully treated group were 0.125 mg/L (3 isolates), 0.25

219   mg/L (1 isolate) and 1 mg/L (3 isolates) (Table 2). These patients were all treated with

220   azithromycin 1 g only. The doxycycline geometric mean MICs were 0.322 mg/L and 0.097

221   mg/L for isolates from the persistently positive group and the successfully treated patient

222   group, respectively (p = 0.032).

223   **Assay reproducibility**

224   To investigate the robustness of the susceptibility testing methodology, 11 (45.8%) isolates

225   chosen at random (8 [8/11, 72.7%] from the persistently infected and 3 [23.1%] from the

226   successfully treated patient groups) were repeat tested on the azithromycin assay and the

227   MICs from both assay runs compared. All (11/11, 100%) repeat MICs were in complete

228   agreement with initial testing. Thirteen (54.2%) isolates chosen at random (5 [5/11, 45.5%]

229   from the persistently infected and 8 [8/13, 61.5%] from the successfully treated patient

230   groups) were repeat tested on the doxycycline assay. Twelve (12/13, 92.3%) of the repeat

231   MICs were in agreement with the initial MIC data. For one isolate in the successfully treated

232   group the repeat MIC for doxycycline (≤0.064 mg/L) was at least four-fold (two dilution

233   steps) lower than the initial MIC (≥0.25 mg/L). During initial validation of the assays the

234   range of antimicrobial concentrations tested were altered as considered appropriate based

235   on the MICs obtained. The initial assay for this isolate had an antibiotic range tested of

236   0.064 – 0.25 mg/L doxycycline whilst later assays were tested up to 1 mg/L doxycycline.

237   Collectively 23/24 (95.8% [confidence interval: 76.9-99.8%]) of the isolates that were

238   retested on either assay gave reproducible MIC.

239

240

Discussion

We have adapted and further developed an assay for phenotypic *in vitro* antimicrobial susceptibility testing of *C. trachomatis*. The assay was used to test clinical isolates sourced from two distinct patient groups, one with persistent *C. trachomatis* infections and the other with *C. trachomatis* infections successfully treated following first-line recommended therapy (i.e. 1 g stat azithromycin). Clinical isolates from both groups were assayed against azithromycin and doxycycline. The assay methodology produced reproducible MICs of both antimicrobials when isolates were retested, with 95.8% of isolates giving identical MICs. The exception was an MIC obtained in a 'failed' repeat assay that was at least four-fold lower than for the initial assay.

MICs of doxycycline for the isolates from patients who had persistent infections were significantly higher than for isolates from successfully treated patients. A number of the patients in the persistently infected group had been treated with doxycycline in addition to azithromycin (Table 2). The doxycycline MICs for these isolates varied from 0.125 mg/L, which is comparable to the majority of the MICs for isolates from the successfully treated group, to >1 mg/L which is significantly less susceptible. However, the doxycycline MICs for two isolates in the successfully treated group were also 1 mg/L and neither of these patients were treated with doxycycline regimens. The significance of these raised MICs is unclear.

There did not appear to be a difference between the MICs of azithromycin for isolates from the two patient groups. Indeed, whilst the majority of patients in the persistently infected group were treated at least twice with 1 g azithromycin stat regimens (Table 2) the MICs for the isolates from these patients were mostly within a two-fold dilution compared with the MICs for the isolates from the successfully treated patients and the 'susceptible' control

264 strain, 314. There were two isolates in the persistently infected group with azithromycin

265 MICs at least two dilutions higher than control strain 314 and the successfully treated

266 patient group. Interestingly, both of these patients had only been treated once with 1 g

267 azithromycin stat regimens. Overall, this suggests that the antibiotic pressure exerted by re-

268 treatment with the same antibiotic did not select for increased MICs (reduced susceptibility)

269 in these isolates. It may be hypothesised that heterotypic resistance induced *in vivo* may

270 account for the similarity of MIC, but difference in clinical outcome seen with these patients

271 if re-infection can truly be excluded, as asserted.

