Identification and characterization of mixed infections of *Chlamydia trachomatis* via high-throughput sequencing

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Precise genotyping is necessary to understand epidemiology and clinical manifestations of *Chlamydia trachomatis* infection with different genotypes. Next-generation high-throughput sequencing (NGHTS) has opened new frontiers in microbial genotyping, but has been clinically characterized in only a few settings. This study aimed to determine *C. trachomatis* genotypes in particular mixed-genotype infections and their association with clinical manifestations and to characterize the sensitivity and accuracy of NGHTS. Cervical specimens were collected from 8,087 subjects from physical examination center (PEC), assisted reproductive technology center (ART) and gynecology clinics (GC) of Chenzhou Hospital of China. The overall prevalence of *C. trachomatis* was 3.8% (311/8087) whereas a prevalence of 2.8, 3.7 and 4.8% was found in PEC, ART and GC, respectively. The most frequent three *C. trachomatis* genotypes were E (27.4%, 83/303), F (21.5%, 65/303) and J (18.2%, 55/303). Moreover, NGHTS identified 20 (6.6%, 20/303) mixed-genotype infections of *C. trachomatis*. Genotype G was more often observed in the subjects with pelvic inflammatory disease than genotype E (adjusted OR = 3.61, 95% CI, 1.02–12.8, *p* = 0.046). Mixed-genotype infection was associated with severe vaginal cleanliness (degree IV) with an adjusted OR of 5.17 (95% CI, 1.03–25.9, *p* = 0.046) whereas mixed-genotype infection with large proportion of minor genotypes was associated with cervical squamous intraepithelial lesion (SIL) with an adjusted OR of 5.51 (95% CI, 1.17–26.01, *p* = 0.031). Our results indicated that NGHTS is a feasible tool to identify *C. trachomatis* mixed-genotype infections, which may be associated with worse vaginal cleanliness and cervical SIL.

**KEYWORDS**

*Chlamydia trachomatis*, next-generation high-throughput sequencing, genotype, mixed-genotype infection, clinical manifestation
Introduction

*Chlamydia trachomatis* is one of the most widespread sexually transmitted diseases (Fu et al., 2022). Approximately, 80% of *C. trachomatis* infections are essentially asymptomatic (Marcone et al., 2012). Persistent *C. trachomatis* infection can cause various sequelae such as urethritis, endometritis, pelvic inflammatory disease (PID), tubal factor infertility, and ectopic pregnancy (Woodhall et al., 2018). *C. trachomatis* includes 19 genotypes (Bax et al., 2011). In general, genotype A, B and C are usually associated with trachoma while genotype D-K primarily cause urogenital infection (Bax et al., 2011). In addition, genotype L1-L3 are the agents of lymphogranuloma venereum (Jurstnad et al., 2001).

The ompA is one of the most variable genes in the *C. trachomatis* genomes and encodes the main outer membrane protein (MOMP) (Nunes et al., 2009). The *ompA* gene contains four highly polymorphic variable sequences VS1–4, which are separated by five constant sequences CS1–5 (Spaargaren et al., 2005). The most variable and discriminatory nucleotide sequences are found in the VS1 and VS2 regions, which make them the suitable target fragments for *C. trachomatis* genotyping (Spaargaren et al., 2005). However, previous studies showed that the recombination and horizontal gene transfer of *ompA* is a natural phenomenon occurring within some *C. trachomatis* strains (Somboonna et al., 2011; Harris et al., 2012; Matić et al., 2016). Although the *ompA* gene of *C. trachomatis* is a single copy gene and may have a lower probability of switching compared to the multi-copy genes, e.g., cryptic plasmid (Joseph et al., 2011), it may not always represent the genetic background of the *C. trachomatis*.

