**Background**

*Chlamydia trachomatis* is the most common bacterial sexually transmitted infection (STI) globally, leading to late sequelae like pelvic inflammatory disease and infertility [1, 2]. Currently, the first-choice treatment for anogenital chlamydia consists of a single 1000 mg dose of azithromycin, or 100 mg doxycycline twice daily for 7 days [3, 4]. No resistance of *C. trachomatis* to either of these drugs has been reported, and a recent randomized controlled trial suggested no inferiority of azithromycin (97 % effective) compared to doxycycline (100 % effective) in urogenital chlamydia infections [5]. However, some studies voice concern about the efficacy of azithromycin as first-choice treatment for anorectal chlamydia [6–9]. Persisting *C. trachomatis* infections could be detected by performing a test of cure (TOC) after treatment. Current chlamydia treatment guidelines recommend a TOC between 3 and 4 weeks after initiation of treatment, in certain patient groups or when symptoms persist [4, 7, 10].
However, up to 90 % of chlamydia infections are asymptomatic, which could lead to persisting infections remaining undetected [4, 11, 12]. Previous reports on the appropriate timing of a TOC using molecular methods are inconsistent, and show (intermittent) persistence of C. trachomatis nucleic acids between 0 and 42 % up to 51 days after treatment [9, 13–19]. Recently, we performed a prospective cohort study on time to clearance for N. gonorrhoeae, using modern RNA- and DNA-based nucleic acid amplification tests (NAATs) [20]. Thirty-seven per cent of the included patients were also coinfected with C. trachomatis. As this study has results of 28 consecutive days for both RNA and DNA, we evaluated the appropriate timing of TOC for anogenital C. trachomatis infections in these coinfected patients.

**Methods**

**Study population and procedure**

In a previously performed cohort study we included patients with anogenital N. gonorrhoeae infection, who visited the STI Outpatient Clinic in Amsterdam, The Netherlands, from March through October 2014 [20]. We collected follow-up data and samples for only one infected anatomical site. The Academic Medical Center Amsterdam medical ethics committee approved the original cohort study (NL45935.018.13), and all patients provided written informed consent. For the current analysis, only patients coinfected with C. trachomatis were included from the cohort.

All patients had received routine treatment for N. gonorrhoeae and C. trachomatis coinfection, consisting of a single intramuscular dose of 500 mg ceftriaxone, plus one oral dose of azithromycin 1000 mg in case of urogenital infection, or doxycycline 100 mg twice daily for at least 7 days in case of anorectal infection. Participants self-collected urine, anal or vaginal NAAT samples: one for RNA-based and one for DNA-based NAAT. Samples were collected pre-treatment and subsequently daily for 28 consecutive days after treatment. We requested participants to abstain from sexual contact or use condoms, refrain from vaginal or rectal douching and keep a study diary. At the end-of-study visit (within 35 days of inclusion) a nurse collected samples from the designated anatomical site for both NAATs.

**NAAT testing for C. trachomatis**

Samples for the RNA-based NAAT were tested using the Aptima Combo 2 assay for C. trachomatis and N. gonorrhoeae (Hologic, San Diego, California). Test sensitivity is 93–98 % and specificity is >99 % [21–23]. Equivocal results were retested using the Aptima CT assay (Hologic). We considered repeatedly equivocal results as positive and excluded samples with repeatedly invalid results.

Samples for DNA-based NAAT were tested using the Cobas 4800 CT/NG assay for C. trachomatis and N. gonorrhoeae (Roche, Basel, Switzerland), and reported as either negative or positive with corresponding cycle threshold (Ct) value. Test sensitivity is 87–97 % and specificity is >99 % [22–24]. Pre-treatment samples with discrepant RNA and DNA results were retested, using the Aptima CT assay (Hologic) for RNA samples, and the Abbott RealTime CT/NG assay (Abbott, Abbott Park, Illinois) for DNA samples.