272 What is clear from the data presented is that much further work is needed to understand

273 the relevance of the MICs obtained from both patients who resolve infection after

274 treatment with first-line therapies and from patients who remain infected. *In vitro*

275 susceptibility testing can only be performed with cultured isolates, which for *C. trachomatis,*

276 are a rare commodity in the current diagnostic environment. Whilst molecular detection of

277 known markers associated with antimicrobial resistance can infer genotypic susceptibility,

278 emerging resistance can only be detected through *in vitro* susceptibility testing. Therefore

279 access to isolates of clinically significant pathogens, such as *C. trachomatis,* is imperative.

280 For many organisms, such as *Neisseria gonorrhoeae*, there are internationally recognised

281 standard protocols for antimicrobial susceptibility testing. No such standardisation exists for

282 *C. trachomatis* [18] and antimicrobial susceptibility testing is particularly complex as it is an

283 obligate intracellular organism requiring tissue cell culture for *in vitro* growth. [18] This,

284 combined with the biphasic nature of the *C. trachomatis* lifecycle (where the extracellular

285 phase is non-replicative), introduces a potential for assay variability not seen for other

286 organisms. Suchland *et al.* (2003) and Wang *et al* (2005) described a range of factors that

287   may influence the MICs for *C. trachomatis in vitro,* such as cell line used, inoculum size and

288   time from where infection occurs to addition of the antimicrobial. Interpretation of the

289   endpoint of the MIC assay can also be problematic. Traditionally, immunofluorescent

290   staining of tissue cultures has been commonly used to identify aberrant chlamydial

291   inclusions, but this method is time-consuming and subjective. In addition, failure to visualise

292   *C. trachomatis* inclusions in *in vitro* cultures does not exclude a viable state that can

293   proliferate once the antibiotic pressure has been removed. [27]

294   To negate subjectivity and to detect all viable organisms, we adapted a method previously

295   described by Storm *et al.* (2005), which monitored the presence of mRNA transcripts in pre-

296   and post-antimicrobial treated *C. trachomatis* cultures. Whilst the efficiency of reverse

297   transcriptase PCR is known to be variable, the reproducibility of the MICs presented in this

298   report indicate that this procedure was standardised as much as possible. The Storm assay

299   was adapted to include detection of a predicted virulence factor gene on the *C. trachomatis*

300   cryptic plasmid in place of the original *C. trachomatis omp2* gene. The cryptic plasmid is

301   constitutively expressed throughout the *C. trachomatis* life-cycle and, whilst the number of

302   copies of the plasmid carried can vary between different strains of *C. trachomatis,* [28] isolates

303   were compared with themselves only. It was assumed that the plasmid copy number

304   remained stable within a strain however it is possible that they may vary during different

305   lifecycle stages and/or when challenged with antimicrobial; investigation of this was beyond

306   the scope of this study but is a recognised potential limitation. We also increased the time

307   that infected cultures were incubated prior to application of the antimicrobials from two

308   hours, as described in Storm *et al*. (2005), to four hours to allow infections to establish more

309   completely before challenge. Clean cell lines were screened for the presence of

310   contaminants prior to inoculation as part of routine tissue culture maintenance and all

311   infected cell line work was carried out in the presence of multiple antimicrobial/antifungal

312   agents. However, as the isolates were clinical in origin it cannot be fully excluded that no

313   other organism was present in the tissue culture at the time of susceptibility testing,

314   Despite development of a reproducible assay, there are limitations to this work.

315   Interpretation of our MIC results was difficult as no susceptibility or resistance breakpoints

316   exist for *C. trachomatis* and there is very limited data [29, 30] regarding the wild-type

317   distributions of susceptibility to azithromycin and doxycycline for circulating strains. Due to

318   the dearth of susceptibility data for this organism, how *in vitro* MICs correlate with

319   treatment success or failure in the patient is poorly understood. Indeed, when the results

320   presented in this report are taken into account i.e. evidence of consistent *in vivo* phenotypic

321   resistance to azithromycin in the patients persistently infected with *C. trachomatis* without

322   evidence of reduced susceptibility of the isolate *in vitro*, the picture becomes even more

323   complex.