At present, the methods for *C. trachomatis* genotyping include Sanger sequencing, polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) (Petrovay et al., 2015; Foschi et al., 2016), hybridization methods (Ruettgert et al., 2011; Gharsallah et al., 2012; Martinez et al., 2015; Brasiliense et al., 2016), real-time PCR using fluorescent probes (Jalal et al., 2007), DNA microarray assay (Gallo Vuallet et al., 2016) and whole genome sequencing (WGS; Brown and Christiansen, 2019). Sanger sequencing is the most widely used technique for genotyping, but is not adequate to detect mixed-genotype infections of *C. trachomatis* since it only provides one consensus sequence (Quint et al., 2007; Ruettgert et al., 2011; Gharsallah et al., 2012). Although PCR-RFLP, hybridization methods and DNA microarray assay can identify mixed-genotype infections to some extent (Ruettgert et al., 2011; Gharsallah et al., 2018), their sensitivity and reliability remain to be improved. In contrast, next-generation high-throughput sequencing (NGHTS) targeting variable regions of *ompA* gene can not only inherit the advantage of *ompA* Sanger sequencing to identify *C. trachomatis* genotypes, but also determine mixed-genotype infections and the proportion of different genotypes in the mixed-genotype infections due to enough sequencing depth and large number of sequencing reads (Kawada et al., 2016), which are more suitable for the detection of co-infections or super-infections of different *C. trachomatis* genotypes.

Indeed, the previous studies of *C. trachomatis*-infected patients or animals have demonstrated the difference of *C. trachomatis* genotypes in virulence and pathogenicity (Ito et al., 1990; Lyons et al., 2004). However, these studies only compared the virulence of different *C. trachomatis* genotypes (Batteiger et al., 1989; Workowski et al., 1994; Ngandjio et al., 2003; Lyons et al., 2004). It remains to elucidate whether mixed-genotype infection of *C. trachomatis* could result in enhanced virulence and may account for divergent clinical outcomes of *C. trachomatis* infection. Few studies have attempted to correlate specific clinical manifestations of genital mixed-genotype infection of *C. trachomatis* in humans because of the technical difficulty to identify mixed-genotype infections (Yan et al., 2018).

In this study, we explored the feasibility of NGHTS to determine *C. trachomatis* genotypes and to identify mixed-genotype infections in a large number of *C. trachomatis*-positive cervical samples in a cross-sectional and observational study. The objective of this study was to characterize the sensitivity and accuracy of NGHTS and to determine *C. trachomatis* genotypes in particular mixed-genotype infections and their association with clinical manifestations.

Materials and methods

Study participants and clinical samples

A total of 8,087 samples of cervical swabs from the physical examination center (PEC), assisted reproductive technology center (ART) and gynecology clinics (GC) of Chenzhou No.1 People’s Hospital in Chenzhou of Hunan Province, China were randomly collected and tested for *C. trachomatis* nucleic acid from March 1, 2019 to July 13, 2021. The hospital is the biggest general hospital in Chenzhou and consists of five campuses to cover the whole city and nearby regions. The 2,950 female subjects from PEC were those for routine annual health examinations while the 1,666 female subjects from ART and 3,471 female subjects from GC were those for diagnosis and treatment of infertility and gynecological diseases, respectively. The inclusion criteria were female, and not pregnant. Cervical swab samples were collected using a 200 mm polyethylene Cervix brush device (Hybribio Corp, Guangzhou, China). The specimens were transferred to a tube containing cervical cell preservation solution provided in the kit and stored at −80°C until analysis. For *C. trachomatis* positive subjects, demographic characteristics, antibiotic usage during the previous 3 months, human papillomavirus (HPV) infection, clinical symptoms, vagina cleanliness, cervical abnormalities were retrospectively collected. To detect HPV infection, DNA was extracted from cervical swab samples within 48h after collection using the QIaAmp mini kit (Qiagen, Hilden, Germany). HPV detection and genotyping were performed by using the Hybribio Rapid Geno-Array test kit (Hybribio Corp, Guangdong) based on
Clinical manifestations and diagnosis

