Infection and Drug Resistance

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ORIGINAL RESEARCH

The Effect of Amoxicillin Pre-Exposure on Treatment Outcomes and Antimicrobial Susceptibility in Patients with Urogenital *Chlamydia trachomatis* Infection

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Purpose: We investigated the influence of amoxicillin pre-exposure on treatment outcomes, *Chlamydia trachomatis* (CT) culture, the presence of drug-resistant genes, minimum inhibitory concentrations (MICs), and fractional inhibitory concentrations (FICs) in CT clinical strains. Additionally, we explored the effect of different antimicrobial combinations on CT.

Patients and Methods: Clinical data of 62 patients with CT infection were recorded. Of these, 33 had pre-exposure to amoxicillin and 29 did not. Among patients with pre-exposure, 17 received azithromycin and 16 received minocycline. Among the patients without pre-exposure, 15 received azithromycin and 14 received minocycline. All patients underwent microbiological cure follow-ups one month after completing the treatment. *23S rRNA* gene mutations, acquisition of *tet*(M) and *tet*(C) were detected using reverse transcription PCR (RT-PCR) and PCR, respectively. The MICs and FICs of azithromycin, minocycline, and moxifloxacin, alone or in combination, were determined using the microdilution and checkerboard methods, respectively.

Results: More cases of treatment failure occurred in pre-exposed patients, in both treatment groups (P <0.05). No *23S rRNA* gene mutations or *tet*(M) and *tet*(C) acquisitions were found. More inclusion bodies were cultured from patients without amoxicillin pre-exposure than from those with pre-exposure (P <0.0001). The MICs of all antibiotics were higher in pre-exposed patients than in those without pre-exposure (P <0.01). The FICs of azithromycin plus moxifloxacin were lower than those of the other antibiotic combinations (P <0.0001). The synergy rate of azithromycin plus moxifloxacin was significantly higher than those of azithromycin plus minocycline and minocycline plus moxifloxacin (P <0.001). The FICs of all antibiotic combinations were comparable between isolates from the two patient groups (all P >0.05).

Conclusion: Pre-exposure to amoxicillin in CT patients may inhibit CT growth and decrease sensitivity of CT strains to antibiotics. Azithromycin plus moxifloxacin may be a promising treatment regimen for genital CT infections with treatment failure.

Keywords: *Chlamydia trachomatis*, amoxicillin, minimal inhibitory concentrations, fractional inhibitory concentrations, persistent infection

Introduction

Chlamydia trachomatis (CT) is a pathogen that causes the most commonly diagnosed bacterial sexually transmitted infection worldwide.[1] Based on the 2018 global sexually transmitted infection surveillance report from the World Health Organization, the global estimate of new CT cases in 2016 was 127 million.[2] In China, the reported incidence rate of genital CT infection increased from 37.18 per 100,000 in 2015 to 55.32 per 100,000 in 2019, with an average rate of increase of 10.44%.[3] CT infections are asymptomatic in 61% of women and 68% of men, which means that these infections often remain undiagnosed and untreated, possibly leading to disease transmission.[4] If left untreated, CT infections can result in pelvic inflammatory disease, infertility, ectopic pregnancy, and chronic pelvic pain in women and

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urethritis and epididymitis in men.[5,6] CT can also causes trachoma which is commonly seen in Africa and the Middle East. Moreover, proctitis can occur in men who have sex with men through receptive anal intercourse.[7]

In China, the antibiotics of azithromycin and doxycycline are the first-line treatments for genital CT infections in adults, with minocycline and moxifloxacin as alternatives.[8] Despite the availability of these treatment options, published data indicate that treatment failure of CT infections is a significant problem. For example, Batteiger et al observed that 13.7% of women experienced treatment failure for CT genital infection despite reporting no post-treatment sexual contact and full medication compliance.[9] A review supported this finding by suggesting that the treatment failure rates of genital CT infections range from 5% to 23%.[10] Further support for this finding is provided in an investigation conducted by Handsfield et al where they strongly suggested that treatment failure may occur in more than 5% of patients.[11]

Two possible factors leading to treatment failure may be persistent CT infection and gene mutation/acquisition imparting drug resistance to the pathogen.[12,13] The gene mutation responsible for azithromycin resistance is frequently caused by mutations in the peptidyl transferase region of *23S rRNA* genes, such as those at positions 2057, 2058, 2059, and 2611 (*Escherichia coli* numbering).[14–16] In addition, resistance to tetracycline is often associated with foreign genomic islands integrated into the chlamydial chromosome.[17,18] The *tet*(C)-resistant *Chlamydia suis* (CS) strains and environmental *Chlamydiae* can transfer tetracycline-resistance genes to CT following co-culture in vitro.[18,19] In addition, there is a high-level of resistance to tetracycline in genital bacteria which is mainly due to the presence of the *tet*(M) gene.[20–22]

Under stress, CT enters a state known as "chlamydial persistence", where the developmental cycle is halted and enlarged, and aberrant reticulate bodies are formed.[23] While in this state, CT exhibits reduced sensitivity to antibiotics, which is one proposed mechanism for treatment failure in humans.[10,24] Amoxicillin induces a state of chlamydial persistence in CT both in vivo and in vitro.[12,25,26] In many cities in China, amoxicillin is available in pharmacies without prescription, which may be convenient for people; however, it also carries the risk of fueling the development of drug resistance.[27] The emergence of drug-resistant strains and their states of persistence is a threat to CT antibiotic monotherapy.