324   Given the move to the use of doxycycline as the preferred first-line therapy for NGU, in

325   which *C. trachomatis* is the most commonly identified pathogen, [31] understanding the

326   relevance of the raised doxycycline MICs in isolates from the persistently infected patient

327   group is important. Particularly as doxycycline may in the future be given as prophylaxis to

328   men who have sex with men as PrEP for bacterial sexually transmitted pathogens [32] and the

329   impact of this increased doxycycline usage on *C. trachomatis* MICs is unknown. The high-

330   level of assay reproducibility suggests that whilst the majority of strain MICs differed by only

331   one doubling dilution, the difference (p=0.006) was unlikely related to the susceptibility

332   testing methodology and an MIC shift towards less-susceptible was observed in the

333　persistently infected patient group. However the root cause of this shift and its impact on

334　clinical outcome is unclear. A larger observational case control study is required to generate

335　data to allow appropriate antimicrobial stewardship. [33] This data may strengthen the case

336　for recommendation of a test of cure in all patient groups.

337　In addition, a number of physiological factors, such as the host inflammatory response, that

338　would form part of natural infection resolution (in addition to antimicrobial therapy) and

339　individual patient pharmacokinetics that cannot be replicated in *in vitro* cell culture systems

340　must also be considered. It is therefore difficult to hypothesise how representative an MIC

341　alone would be as a marker of likelihood of treatment success. There are also few data

342　available regarding how *in vitro* culturing of isolates affects the organisms' susceptibility to

343　antimicrobials. The patients who were persistently infected with *C. trachomatis* were

344　exposed to a minimum of two rounds of antimicrobial therapy, but viable organisms

345　remained. These patients were thought unlikely to have been re-infected, but this cannot be

346　excluded completely. Antimicrobial susceptibility assays were carried out secondary to the

347　primary isolation from the clinical specimen. As a result, it was necessary to re-culture each

348　isolate from an archived aliquot. It is possible that multiple passages in tissue culture in the

349　absence of antimicrobial challenge could have affected the MIC obtained especially if

350　surviving antimicrobial therapy in the patient led to a fitness cost. The authors recognise

351　this as a weakness of the study and would recommend progressive processing through

352　primary isolation and antimicrobial susceptibility testing to limit time in culture as an ideal.

353　Further to this, the length, complexity and cost of the testing procedure is not amenable to

354　large-scale phenotypic testing over a wide antibiotic concentration range. Processing of

355　isolates from recovery from archive to obtaining MIC results took on average 15 working

356 days. Nevertheless, we determined MICs of two therapeutically-relevant antibiotics for 24

357 clinical *C. trachomatis* strains. Any large-scale antimicrobial resistance surveillance in *C.*

358 *trachomatis* would need to take advantage of molecular techniques to screen for genetic

359 markers of reduced susceptibility in addition to *in vitro* susceptibility testing if reliable

360 indicators could be identified. Reassuringly, in a recent large-scale genome sequencing

361 study of global *C. trachomatis* isolates, no known molecular markers of antimicrobial

362 resistance were detected. [34]

363 In summary, a reproducible method for phenotypic antimicrobial susceptibility testing of *C.*

364 *trachomatis* has been described. The assay was employed for the analysis of a small number

365 of clinical isolates from two groups of patients who had very different treatment outcomes.

366 The azithromycin MICs for the majority of strains within the persistently-positive group

367 were comparable with those for strains in the successfully treated group. However, the

368 MICs of doxycycline were higher in the persistently infected than in the successfully treated

369 patient group. Antimicrobial susceptibility testing and interpretation of elevated MICs in *C.*

370 *trachomatis* is extremely challenging in the absence of breakpoints. Further work to

371 generate wild-type and treatment failure distribution data should be undertaken.

377

382   **Transparency**

383   The authors have no competing interests to declare.

384

385

386

387

388

389

390

391

392

393

394

395

396

397

398

399

400

401   Reference List

402

1. Public Health England. Sexually Transmitted Infections and Chlamydia Screening in England, 2016. In: 2017.

2. BASHH. UK National Guideline for the Mangament of Genital Tract Infection with Chlamydia trachomatis. In: 2006.

3. Geisler WM, Uniyal A, Lee JY, *et al*. Azithromycin versus Doxycycline for Urogenital Chlamydia trachomatis Infection. *N Engl J Med* 2015; **373**: 2512-21.