Asymptomatic \textit{C. trachomatis} infection was defined as positive for \textit{C. trachomatis} nucleic acid without symptoms, such as painful sexual intercourse, abnormal vaginal discharge, urethritis, irregular vaginal bleeding, or bleeding after sexual intercourse and genital warts (Chen et al., 2020). Vaginosis was diagnosed according to Amsel criteria (Carr et al., 1998). There are many methods to evaluate the vaginal microenvironment. In China, the vaginal cleanliness grade is also used to comprehensively evaluate the status of the vaginal microenvironment (Group, C.M.A.O.a.G.B.I.D.C, 2016), and has been widely accepted for gynecological studies (Yue et al., 2015; Yu et al., 2018). Vaginal cleanliness is classified as I, II, III and IV grades according to bacterium vaginae, Coccus, epithelial cell and leukocytes (Supplementary Table S1). Class I and II are considered normal while class III and IV as abnormal (Bao et al., 2015), and Grade IV vaginal cleanliness is regarded as severe vaginal cleanliness. The vaginal cleanliness is characterized by microscopy and bacterial morphology, and cannot identify the specific species of bacterium vaginae. PID is defined as tenderness with adnexal, cervical motion, and uterine tenderness (Dean et al., 1995). Cervicitis is determined by evaluating and scoring the clinical findings at the time of speculum examination. A score of \( \geq 3 \) is defined as cervicitis while \( \leq 2 \) as no cervicitis (Supplementary Table S1; Batteiger et al., 1989). Colposcopy screening is performed using a digital electronic colposcopy (SLC-3000, Philips, Shenzhen, China) following a standard procedure (Chen et al., 2020). According to the standard and terminology of the American Society for Colposcopy and Cervical Pathology (ASCCP) (Khan et al., 2017), colposcopy impression includes benign, low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and cancer. LSIL and HSIL represent Grade 1 (minor) and Grade 2 (major) abnormal colposcopy findings defined by the International Federation for Cervical Pathology and Colposcopy (IFCPC) nomenclature, respectively (Bornstein et al., 2012). The samples for cytology analysis are harvested by using polyethylene cervix brush device and cervical cell preservation solution (Hybribio Corp, Guangzhou, China). Sectioning and staining are conducted in all-in-one machine (Dacheng, Guangzhou, China). The cytology results are plotted in a table and categorized according to the Bethesda system (TBS) (de Oliveira et al., 2020). The following variables are considered: no malignancy, ASC-US, LSIL, ASC-H, HSIL, squamous cell carcinoma, atypical glandular cells (AGC), adenocarcinoma, and other malignant neoplasms.

Detection and genotyping of \textit{Chlamydia trachomatis} by PCR and sanger sequencing

DNA was extracted from the cervical swabs using QIAamp DNA Minikit QIAgen (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The isolated DNA was stored at \(-80^\circ\text{C}\) until use for PCR and sequencing. A 200 bp conserved cryptic plasmid Pgp2 fragment of \textit{C. trachomatis} was amplified by PCR for diagnosis of \textit{C. trachomatis} infection with the primers of CT-d-F and CT-d-R (Supplementary Table S2). PCR was carried out in 25\(\mu\)l reaction mixture in a thermal cycler with the following reaction conditions: 95\(^\circ\text{C}\) for 2 min, followed by 35 cycles of 95\(^\circ\text{C}\) for 15 s, 55\(^\circ\text{C}\) for 30 s and 72\(^\circ\text{C}\) for 40 s with a final elongation at 72\(^\circ\text{C}\) for 5 min.