Minimum inhibitory concentrations (MICs) are usually assessed for the detection of CT antibiotic resistance, and fractional inhibitory concentrations (FICs) are used to determine the interaction of two combined drugs.[28] In a persistent state, various *Chlamydia* species can survive at antibiotic concentrations well above their MICs, which might be a cause of treatment failure.[29,30] Treatment failure of CT with antimicrobial monotherapy has led to recommendations for dual antibiotic therapy. CT strains that are resistant to azithromycin are unlikely to exhibit cross-resistance with moxifloxacin because of their different inhibitory mechanisms.[31] For this reason, azithromycin combined with moxifloxacin is considered to be an alternative treatment option.[32]

To date, there is no published data comparing treatment outcomes of CT clinical isolates from patients with and without previous exposure to amoxicillin. MIC distributions and resistance gene mutation/acquisition in clinical strains with and without amoxicillin exposure before the initiation of standard treatment have not yet been investigated. Furthermore, there is a lack of data on FICs for the different dual antibiotic regimens in the treatment of clinical CT strains.

In this study, our aim was to examine the effect of amoxicillin exposure and determine its influence on the following: treatment outcomes of patients with CT infection, CT culture, the existence of drug-resistant genes, MICs, and FICs in clinical CT strains. We also explored the effects of different antimicrobial combinations on the FICs of CT.

Materials and Methods

Patients

Clinical data was collected from 62 patients with urogenital CT infection and successful CT culture in vitro. This data was collected at the Sexually Transmitted Disease Clinic of the General Hospital of Tianjin Medical University between 2009 and 2013. Signed informed consent was obtained from all participants. This study adhered to the tenets of the Declaration of Helsinki, and ethical approval was granted by the Ethical Committee of the General Hospital of Tianjin Medical University (No.IRB2023-WZ-023).

Patients were included in the study on the basis of the following criteria: the diagnosis was in line with the diagnostic criteria for *Chlamydia* urogenital infection as described in our previous study;[16] patients with previous amoxicillin exposure took amoxicillin within two weeks of the initiation of azithromycin or minocycline treatment; the patients strictly complied with the standard antibiotic treatment and had no sexual contact during their treatment course and follow-ups. Patients were excluded on the basis of the following criteria: patients who did not adhere to the standard antibiotic regimens, patients who had sexual contact during the follow-up period, pregnant female patients, patients that were exposed to antibiotics other than amoxicillin two weeks prior to receiving the standard treatment regimen, or patients with comorbidities, such as serious cardiovascular disease or infection.

Standard Treatment Regimen

Of the 62 patients included in this study, 33 had a history of amoxicillin exposure prior to the commencement of their standard treatment regimen. The remaining 29 patients did not have pre-exposure to amoxicillin. Among the 33 patients with amoxicillin pre-exposure, 17 were treated with azithromycin and 16 with minocycline. Among the 29 patients without amoxicillin pre-exposure, 15 were treated with azithromycin and 14 with minocycline.

The standard antibiotic treatment regimens for CT infection used in this study were the following: azithromycin treatment (Pfizer Pharmaceutical Co, Ltd. Dalian, China) involved administering 1.0 g once for the first day, and 0.5 g once a day for the following two days. Minocycline treatment (Hanhui Pharmaceutical Co, Ltd. Hanzhou, China), involved administering 100 mg twice daily for 10 days.[8] The study design is illustrated in Figure 1. Microbiological cure was evaluated one month after the treatment course using the PCR- Fluorescence Probing assay (Daan Gene Co, Ltd. Guangzhou, China). If the PCR test result at follow-up was positive, the treatment was considered a failure.

Detection of Resistance-Related Genes in Clinical Isolates

Reverse transcription PCR (RT-PCR) was used to detect mutations in the *23S rRNA* gene of CT, and PCR to detect the *tet* (C) and *tet*(M) genes. The procedures followed, specific primers used, and reaction conditions under which the