4. Kong FY, Tabrizi SN, Law M, *et al*. Azithromycin versus doxycycline for the treatment of genital chlamydia infection: a meta-analysis of randomized controlled trials. *Clin Infect Dis* 2014; **59**: 193-205.

5. Kong FY, Hocking JS. Treatment challenges for urogenital and anorectal Chlamydia trachomatis. *BMC Infect Dis* 2015; **15**: 293.

6. Kong FY, Tabrizi SN, Fairley CK, *et al*. Higher organism load associated with failure of azithromycin to treat rectal chlamydia. *Epidemiol Infect* 2016; **144**: 2587-96.

7. Horner PJ. Azithromycin antimicrobial resistance and genital Chlamydia trachomatis infection: duration of therapy may be the key to improving efficacy. *Sex Transm Infect* 2012; **88**: 154-6.

8. Bhengraj AR, Srivastava P, Mittal A. Lack of mutation in macrolide resistance genes in Chlamydia trachomatis clinical isolates with decreased susceptibility to azithromycin. *Int J Antimicrob Agents* 2011; **38**: 178-9.

9. Bhengraj AR, Vardhan H, Srivastava P, *et al*. Decreased susceptibility to azithromycin and doxycycline in clinical isolates of Chlamydia trachomatis obtained from recurrently infected female patients in India. *Chemotherapy* 2010; **56**: 371-7.

10. Misyurina OY, Chipitsyna EV, Finashutina YP, *et al*. Mutations in a 23S rRNA gene of Chlamydia trachomatis associated with resistance to macrolides. *Antimicrob Agents Chemother* 2004; **48**: 1347-9.

11. Somani J, Bhullar VB, Workowski KA, *et al*. Multiple drug-resistant Chlamydia trachomatis associated with clinical treatment failure. *J Infect Dis* 2000; **181**: 1421-7.

12. Afrakhteh M, Mahdavi A, Beyhaghi H, *et al*. The prevalence of Chlamydia trachomatis in patients who remained symptomatic after completion of sexually transmitted infection treatment. *Iran J Reprod Med* 2013; **11**: 285-92.

13. Dukers-Muijrers NH, Speksnijder AG, Morre SA, *et al*. Detection of anorectal and cervicovaginal Chlamydia trachomatis infections following azithromycin treatment: prospective cohort study with multiple time-sequential measures of rRNA, DNA, quantitative load and symptoms. *PLoS One* 2013; **8**: e81236.

14. Gotz HM, Bom RJ, Wolfers ME, *et al*. Use of Chlamydia trachomatis high-resolution typing: an extended case study to distinguish recurrent or persistent infection from new infection. *Sex Transm Infect* 2014; **90**: 155-60.

440   15. Batteiger BE, Tu W, Ofner S, *et al*. Repeated Chlamydia trachomatis genital infections in
441   adolescent women. *J Infect Dis* 2010; **201**: 42-51.

442   16. Lanjouw E, Ouburg S, de Vries HJ, *et al*. Background review for the '2015 European guideline
443   on the management of Chlamydia trachomatis infections'. *Int J STD AIDS* 2015; **0**: 1-25.

444   17. Michel CE, Sonnex C, Carne CA, *et al*. Chlamydia trachomatis load at matched anatomic sites:
445   implications for screening strategies. *J Clin Microbiol* 2007; **45**: 1395-402.

446   18. Wang SA, Papp JR, Stamm WE, *et al*. Evaluation of antimicrobial resistance and treatment
447   failures for Chlamydia trachomatis: a meeting report. *J Infect Dis* 2005; **191**: 917-23.

448   19. Horner P. The case for further treatment studies of uncomplicated genital Chlamydia
449   trachomatis infection. *Sex Transm Infect* 2006; **82**: 340-3.

450   20. Jones RB, Van Der Pol B, Martin DH, *et al*. Partial characterization of Chlamydia trachomatis
451   isolates resistant to multiple antibiotics. *J Infect Dis* 1990; **162**: 1309-15.