**Statistical analysis**

The primary endpoint, clearance of C. trachomatis using RNA- or DNA-based NAAT, was defined as three or more consecutive negative results following a positive result. We allowed one missing sample between the last positive and the first negative result. Reinfection was defined as positive test results on three or more consecutive days after clearance; tests had to be positive for both RNA and DNA on at least 1 day. To analyse differences we compared patients grouped by anatomical site using Chi-square, Fisher’s exact or Kruskal-Wallis testing. Time to clearance was analysed with Kaplan-Meier curves, log-rank testing and Cox regression analysis. If we could not determine the exact day of clearance due to missing samples, the patient was excluded from this analysis.

The secondary endpoint, intermittent presence of RNA or DNA (“blip”), was defined as a positive test following clearance, not due to reinfection. Only positive results after the three consecutive negative tests results, used to define clearance, were eligible as a blip. We used logistic regression with generalized estimated equation (GEE) models to identify characteristics associated with blips. All analyses were performed using Stata (version 13; StataCorp, College Station, Texas).

**Results**

**Participants**

Out of 462 patients with anogenital gonorrhoea diagnosed at our STI clinic from March through October 2014, 77 were included in the original cohort. Twenty-six patients (34 %) had a coinfection with C. trachomatis of whom three were lost to follow-up. The remaining 23 patients were included in the current analysis.

**Baseline characteristics**

We included nine women, all with endocervical infections, and 14 men, of whom seven had a urethral and seven a rectal infection; 71 % were men who have sex with men (MSM, Table 1). The median age was 24 years (interquartile range [IQR]: 20–35 years); women were
significantly younger (median 20 years) compared to men (median 32 years, \( P < 0.001 \)). Five men (22 \%) were HIV-positive, and four (80 \%) were on antiretroviral therapy, of whom three had \( CD_4 \) cell counts of \( \geq 500 \) cells/mm\(^3\). A previous chlamydia infection was reported by 12 patients (52 \%), and 13 (57 \%) currently experienced symptoms. The median time between diagnosis and inclusion was 8 days (range 0–12). At inclusion 16 patients (70 \%) received ceftriaxone with azithromycin, and seven (30 \%) received ceftriaxone with doxycycline.

**Behaviour after inclusion**

The median number of collected samples was 28 (range 25–28, Table 2). Forty-eight per cent of patients missed at least one sample. Rectal or vaginal douching was reported by four of 16 patients with rectal or endocervical chlamydia (25 \%). Sexual contact at some point during the 28 days of follow-up was reported by 12 patients (52 \%), and condomless sex by five patients (22 \%).

**Table 1 Baseline characteristics of 23 patients with *Chlamydia trachomatis* and *Neisseria gonorrhoeae* coinfection at inclusion**

| Characteristics                        | Total (N (%)\(^a\)) | Urethra (N (%)\(^a\)) | Rectum (N (%)\(^a\)) | Endocervix (N (%)) | \( P \) |
|----------------------------------------|---------------------|-----------------------|-----------------------|--------------------|--------|
| Total                                   | 23                  | 7 (30.4)              | 7 (30.4)              | 9 (39.1)           |        |
| Gender                                  |                     |                       |                       |                    |        |
| Male                                    | 14 (60.9)           | 7 (100.0)             | 7 (100.0)             | 0 (0.0)            |        |
| Female                                  | 9 (39.1)            | 0 (0.0)               | 0 (0.0)               | 9 (100.0)          |        |
| Median age, in years (IQR)              | 24 (20–35)          | 29 (24–35)            | 40 (24–44)            | 20 (19–23)         | 0.003  |
| Ethnicity                               |                     |                       |                       |                    | 1.00   |
| Dutch                                   | 11 (47.8)           | 3 (42.9)              | 4 (57.1)              | 4 (44.4)           |        |
| Non-Dutch                               | 12 (52.2)           | 4 (57.1)              | 3 (42.9)              | 5 (55.6)           |        |
| Sexual risk group                       |                     |                       |                       |                    |        |
| MSM                                     | 10 (43.5)           | 3 (42.9)              | 7 (100.0)             | 0 (0.0)            |        |
| Hetero male                             | 4 (17.4)            | 4 (57.1)              | 0 (0.0)               | 0 (0.0)            |        |
| Female                                  | 9 (39.1)            | 0 (0.0)               | 0 (0.0)               | 9 (100.0)          |        |
| HIV positive                            | 5 (21.7)            | 1 (14.3)              | 4 (57.1)              | 0 (0.0)            | 0.02   |
| Using cART                              | 4 (80.0)            | 0 (0.0)               | 4 (100.0)             | -                  | 0.20   |
| \( CD_4 \) cell count (cells/mm\(^3\))|                     |                       |                       |                    | 1.00   |
| \( > 499 \)                             | 1 (20.0)            | 0 (0.0)               | 1 (25.0)              | -                  |        |
| \( > 500 \)                             | 4 (80.0)            | 1 (100.0)             | 3 (75.0)              | -                  |        |
| Previous chlamydia episode              | 12 (52.2)           | 3 (42.9)              | 4 (57.1)              | 5 (55.6)           | 1.00   |
| Chlamydia in preceding 6 months         | 3 (13.0)            | 0 (0.0)               | 1 (14.3)              | 2 (22.2)           | 0.75   |
| Symptoms or signs at examination\(^{abc}\) | 13 (56.5)          | 6 (85.7)              | 3 (42.9)              | 4 (44.4)           | 0.23   |
| Median time to inclusion, days (IQR)    | 8 (0–12)            | 0 (0–0)               | 10 (7–13)             | 9 (8–12)           | 0.003  |
| Treatment at inclusion\(^d\)           |                     |                       |                       |                    | 0.001  |
| Ceftriaxone + azithromycin              | 16 (69.6)           | 7 (100.0)             | 1 (14.3)              | 8 (88.9)           |        |
| Ceftriaxone + doxycycline               | 7 (30.4)            | 0 (0.0)               | 6 (85.7)              | 1 (11.1)           |        |