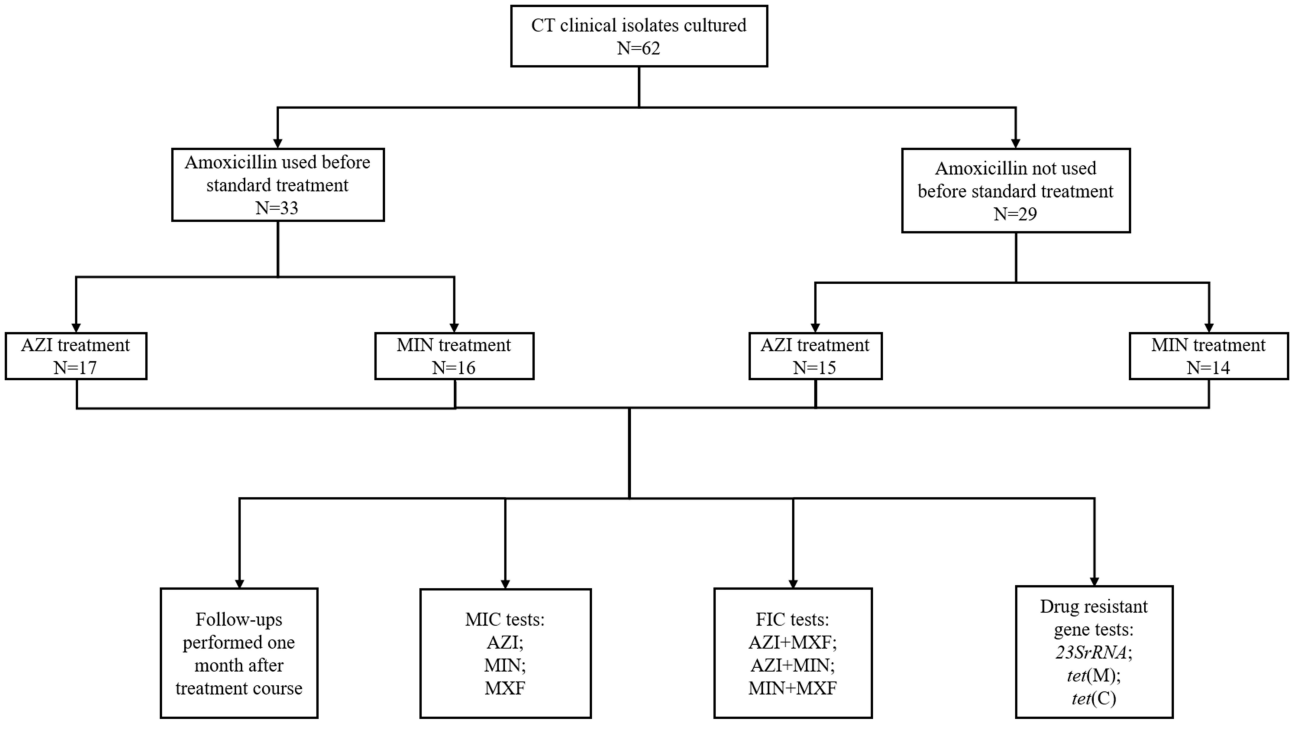

Figure 1 CT clinical sample groups and the laboratory tests that were conducted on the clinical strains.
Abbreviations: CT, *Chlamydia trachomatis*; MIC, minimal inhibitory concentration; FIC, fractional inhibitory concentration; AZI, azithromycin; MINO, minocycline; MXF, moxifloxacin.

experiments were conducted are described in previous publications.[16,33] The Applied Biosystems™ Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems, Inc., Foster City, CA, USA) was used for amplification. The PCR reaction system (25 μL) contained 12.5 μL 2×Taq PCR Master Mix (Tiangen Co, Ltd. Beijing, China), 2.5 μL forward primer, 2.5 μL reverse primer, 5 μL DNA template (100–200ng), and 2.5 μL double distilled water. The PCR products were analyzed via agarose gel electrophoresis. The nucleotide sequences were compared with those of the reference CT serotype D *23S rRNA* (GenBank accession number: AE001273). The length of the amplification products for the 23S rRNA of CT, *tet* (M) and *tet*(C) genes were 725, 305, and 525bp, respectively.

Culture Method of Clinical Strains

Clinical samples of urethral and cervical swabs were mechanically disrupted by vortexing with three glass beads for 5 min, followed by centrifugation at 500 × g for 5 min. The suspension was then inoculated into confluent monolayers of McCoy cells in 24-well plates. The D-UW-5/Cx reference strain (ATCC®-VR-885) purchased from the American Type Culture Collection was used as a control. Centrifugation of the plates at 1200 × g for 1 h at 32 °C was performed, and the plates were incubated for 2 h at 37 °C in 5% CO_2. The supernatant was then removed, and the infected monolayers were overlaid with Dulbecco's modification of Eagle's medium containing 10% (v/v) fetal bovine serum (Haoyang Technological Co, Ltd. Tianjin, China) and 1 μg/mL cycloheximide (Bailingwei Technological Co, Ltd. Beijing, China). Then, the plates were incubated for 48 h at 37 °C in 5% CO_2. After fixation with methanol for 10 min and staining with iodine solution, the CT inclusion bodies were observed under a microscope (Olympus CKX53, Olympus, Tokyo, Japan). All clinical swabs were inoculated in duplicates for the first passage. One well was used for microscopy and the other well, for CT strain collection. Then, the collected CT strain was inoculated in triplicate to process successive passages (the second passage). After inoculation for 48 hours, the CT inclusions were stained with iodine. Three trained personnel independently counted the inclusion bodies of 10 fields at a magnification of 200× in each well. A two-passage culture method was applied in all clinical swabs because we found that there were only a small amount of inclusions formed in the clinical swabs in the first passage; thus, the sensitivity of CT iodine staining could be increased with multiple passages.[34] The successfully cultured CT strains were stored in sucrose phosphate glutamate (218 mM sucrose, 3.76 mM KH_2PO_4, 7.1 mM K_2HPO_4, and 5 mM GlutaMAX-100—all purchased from the Solarbio Technological Co, Ltd. Beijing, China) storage medium at −80 °C for further use.