452   21. Lefevre JC, Lepargneur JP, Guion D, *et al*. Tetracycline-resistant Chlamydia trachomatis in
453   Toulouse, France. *Pathol Biol (Paris)* 1997; **45**: 376-8.

454   22. Suchland RJ, Geisler WM, Stamm WE. Methodologies and cell lines used for antimicrobial
455   susceptibility testing of Chlamydia spp. *Antimicrob Agents Chemother* 2003; **47**: 636-
456   42.

457   23. Storm M, Gustafsson I, Herrmann B, *et al*. Real-time PCR for pharmacodynamic studies of
458   Chlamydia trachomatis. *J Microbiol Methods* 2005; **61**: 361-7.

459   24. Pitt RA, Alexander S, Horner PJ, *et al*. Presentation of clinically suspected persistent chlamydial
460   infection: a case series. *Int J STD AIDS* 2013; **24**: 469-75.

461   25. Dugan J, Rockey DD, Jones L, *et al*. Tetracycline resistance in Chlamydia suis mediated by
462   genomic islands inserted into the chlamydial inv-like gene. *Antimicrob Agents*
463   *Chemother* 2004; **48**: 3989-95.

464   26. Chen CY, Chi KH, Alexander S, *et al*. The molecular diagnosis of lymphogranuloma venereum:
465   evaluation of a real-time multiplex polymerase chain reaction test using rectal and
466   urethral specimens. *Sex Transm Dis* 2007; **34**: 451-5.

467   27. Beatty WL, Byrne GI, Morrison RP. Morphologic and antigenic characterization of interferon
468   gamma-mediated persistent Chlamydia trachomatis infection in vitro. *Proc Natl Acad*
469   *Sci U S A* 1993; **90**: 3998-4002.

470   28. Pickett MA, Everson JS, Pead PJ, *et al*. The plasmids of Chlamydia trachomatis and
471   Chlamydophila pneumoniae (N16): accurate determination of copy number and the
472   paradoxical effect of plasmid-curing agents. *Microbiology* 2005; **151**: 893-903.

473   29. Ljubin-Sternak S, Mestrovic T, Vilibic-Cavlek T, *et al*. In vitro susceptibility of urogenital
474   Chlamydia trachomatis strains in a country with high azithromycin consumption rate.
475   *Folia Microbiol (Praha)* 2013; **58**: 361-5.

476   30. Mestrovic T, Ljubin-Sternak S, Sviben M, *et al*. Antimicrobial Sensitivity Profile of Chlamydia
477   trachomatis isolates from Croatia in McCoy Cell Culture System and Comparison with
478   the Literature. *Clin Lab* 2016; **62**: 357-64.

479   31. Horner P, Blee K, O'Mahony C, *et al*. 2015 UK National Guideline on the management of non-
480   gonococcal urethritis. *Int J STD AIDS* 2016; **27**: 85-96.

481   32. Bolan RK, Beymer MR, Weiss RE, *et al*. Doxycycline prophylaxis to reduce incident syphilis
482   among HIV-infected men who have sex with men who continue to engage in high-risk
483   sex: a randomized, controlled pilot study. *Sex Transm Dis* 2015; **42**: 98-103.

484   33. NICE Medicines and Prescribing Centre. Antimicrobial stewardship: systems and processes for
485   effective medicine use. In. *Full guideline; Methods, evidence and recommendations*,
486   2015.

487   34. Hadfield J, Harris SR, Seth-Smith HMB, *et al*. Comprehensive global genome dynamics of
488   Chlamydia trachomatis show ancient diversification followed by contemporary mixing
489   and recent lineage expansion. *Genome Res* 2017; **27**: 1220-9.