For genotyping, \textit{C. trachomatis ompA} fragment VS1–VS4 was first amplified by nested PCR using the outer primers CT1 and CT2 followed by the amplification of a 580 bp VS1–VS2 fragment using the inner primers CT3 and CT4. First round PCR was carried out in 25\(\mu\)l reaction mixture with the following reaction conditions: 95\(^\circ\text{C}\) for 5 min, followed by 25 cycles of 95\(^\circ\text{C}\) for 60 s, 55\(^\circ\text{C}\) for 60 s and 72\(^\circ\text{C}\) for 80 s, with a final elongation at 72\(^\circ\text{C}\) for 10 min. The reaction conditions of the second round PCR were: 95\(^\circ\text{C}\) for 5 min, followed by 35 cycles of 95\(^\circ\text{C}\) for 30 s, 55\(^\circ\text{C}\) for 30 s and 72\(^\circ\text{C}\) for 30 s, with a final elongation at 72\(^\circ\text{C}\) for 10 min. All the primer sequences were listed in the Supplementary Table S2. The \textit{C. trachomatis} strain (ATCC VR-348B) was used as positive control and DNase-free water as negative control in PCR. PCR products for \textit{C. trachomatis ompA} gene were sent out for Sanger sequencing in Ruibo Biotech (Guangzhou, China). Genotypes of \textit{C. trachomatis} were determined by BLAST as previously described (Yang et al., 2010).

Identification of mixed-genotype infections of \textit{Chlamydia trachomatis} using NGHTS

For \textit{C. trachomatis} genotyping through NGHTS, a 448 bp fragment of \textit{C. trachomatis} ompA gene VS1–VS2 region was amplified using nested PCR with the following primers (Supplementary Figure S1): outer primes ompA CT-HTS-F-outer/CT-HTS-R-outer and inner primers CT-HTS-F-inner/CT-HTS-R-inner with barcode (Supplementary Table S2). The first round PCR was carried out in 25\(\mu\)l reaction mixture, with 12.5\(\mu\)l of forward and reverse primers (10 pmol/ul), and the following reaction conditions: 95\(^\circ\text{C}\) for 3 min, followed by 25 cycles of 95\(^\circ\text{C}\) for 15 s, 55\(^\circ\text{C}\) for 30 s and 72\(^\circ\text{C}\) for 40 s, with a final elongation at 72\(^\circ\text{C}\) for 5 min. The second round

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PCR was carried out in 50μl reaction volume with 25μl of Phanta® Max Super-Fidelity DNA polymerase (Vazyme Biotech, Nanjing, China), 1μl of forward and reverse primers (10 pmol/μl), and 2μl first-round PCR product. Thermal cycling consisted of initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 15s, annealing at 55°C for 15s, and elongation at 72°C for 30s with final incubation at 72°C for 5 min. The second-round PCR products were purified using universal DNA Purification Kit (TIANGEN Biotech, Beijing, China) according to the manufacturer’s manual, and quantified using GENOVA NANO (Bibby Scientific Ltd., Stone, United Kingdom). Finally, 10 samples were mixed at 1μg of purified DNA per sample and confirmed by electrophoresis on 2% agarose gel.

Sequencing libraries were generated using NEB Next® UltraTM DNA Library Prep Kit for Illumina (NEB, Massachusetts, United States) following the manufacturer’s recommendations and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific, Massachusetts, United States) and Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, United States). Finally, the library was sequenced on an Illumina NovaSeq 6000 and 250bp paired-end reads were generated. To make the analysis results more accurate and reliable, the original data were first spliced and filtered to obtain clean data. The clean data was obtained using fastp software, with the following criterion (Novogene Technology Co., LTD, Beijing, China): when the N in any sequencing read exceeds 10% of the total number of reads, or the number of bases with low quality (Q ≤5) in any sequencing reads exceeds 50% of the total number of reads, or sequencing read contains adapter sequences, these reads are eliminated. Paired-end reads from the original DNA fragments were merged by using the FLASH program, which inputs a fastq library of paired-end reads (reads1 and reads2) in which some of the reads overlap the read generated from the opposite end of the same DNA fragment, and merged the fragments based on the correct overlap between the paired-end reads (Magoč and Salzberg, 2011). The sequences of different samples were extracted based on the specific barcode sequences. Burrows-Wheeler transform (BWA 0.7.17; Li and Durbin, 2009) with the default parameters was used for aligning all the clean sequence data with the reference sequences of C. trachomatis genotype A-K and L1-L3. Based on the results of sequence alignment, the genotype of each read was determined and the composition of different genotypes was calculated. The proportion of minor genotype >1% is defined as a mixed-genotype infection (Quer et al., 2015). The genotype with the large proportion is considered to be the major genotype in the case of a mixed-genotype infection identified. The reference sequences used in this study included A/Sa1 (M58938), B/IU1226 (AF063208), C/TW3 (M17343), D/ UW3 (AE001338), E/Bour (X52557), F/IC-Cal3 (X52080), G/UW57 (AF063199), H/UW4 (X16007), I/UW-12 (AF063200), J/UW36 (AF063202), K/UW31 (AF063204), L1/440 (M36533), L2/434 (M14738), and L3/404 (X55700).