In vitro Drug Sensitivity Determination

The antimicrobials tested were azithromycin, minocycline, and moxifloxacin (Solarbio Technological Co., Ltd. Beijing, China). High-concentration stock solutions of the three antimicrobials were prepared and diluted to different concentrations for in vitro susceptibility testing, which was performed following the microdilution method with confluent McCoy cells grown in 96-well microtiter plates, as described previously.[16]

Briefly, each clinical strain yielding 1×10^4 inclusion-forming units/mL (IFU/mL) was added to confluent McCoy cell monolayers. IFU/mL was determined using the following equation: $\mathrm{IFU/mL} = \frac{\text{average no .inclusions per field}\times\text{dilution factor}\times\text{no .fields per well}}{\text{volume inoculum}}$.[35] The plates were centrifuged at 1200 × g at 32 °C for 1 h and were then incubated at 37 °C, 5% CO_2 for 2 h to facilitate infection. Then, after the aspiration of the supernatant, the McCoy cell monolayers with CT were overlaid with 0.1 mL of growth medium containing 1 μg/mL cycloheximide and serial two-fold dilutions of antibiotics. The final concentrations of azithromycin, minocycline, and moxifloxacin used were 0.125–2.0 μg/mL, 0.004–0.256 μg/mL, and 0.03–0.24 μg/mL, respectively. CT without any antibiotics was used as the positive control, McCoy cell monolayers without CT were included as the negative control, and the D-UW-5/Cx reference strain with different antibiotics was used in the assays. After incubation at 37 °C in 5% CO_2 for 48 h, the supernatant was discarded, and CT inclusion bodies were stained with iodine dye and observed under a microscope. The lowest antimicrobial concentration at which no CT inclusion bodies were detected was defined as the MIC.

The checkerboard method was used to compare the in vitro efficacy of the antibiotic combinations against CT. The FIC value was used to determine the interaction between two antimicrobial drugs in combination. The procedures were

similar to that of the in vitro susceptibility tests with a single antibiotic. The only difference was that the first antibiotic in the combination was serially diluted along the ordinate, whereas the second antibiotic was diluted along the abscissa of the plates. The microtiter wells inoculated with CT were overlaid with two kinds of two-fold serially diluted antibiotics: azithromycin plus moxifloxacin, azithromycin plus minocycline, or minocycline plus moxifloxacin. The FICs were calculated as follows: FIC = FIC A + FIC B, where FIC A is the MIC of drug A in the combination divided by the MIC of drug A alone, and FIC B is the MIC of drug B in the combination divided by the MIC of drug B alone. The combination is considered synergistic when the FIC is ≤0.5, indifferent when 0.5<FIC<2, and antagonistic when the FIC is ≥2. All tests were performed in triplicate.[36]

Statistical Analysis

IBM SPSS Statistics (v.23.0; IBM Corp, Armonk, NY, USA) and GraphPad Prism 9.0 (Insightful Science Company, San Diego, CA, USA) were used for the statistical analysis of the data obtained in this study. Categorical variables were compared using the Pearson's chi-squared test or Fisher's exact test. An unpaired *t*-test was used to analyze the differences in inclusion body counts. Mann–Whitney *U*-tests were used to analyze the differences in MIC and FIC distributions between the different patient groups. The Friedman test, followed by Bonferroni's multiple comparison test, was used to calculate the FIC differences among the different antibiotic combination groups. Data are presented as the mean± standard deviation (SD) or median and interquartile range (IQR; 25th percentile to 75th percentile). $P < 0.05$ was considered statistically significant.

Results

During the study period, 62 patients with uncomplicated urogenital CT infection were identified. The baseline characteristics of patients with and without amoxicillin exposure before standard treatment are presented in Table 1. There were no significant differences in age, sex, occupation, education, marriage, number of extramarital sexual partners, last extramarital sexual activity time, or antibiotic regimen between the two patient groups (all P >0.05, Table 1). More cases

Table 1 Baseline Characteristics of the Patients with *Chlamydia Trachomatis* Infection with and without Previous Amoxicillin Exposure (n=62)

Characteristics	Groups	Previous Amoxicillin Exposure		χ^2	*P* value[a]
		Yes, Cases n(%)	No, Cases n(%)		
Age	≤29 years	8(24.24)	11(37.93)	1.55	0.46
	30–49 years	22(66.67)	15(51.72)		
	≥50 years	3(9.09)	3(10.34)		
Sex	Male	25(75.76)	22(75.86)	0	>0.99
	Female	8(24.24)	7(24.14)		
Occupation	Worker	9(27.27)	11(37.93)	10.52	0.31
	Self-employed	6(18.18)	9(31.03)		
	Teacher	1(3.03)	0(0)		
	Manager	2(6.06)	0(0)		
	Driver	2(6.06)	0(0)		
	Civil servant	6(18.18)	4(13.79)		
	Student	1(3.03)	2(6.9)		
	Farmer	3(9.09)	0(0)		
	Engineer	1(3.03)	0(0)		
	Officer	2(6.06)	3(10.34)		
Education	Primary school	3(9.09)	1(3.45)	4.91	0.43
	Middle school	6(18.18)	10(34.48)		
	High school	7(21.21)	7(24.14)		

(*Continued*)

Table 1 (Continued).

Characteristics	Groups	Previous Amoxicillin Exposure		χ^2	*P* value[a]
		Yes, Cases n(%)	No, Cases n(%)		
	Vocational school	9(27.27)	5(17.24)		
	Undergraduate school	6(18.18)	6(20.69)		
	Graduate school	2(6.06)	0(0)		
Marriage	Unmarried	4(12.12)	6(20.69)	0.32	0.57
	Married	29(87.88)	23(79.31)		
No. of extramarital sex partners	0	8(24.24)	11(37.93)	1.46	0.69
	1–2	17(51.52)	13(44.83)		
	3–4	3(9.09)	2(6.90)		
	≥5	5(15.15)	3(10.34)		
Last extramarital sexual activity time	≤1 month	8(24.24)	3(10.34)	7.90	0.16
	1 month - 3 months	6(18.18)	4(13.79)		
	3 months - 6 months	6(18.18)	2(6.90)		
	6 months - 1 year	2(6.06)	7(24.14)		
	≥ 1 year	3(9.09)	2(6.90)		
	None	8(24.24)	11(37.93)		
Antibiotic regimen	Azithromycin	17(51.52)	15(51.72)	0	0.99
	Minocycline	16(48.48)	14(48.28)		

Notes: [a]*P* values were computed using Pearson's chi-squared or Fisher's exact test.

of treatment failure were found in patients with previous amoxicillin exposure than in those without previous amoxicillin exposure in both the azithromycin-treated and minocycline-treated groups (P <0.05, Table 2).