490   35. Pantchev A, Sting R, Bauerfeind R, *et al*. Detection of all Chlamydophila and Chlamydia spp. of
491   veterinary interest using species-specific real-time PCR assays. *Comp Immunol*
492   *Microbiol Infect Dis* 2010; **33**: 473-84.
493
494

495

496

497

498

499

500

501

502

503

504

505

506

507   Table 1. Primer and probe sequences used for RT-PCR

	Sequence 5'-3'	Reference

Ct-Forward Ct-Reverse Ct-Probe	GGA TTG ACT CCG ACA ACG TAT TC ATC ATT GCC ATT AGA AAG GGC ATT FAM-TTA CGT GTA GGC GGT TTA GAA AGC GG-BHQ-1	Chen[26]
McCoy-Forward McCoy-Reverse McCoy-Probe	TCA CCC ACA CTG TGC CCA TCT ACG A TGG TGA AGC TGT AGC CAC GCT JOE-TAT GCT CTC CCT-(TAMRA)-CAC GCC ATC CTG CGT	Storm[23]
R19-Forward R19-Reverse R19-Probe	CCT GCC GAA CTG AAA CAT CTT A CCC TAC AAC CCC TCG CTT CT Cy5-CGA GCG AAA GGG GAA GAG CCT AAA CC-BHQ3	Modified from Pantchev[35]

508

509

510

511

512

513

514

515

516

			Treatment prescribed			MIC (mg/L)	
		omp1 genotype	Azithromycin 1 g stat	Doxycycline 100 mg bd 7 days	Other	Azithromycin	Doxycycline
Persistently infected	Cat. 1	Pt.1	G	X1	X2		≤0.125	0.125
		Pt.2	E	X2			≤0.125	>1
		Pt.3	E	X3	X1(14 days)		0.125	0.125
		Pt.4	E	X1	X1		2	0.125
		Pt.5	J	X2	X1	500 mg stat azithromycin then unknown dose od 4 days	≤0.125	0.125
	Cat. 2	Pt.6	D	X2			≤0.125	0.125
		Pt.7	G	X2	X1		≤0.125	0.125
	Cat. 3	Pt.8	E	X2			≤0.125	0.125
		Pt.9	E	X2			0.25	1
		Pt.10	E	X2	X1		≤0.125	>1
		Pt.11	E	X1		500 mg erythromycin qd 7 days	0.5	>1
Successfully treated controls		Ctrl.1	F	x1			≤0.125	≤0.064
		Ctrl.2	E	x1			≤0.125	≤0.064
		Ctrl.3	E	x1			≤0.125	0.125
		Ctrl.4	E	x1			≤0.125	≤0.064
		Ctrl.5	E	x1			≤0.125	0.125
		Ctrl.6	D	x1			≤0.125	≤0.064
		Ctrl.7	E	x1			≤0.125	1
		Ctrl.8	F	x1			≤0.125	0.125
		Ctrl.9	E	x1			≤0.125	0.064
		Ctrl.10	D	x1			≤0.125	0.064
		Ctrl.11	D	x1			0.25	0.25
		Ctrl.12	E	x1			≤0.125	1
		Ctrl.13	E	x1			≤0.125	≤0.064

Control strains	314*	D				≤0.125	-
	R19[Δ]	N/A				-	≥1

519   * isolate from a successfully treated patient, [Δ] tetracycline resistant *C. suis* strain R19[25].

520   *Persistently infected patients were categorised based on their likelihood of re-infection through a self-declared*
521   *sexual behaviour questionnaire. Cat. 1 – no sexual contact since initial diagnosis, Cat. 2 – protected sexual*
522   *contact only and Cat. 3 – unprotected sexual contact with a regular partner who had also tested positive and*
523   *had been treated or a partner that did not test positive. Stat – statim, od – once daily, bd – bi-daily, qd –*
524   *quarter-daily, N/A – not applicable.*

525   Table 3. MICs of azithromycin and doxycycline obtained from isolates from two different *C.*
526   *trachomatis*-infected patient cohorts.

			Persistently infected (n=11)	Successfully treated (n=13)	
Azithromycin	≤0.25		9	13	n^o
			81.8	100	%
	>0.25		2	0	
			18.2	0	
Doxycycline	≤0.064		0	7	
			0	53.9	
	>0.064		11	6	
			100	46.2	

527   Geometric mean of MICs: Azithromycin - 0.127 mg/L (persistently infected) and 0.071 mg/L
528   (successfully treated), Doxycycline – 0.322 mg/L (persistently infected) and 0.097 mg/L (successfully
529   treated).