\( IQR \) interquartile range, \( MSM \) men who have sex with men, \( HIV \) human immunodeficiency virus, \( cART \) combination antiretroviral therapy  
\(^a\)Unless otherwise indicated  
\(^b\)Symptoms included: discharge, itch, burning, frequent or painful urination, bleeding, abdominal pain, pain during sex, anal cramps or pain, and changed defecation  
\(^c\)Signs included: red urethra, discharge, bleeding, fragile mucosa, swelling or anal ulcerations  
\(^d\)1 patient was negative for *Chlamydia trachomatis* at the initial visit and therefore received ceftriaxone mono-therapy. The test at inclusion was positive and doxycycline was started 6 days after inclusion; therefore the start of the study for the *C. trachomatis* analysis in this patient was day 6, and the treatment was ceftriaxone + doxycycline

**Clearance of *C. trachomatis* RNA and DNA**

During the 28 days of follow-up all patients cleared *C. trachomatis* RNA, and none experienced a reinfection (Table 2). Because of missing samples in the days around clearance, we could not determine the exact day of clearance for two patients. The median time to clearance for the remaining 21 patients was 7 days (range 1–13), and 95 \% of patients had cleared RNA at day 13 (Fig. 1a). One patient was post-hoc excluded from the DNA analysis because of a negative pre-treatment DNA result for *C. trachomatis*. All other patients cleared *C. trachomatis*
DNA during follow-up, and there were no reinfections. We could not determine the exact day of clearance for one patient (Table 2). The median time to clearance of the 21 patients was 6 days (range 1–15), and 95 % of patients had cleared DNA at day 14 (Fig. 1b).

Because of the small sample size the power to detect associations with clearance is very limited. Cox regression analyses showed no significant associations with clearance, and Kaplan-Meier curves with log-rank testing showed no significant difference in clearance by anatomical site or treatment.

**Table 2** Behaviour after inclusion and clearance of *Chlamydia trachomatis* based on RNA and DNA testing, by anatomical site