The *23S rRNA, tet*(M), and *tet*(C) amplification products were analyzed using agarose gel electrophoresis (Figure 2). No gene mutations were found at positions 2057, 2058, 2059, 2452, or 2611 in the peptidyl transferase region of *23S rRNA*, and no *tet*(M) and *tet*(C) genes were found in any of the patients.

The inclusion bodies of clinical strains were stained with iodine (Figure 3A). More inclusion bodies were cultured from clinical samples of patients without prior amoxicillin exposure than from those with prior amoxicillin exposure (mean number of inclusion bodies per field ± SD: 21.90±4.86 versus 9.40±4.09; P <0.0001, Figure 3B). The mean numbers of inclusion bodies per field ± SD in the different replicates and personnel counting in the two patient groups are presented in Supplementary Table 1.

The MICs of azithromycin, minocycline, and moxifloxacin in the D-UW-5/Cx reference strain were 0.25 μg/mL, 0.032 μg/mL, and 0.06 μg/mL, respectively. The comparison of the MICs of azithromycin, minocycline, and moxifloxacin between CT clinical strains with and without previous amoxicillin exposure is presented in Table 3. The MICs of azithromycin, minocycline, and moxifloxacin were higher in CT clinical strains with amoxicillin pre-exposure than in those without (P <0.01; Table 3). The MIC distribution of the CT clinical strains in the two patient groups is presented in Supplementary Table 2.

Table 2 Treatment Outcomes of the *Chlamydia Trachomatis* Clinical Isolates from Two Different Amoxicillin Exposure Groups (n=62)

Amoxicillin Pre-Exposure	AZI-Treated		*P* value[a]	MIN-Treated		*P* value[a]
	Success Cases n(%)	Failure Cases n(%)		Success Cases n(%)	Failure Cases n(%)	
Yes	10(31.25)	7(21.88)	0.04	9(30)	7(23.33)	0.04
No	14(43.75)	1(3.13)		13(43.33)	1(3.33)	

Notes: [a] *P* values were computed using Fisher's exact test.
Abbreviations: AZI, azithromycin; MIN, minocycline.

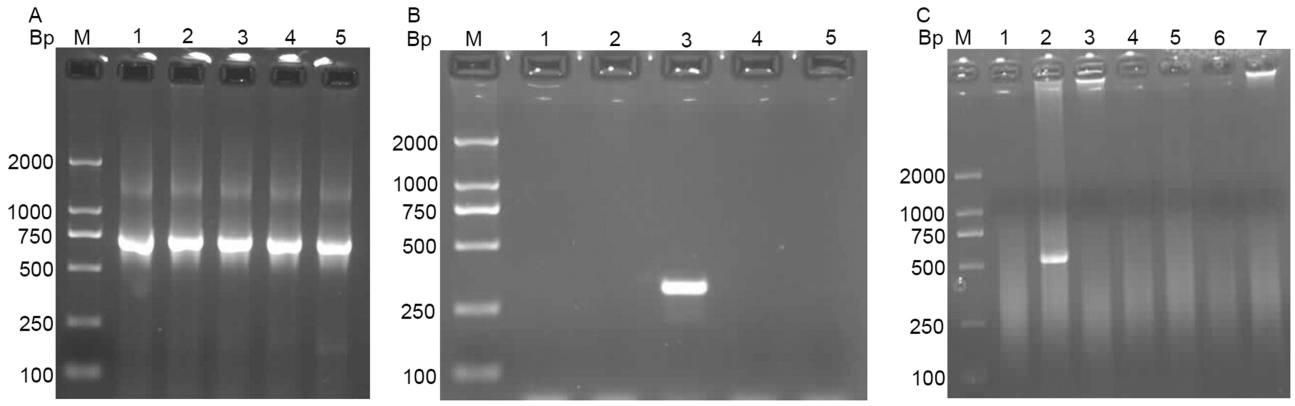

Figure 2 Agarose gel electrophoresis results of *23S rRNA* RT-PCR, *tet*(M), and *tet*(C) PCR products. (**A**) Lane M is the DNA marker, lane 1 is the *23S rRNA* detection of the reference strain, and lanes 2–5 are the *23S rRNA* detection of the four different clinical isolates. (**B**) Lane M is the DNA marker, lanes 1–2 and 4–5 are the clinical strains which are negative for *tet*(M) and lane 3 is the control strain which is positive for *tet*(M). (**C**) Lane M is the DNA marker, lanes 1 and 3–7 are the clinical strains which are negative for *tet*(C) and lane 2 is the positive control of tetracycline-resistant *Chlamydia suis* MS08 strain which is positive for *tet*(C).

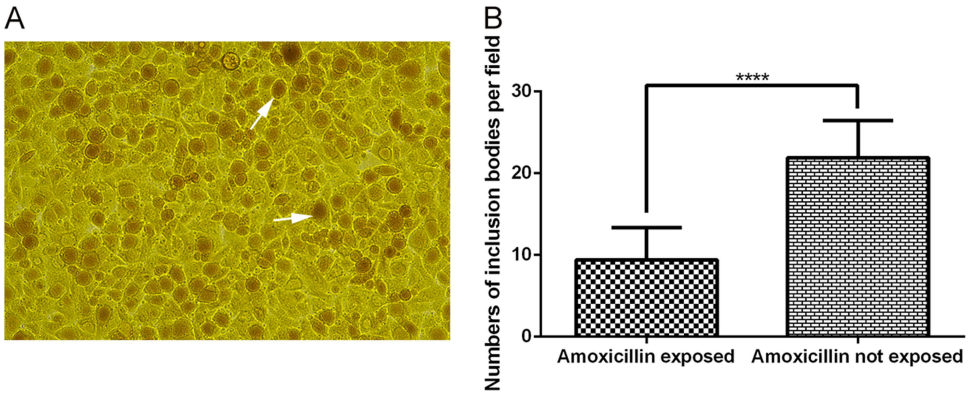

Figure 3 Comparison of the number of inclusion bodies observed in CT clinical strains cultured from patients with or without previous amoxicillin exposure. (**A**) The white arrows indicate the brown CT inclusion bodies stained by iodine (magnification 200×). (**B**) The comparison of the number of inclusion bodies per field between the two patient groups. **** P <0.0001.
Abbreviation: CT, *Chlamydia trachomatis*.

The FIC values of the D-UW-5/Cx reference strain were 0.75, 2.50, and 2.50 in the azithromycin plus moxifloxacin, azithromycin plus minocycline, and minocycline plus moxifloxacin groups, respectively. The median FIC values of all CT clinical strains were 0.75, 2.50, and 3 in the azithromycin plus moxifloxacin, azithromycin plus minocycline, and minocycline plus moxifloxacin groups, respectively (Figure 4). In all patients, the FIC values of azithromycin plus moxifloxacin were lower than those of azithromycin plus minocycline and minocycline plus moxifloxacin (P <0.0001; Figure 4). The synergy rate of azithromycin plus moxifloxacin (24.19%) was significantly higher than those of azithromycin plus minocycline (0) and minocycline plus moxifloxacin (0) (P <0.001; Table 4). No antagonistic interactions were observed

Table 3 Comparison of MICs of AZI, MIN, and MXF in *Chlamydia Trachomatis* Clinical Strains with and without Previous Amoxicillin Exposure

Antibiotics	Previous Amoxicillin Exposure Median MIC (IQR)(μg/mL)	No Previous Amoxicillin Exposure Median MIC (IQR)(μg/mL)	U	*P* value[a]
AZI	0.5 (0.25–1)	0.25 (0.125–0.5)	246	0.001
MIN	0.128 (0.064–0.128)	0.032 (0.032–0.064)	193.5	<0.001
MXF	0.12 (0.12–0.24)	0.06 (0.06–0.12)	106.5	<0.001

Notes: [a] *P* values were computed using the Mann–Whitney *U*-test.
Abbreviations: MIC, minimal inhibitory concentration; IQR, interquartile range; AZI, azithromycin; MIN, minocycline; MXF, moxifloxacin.

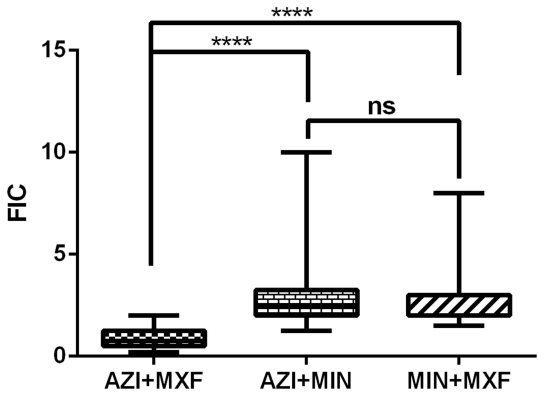

Figure 4 Comparison of FICs among different antibiotic combinations. ****P <0.0001.
Abbreviations: FIC, fractional inhibitory concentration; AZI, azithromycin; MINO, minocycline; MXF, moxifloxacin; ns, non- significant.

in the azithromycin plus moxifloxacin combination (Table 4). The FICs of azithromycin plus moxifloxacin, azithromycin plus minocycline, and minocycline plus moxifloxacin were comparable between isolates from the two patient groups (all P >0.05, Table 5).

Discussion

In this study, we analyzed data from 62 patients with CT infection. According to follow-up data, the occurrence of treatment failure was higher in the group with pre-exposure to amoxicillin than in the group without amoxicillin pre-exposure. We found that 21.88% of the patients treated with azithromycin and 23.33% of the patients treated with minocycline experienced treatment failure following previous amoxicillin exposure. Although there is a lack of data available on the percentage of treatment failure in patients with previous amoxicillin exposure, some experts have found

Table 4 Comparison of Effects of Different Antibiotic Combinations Applied in the *Chlamydia Trachomatis* Clinical Strains (n=62)

Effects	AZI+MXF Cases n(%)	AZI+MIN Cases n(%)	MIN+MXF Cases n(%)	χ^2	*P* value[a]
Synergy[b]	15(24.19)	0(0)	0(0)	72.87	<0.001
Indifference[c]	47(75.81)	30(48.39)	25(40.32)		
Antagonism[d]	0(0)	32(51.61)	37(59.68)		

Notes: [a]*P* value was computed using the Chi square test. [b] Synergy was defined as FIC ≤0.5, [c] indifference was defined as 0.5<FIC<2, and [d] antagonism was defined as FIC ≥2. Paired comparison testing results showed that the synergy rate of AZI +MXF (24.19%) was significantly higher than those of AZI+MIN (0) and MIN+MXF (0) (P <0.001).
Abbreviations: FIC, fractional inhibitory concentration; AZI, azithromycin; MXF, moxifloxacin; MIN, minocycline.