| Characteristics                          | Total n (%) | Urethra n (%) | Rectum n (%) | Endocervix n (%) | P     |
|------------------------------------------|-------------|---------------|--------------|------------------|-------|
| Patients                                 | 23          | 7 (30.4)      | 7 (30.4)     | 9 (39.1)         |       |
| Behaviour after inclusion                |             |               |              |                  |       |
| Median no. of samples collected (range)  | 28 (25–28)  | 28 (26–28)    | 28 (26–28)   | 27 (25–28)       | 0.01  |
| Patients with missed samples             | 11 (47.8)   | 1 (14.3)      | 2 (28.6)     | 8 (88.9)         | 0.009 |
| Median no. of missed samples (IQR)       | 1 (1–3)     | 2 (2–2)       | 1.5 (1–2)    | 1 (1–3)          | 0.86  |
| Rectal/vaginal douching                  | 4 (25.0)    | -             | 3 (42.9)     | 1 (11.1)         | 0.26  |
| Sexual contact                           | 12 (52.2)   | 3 (42.9)      | 4 (57.1)     | 5 (55.6)         | 1.00  |
| Condomless sex                           | 5 (21.7)    | 1 (14.3)      | 2 (28.6)     | 2 (22.2)         | 1.00  |
| RNA clearanceb                           |             |               |              |                  |       |
| Clearance during follow-up               | 23 (100.0)  | 7 (100.0)     | 7 (100.0)    | 9 (100.0)        |       |
| Day of clearance definablec              | 21 (91.3)   | 7 (100.0)     | 6 (85.7)     | 8 (88.9)         | 1.00  |
| Median time to clearance, days (range)   | 7 (1–13)    | 5 (1–13)      | 6.5 (5–9)    | 8 (6–13)         | 0.20  |
| Blipsd                                   |             |               |              |                  |       |
| Samples at risk for blip                 | 411         | 140           | 126          | 145              |       |
| Number of blips                          | 18          | 0             | 12           | 6                |       |
| Number of patients                       | 8 (34.8)    | 0 (0.0)       | 3 (42.9)     | 5 (55.6)         | 0.09  |
| Median time to first blip from being at risk, days (range) | 1 (1–16) | - | 1 (1–2) | 4.5 (1–16) | 0.61 |
| DNA clearanced                           |             |               |              |                  |       |
| Clearance during follow-up               | 22 (100.0)  | 6 (100.0)     | 7 (100.0)    | 9 (100.0)        |       |
| Day of clearance definablef              | 21 (95.5)   | 6 (100.0)     | 7 (100.0)    | 8 (88.9)         |       |
| Median time to clearance, days (range)   | 6 (1–15)    | 6 (1–14)      | 5 (2–9)      | 7.5 (5–15)       | 0.08  |
| Blipsf                                   |             |               |              |                  |       |
| Samples at risk for blip                 | 403         | 117           | 138          | 144              |       |
| Number of blips                          | 7           | 0             | 0            | 7                |       |
| Number of patients                       | 5 (22.7)    | 0             | 0            | 5 (55.6)         | 0.01  |
| Median time to first blip from being at risk, days (range) | 3 (2–8) | - | - | 3 (2–8) |       |
| Mean Ct-value (range)                    | 38.6 (35.3–41.7) | - | - | 38.6 (35.3–41.7) |       |

**Blips after clearance of C. trachomatis**

After clearance of RNA, eight patients experienced 18 blips (Table 2). After clearance of DNA, five patients experienced seven blips, of which three (all vaginal samples) coincided with RNA blips (Table 2). Among the patients with blips, sex after clearance was reported by five (RNA) and three (DNA) patients. We observed both RNA and DNA blips among vaginal samples, while we observed only RNA blips among rectal samples, and no blips among urine samples.

When analysing all samples after clearance (411 for RNA and 403 for DNA), the median number of days at
Discussion

In this study we analysed the time to clearance of anogenital *C. trachomatis* after treatment in patients coinfected with *N. gonorrhoeae*, using modern RNA- and DNA-based NAATs and daily collected samples. The median time to clearance was 7 days for RNA and 6 days for DNA. Ninety-five per cent of patients had cleared *C. trachomatis* RNA and DNA after 13 and 14 days, respectively.

Several previous studies reported on in vivo clearance of *C. trachomatis* after treatment, but used different molecular testing methods, and a sampling frequency of no more than twice a week. Some studies observed clearance of DNA within 3 weeks using ligase chain reaction or in-house PCR methods [13, 14], while other studies reported 5–25 % DNA persistence after 3–4 weeks [9, 15, 16, 25]. The exact time to clearance of RNA, when tested by NAAT, was also previously unknown. Sena et al. reported 12 % RNA persistence after 4 weeks in men, while Dukers et al. reported 42 % intermittent positive results up to 51 days [9, 19]. In addition, a recent study reported 24 % positivity in 180 patients after 6 months, but no data on re-exposure or reinfections were reported [26]. Since none of these previous studies reported results from consecutive days, the exact time to clearance could not be determined, and prolonged persistence could not be distinguished from blips. In addition, none reported data on events that could have caused reinfection, like was done in the current study. Although no previous studies have been performed on the clearance of *C. trachomatis* in patients coinfected with *N. gonorrhoeae*, our results confirm the assumption that *C. trachomatis* RNA and DNA are cleared after 2 weeks [14, 17, 25].