Table 5 Comparison of FIC Between the Groups with and without Previous Amoxicillin Exposure

Antibiotic Combination	Previous Amoxicillin Exposure	No Previous Amoxicillin Exposure	U	*P* value[a]
	Median of FIC (IQR)	Median of FIC (IQR)		
AZI+MXF	0.75 (0.56–1.13)	0.75 (0.5–1.25)	414	0.36
AZI+MIN	2.25 (2–3)	3 (2–4)	432	0.50
MIN+MXF	2.5 (2–4)	3 (2–3)	458	0.76

Notes: [a]*P* values were computed using the Mann–Whitney *U*-test.
Abbreviations: FIC, fractional inhibitory concentration; AZI, azithromycin; MXF, moxifloxacin; MIN, minocycline; IQR, interquartile range.

that the azithromycin therapy failure rate increased from 9% to 22% in mice infected with amoxicillin-stressed *Chlamydiae*, which is similar to our results.[12]

There are several reasons for treatment failure in genital CT infection, including the lack of compliance with the treatment regimen, re-infection due to sexual contact, drug resistance gene mutation/acquisition or persistent CT infection.[16] In our study, patients who did not adhere to the treatment regimen or had the risk of re-infection due to sexual contact during the follow-up period were excluded. Therefore, drug resistance gene mutation/acquisition or persistent infection of CT were considered critical factors leading to treatment failure.

CS has been reported to show antibiotic resistance through the acquisition of a *tet*(C) gene via horizontal gene transfer (HGT).[33,37] Since both CS and CT infect the human conjunctiva and rectum,[38–40] close contact to each other could increase the in vivo chances for HGT of *tet*(C) from CS to CT. Although the in vivo transfer of *tet*(C) gene from CS to CT has not been reported,[39–41] urogenital CT might still have a small possibility of *tet*(C) acquisition from CS in vivo. Joseph et al suggested that the *tet*(C) gene in CS is acquired from Betaproteobacteria.[42] *Neisseria gonorrhoeae* (NG) is a Gram-negative pathogen belonging to Betaproteobacteria that causes human urogenital infection.[43] CT and NG are common bacterial causes of sexually transmitted diseases with high incidence of co-infection in urogenital tracts.[44] Also, the t*et*(M) gene was found in most of the tetracycline-resistant NG isolates.[45,46] *Mycoplasma hominis* (MH) is an opportunistic pathogen detected on the mucosal membranes of human urogenital tracts.[47] Chalker et al found that all tetracycline resistance in MH was mediated by the *tet*(M).[48] Li et al reported that nearly 20% of the vaginal swabs from a fertility clinic in China were positive for CT or *Mycoplasma* spp. All vaginal swabs tested were positive for *tet*(M), which could present in vaginal microbes such as CT, *Mycoplasma* spp, or lactobacilli.[49] We speculated that contact between CT and NG, MH, or other bacteria in the urogenital sites might enable the transfer of *tet*(M) to CT. Therefore, both *tet*(C) and *tet*(M) genes were detected among the clinical CT isolates in our study. No gene mutations in the *23S rRNA* and no acquisition of the *tet*(C) and *tet*(M) were detected in the patients included in our study. These results were consistent with other studies showing that resistance genes are uncommon in clinical strains of CT.[50–52] In contrast, the study performed by Shao et al found acquisition of *tet*(M) and 23S rRNA gene mutations in the treatment-failure group, including the mutations A2057G, C2452A, and T2611C.[16] The reason for this difference may be that the samples we collected were from different years, and our sample size was smaller. Besides, we speculate that persistent CT infection could also have contributed to treatment failure.

In our study, the number of inclusion bodies in patients with amoxicillin pre-exposure were lower than in patients without amoxicillin pre-exposure. It has been inferred that the accumulation of antibiotics in patients can limit CT growth during early passages.[34] In addition, some experts have reported that in vitro exposure to several β-lactam antibiotics causes the reticulate body of CT to convert to the aberrant reticulate body phenotype found in a persistent state.[25] Therefore, the administration of amoxicillin can cause CT to enter a persistent state. In this state, CT is viable but non-cultivable; thus, no typical inclusions can be formed, resulting in a reduction of the number of inclusion bodies.[23]

Furthermore, persistent infection can reduce antibiotic sensitivity and therefore, is a proposed mechanism for treatment failure in humans. It has been reported that entry into aberrant reticulate bodies has increased the resistance of CT to azithromycin and doxycycline.[53,54] Panzetta et al found that post-gonococcal urethritis was caused by the persistence of CT infection as a result of β-lactam or cephalosporin use for the clearance of NG.[23] Augenbraun et al observed the reactivation of CT infection after treatment with a β-lactam or cephalosporin regimen in gonorrhea patients co-infected with CT.[55] In our study, we acquired similar results showing that CT still occurred in some patients with pre-exposure to amoxicillin, even after regular treatment.

Many common pathogenic bacteria have internationally recognized standards for antimicrobial susceptibility testing. Due to the challenges associated with CT culturing and the lack of data on the susceptibility of CT against different antibiotics, a clear interpretation of MIC results is challenging. Therefore, there is no recognized standardization of antimicrobial susceptibility testing for CT, making it impossible to determine whether CT is resistant to certain antibiotics. Consequently, we can only obtain the relative activity of an antimicrobial agent against CT, which is determined by comparing MIC values. In our study, we found that the MICs of CT in the patients with previous exposure to amoxicillin were higher than in those without, which demonstrates that the application of amoxicillin in patients might make CT less susceptible to standard antibiotic regimens. These results are consistent with the in vitro

results presented by Wyrick et al where they showed that pre-exposure of human endometrial epithelial cells (HEC-1B) infected with CT to penicillin made CT resistant to azithromycin.[53] By examining the contents of the ultrastructural inclusion bodies of CT, they demonstrated that penicillin could cause CT to enter a state of persistent infection with abnormal reticulate bodies that are phenotypically resistant to azithromycin.

In a persistent state of infection, CT transforms into a large aberrant body that is viable but has a near-static metabolism. This significantly increases its resistance to antibiotics that inhibit protein synthesis (eg, tetracyclines and macrolides).[56] Bhengraj et al found that the MIC values for azithromycin and doxycycline from isolated CT strains from persistently infected female patients were higher than those from a sensitive control strain.[57] Amoxicillin exposure may exert pressure on CT and lead to persistent infection, resulting in elevated MICs and treatment failure. However, Reveneau et al found that azithromycin was effective in eradicating interferon (IFN)-induced (specifically IFN-γ) persistent chlamydial infections. The mechanisms by which IFN-γ and penicillin induce persistent CT infection differ and therefore, can lead to contrasting azithromycin treatment efficacies.[54]

Based on the above data, it is necessary to identify alternative therapies for patients with CT infection and previous amoxicillin exposure. Qi et al reported that dual therapy with two different antibiotics may be a possible alternative solution.[32] In this study, we found that more CT strains exhibited synergistic effects when treated with the azithromycin plus moxifloxacin combination than when treated with either of the two other dual therapy combinations (Table 4). In Table 5, it can be seen that there was no statistical difference between the FIC values of different antibiotic combinations with or without previous amoxicillin exposure. Thus, unlike the MICs of a single antibiotic, the pre-exposure of amoxicillin did not influence the FICs of dual antimicrobial therapies.

In our study, synergy was only found in the azithromycin plus moxifloxacin combination group. In contrast, more than half of the clinical strains in the azithromycin plus minocycline and minocycline plus moxifloxacin groups displayed antagonism. The effects of these three combinations have also been investigated by other researchers. For example, Wang et al found that 9.76% of clinical strains showed synergistic effects in the azithromycin plus moxifloxacin combination, whereas most of the clinical CT strains (90.24%) exhibited antagonistic effects in the azithromycin plus minocycline combination.[58] They also observed an antagonistic effect in the moxifloxacin plus minocycline combination in 85.37% of strains and no synergistic effect was observed in the moxifloxacin plus minocycline combination groups.[59] Singh et al found that the azithromycin plus moxifloxacin combination presented synergistic effects in 16.8% of NG strains, without any antagonism.[60] They speculated that this may be because the synergy between the different mechanisms of action of the antibiotics produced a more successful antimicrobial effect than each antibiotic in its individual capacity.[60] Thus, they considered the azithromycin plus moxifloxacin combination a potential new candidate for therapy against NG isolates.[60] Based on the above data, the combination of azithromycin plus moxifloxacin could be considered a promising alternative treatment option for persistent CT infection. However, 75.81% of patient samples exhibited indifference (neither synergy nor antagonism) in vitro with the azithromycin plus moxifloxacin combination. Thus, further in vivo studies are necessary to explore the effects of the azithromycin plus moxifloxacin combination since host pharmacokinetics can also influence treatment efficacy.

Our study has some limitations. Firstly, a randomized multicenter study with a larger sample size is required to obtain more objective and reliable results. Secondly, the effects of the antibiotic combinations on CT were not evaluated in vivo. Thirdly, we did not employ techniques such as electron microscopy to prove the existence of persistent CT in the clinical samples. Finally, the detection of inclusion bodies with iodine staining highly depends on the subjective assessment of the person evaluating the stained cells and may lack specificity and sensitivity compared with fluorescent staining with monoclonal antibodies.

Conclusion

Based on this study, it can be inferred that previous exposure to amoxicillin in CT-infected patients could inhibit the CT growth and decrease CT sensitivity to the recommended standard antibiotic treatment regimen. The persistent state of CT induced by amoxicillin is a likely explanation for treatment failure. Also, azithromycin plus moxifloxacin may be a promising treatment regimen for genital CT infection exhibiting failure in response to standard treatment.

Further research could include the repetition of this study with a larger, randomized sample group using more specialized techniques such as electron microscopy to prove persistent infection and fluorescent staining for inclusion

body counting. Another possibility would be to evaluate the efficacy of dual antibiotic therapies in vivo using an animal model with persistent CT infection.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Informed Consent

Before undergoing the treatment, all patients provided written informed consent for their data to be published in the article. The study adhered to the principles of the World Medical Association's Declaration of Helsinki, and ethics approval for this study was granted by the Ethics Committee of the General Hospital of Tianjin Medical University (No. IRB2023-WZ-023).

Consent for Publication

Consent for publication was included in the informed consent obtained from each participant.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

Authors declare no competing interests for this study.

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