Intermittent positive results or blips have been previously described by several studies; between 5 and 18 % of patients had a positive test result following a previous negative result after treatment [9, 14, 17–19]. We report an overall risk of blips after clearance of almost 2 % for DNA and 4 % for RNA, and no treatment failures or reinfections. The slightly higher sensitivity of the RNA test could explain the higher frequency of RNA blips, compared to DNA blips. The fact that all but one of the pre-treatment samples initially diagnosed by RNA testing were also positive for DNA, makes it unlikely that the different sensitivity is of clinical importance in diagnosing chlamydia. On the other hand, when performing a TOC, higher sensitivity could result in more positive results. The implications of this should be clarified in larger studies. Unfortunately, NAAT testing only gives information about the presence of genetic material, but not on the viability of the pathogen or whether this is still infectious. Therefore blips could be the result of deposition of viable or non-viable genetic material by a
sexual partner, release of genetic material by degrading cells, or possibly the presence of elementary bodies that hold genetic components of \textit{C. trachomatis} \cite{18, 27}. The origin of blips needs to be further examined in future research. Due to the small sample size we could not identify characteristics associated with the occurrence of blips. However, the occurrence of blips, as well as reinfections, could explain the higher positivity rates reported by some studies, especially in those with very long follow-up and limited sampling \cite{9, 15, 18, 19, 26}.

Our study has several limitations. It was performed at a single centre in a high-incidence population, which may limit the generalizability. We selected patients from a cohort infected with \textit{N. gonorrhoeae}, so this concerns a population coinfected with \textit{N. gonorrhoeae} and \textit{C. trachomatis}, which may influence both time to clearance and the occurrence of blips. Because all patients were coinfected with \textit{N. gonorrhoeae}, they were also treated with ceftriaxone. Since ceftriaxone is not effective against chlamydia, it is unlikely that treatment with ceftriaxone influenced the clearance of \textit{C. trachomatis}. Nevertheless, confirmation of our results in chlamydia monoinfected patients is warranted.

**Conclusions**

Our results are the first to show that \textit{C. trachomatis} RNA and DNA are cleared within 14 days of initiating treatment, using daily testing. Despite the small sample size, our results suggest that if a TOC is indicated in patients with \textit{C. trachomatis} and \textit{N. gonorrhoeae} coinfection, it is best performed after at least 2 weeks. Positive results obtained more than 2 weeks after initiation of treatment should be evaluated carefully, as these probably represent blips, and do not necessarily indicate treatment failure or reinfection. To exclude blips as the cause of a positive TOC, we recommend to obtain a new sample for retesting.

**Abbreviations**

95%-CI: 95% confidence interval; DNA: Deoxyribonucleic acid; GEE: Generalized estimated equation; HIV: Human immunodeficiency virus; IQR: Interquartile range; MSM: Men who have sex with men; NAAT: Nucleic acid amplification test; OR: Odds ratio; PCR: Polymerase chain reaction; RNA: Ribonucleic acid; STI: Sexually transmitted infection; TOC: Test of cure.

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**Availability of data and materials**

Due to the small sample size and possible identification of patients the data are not openly available. Data are available by request to the corresponding author.

**Authors’ contributions**

CMW, MFSL, MU, APD and HJCV designed the study. CMW coordinated the study implementation, included patients and collected samples. APD and RS supervised the laboratory testing. CMW analysed the data. MFSL provided statistical advice for the data analysis. All authors interpreted the data. CMW wrote the first draft of the manuscript. All authors critically reviewed the manuscript and approved the final version.

**Competing interests**

Hologic provided Aptima test materials and kits in kind. Roche provided Cobas test materials and kits in kind.

**Consent for publication**

Not applicable.
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