Detection limit of NGHTS for identifying mixed genotypes

A 456 bp fragment of theompA gene was amplified from the clinical samples infected with C. trachomatis genotype B, D, E, F, G, H, J and K, and cloned into the pUC57 vector (Tsingke Biotech Corp, Beijing, China). The plasmid DNA was purified and quantified using a GENOVA NANO (Bibby Scientific Ltd., Stone, United Kingdom). The DNA copy number was calculated using the following formula: DNA copy number (copy number/μL) = [6.02 × 10^{22} × plasmid concentration (ng/μL) × 10^{-7}] / [DNA in length x 660]. A serial 10-fold diluted plasmid DNAs for C. trachomatis genotype B, D, E, F, G, H, J and K were used to determine the low detection limit of NGHTS. Furthermore, the mixtures of different plasmid DNAs of C. trachomatis genotype of F/G, E/J and E/F were prepared at the ratio of 50/50, 30/70, 20/80, 10/90, 2.5/97.5, 1/99, and amplified and sequenced to assess the sensitivity and accuracy of NGHTS in distinguishing mixed C. trachomatis genotypes.

Amplification of minor genotypes using genotype-specific primers in the samples infected with mixed Chlamydia trachomatis genotypes

The genotype-specific primers for minor genotypes were designed using DNASTAR software (DNASTAR Inc., Madison, WI, United States) according to the sequence difference between C. trachomatis genotypes (Supplementary Table S3). For the verification of the samples with mixed C. trachomatis genotypes, C. trachomatis ompA fragment VS1–VS2 was first amplified using the outer primers ompA CT-HTS-F-outer/CT-HTS-R-outer followed by the amplification using the sample-genotype-specific primers. For each sample with mixed-genotype infections, the genotype-specific primer was designed to match the sequence of minor genotype but not the major genotype, especially in the 3′ end. The PCR products were detected by electrophoresis on 2% agarose gel and sent to Ruibo Biotech (Guangzhou, China) for Sanger sequencing. Genotype verification was conducted by using the BLAST program as previously described (Yang et al., 2010).

Detection of bacterial load of Chlamydia trachomatis

A real-time quantitative PCR (qPCR) assay was adapted to determine C. trachomatis bacterial loads using primers of the Pgp2 gene (Supplementary Table S2). qPCR was carried out in 20μl reaction volume with 10μl of TB Green Fast qPCR Mix (Takara Bio Inc., Shiga, Japan), 0.8μl of forward and reverse primers (10 pmol/μl), 6.4μl of H₂O and 2μl DNA template. The following are the reaction conditions with LightCycler 480 System (Roche Diagnostics GmbH, Mannheim, Germany): 95°C for 30s,
followed by 40 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 30 s. The bacterial loads were calculated according to the standard curve.

Statistical analysis

Statistical analysis was done using SPSS 25.0 software (IBM). Continuous variables were presented as mean ± SE and tested by t-test whereas categorical variables were expressed as numbers and tested by Chi-square tests. Association of \( C.\ trachomatis \) genotype or mixed-genotype with clinical manifestations was explored by multivariate logistic regression analysis and presented as odds ratio (OR).

Propensity scores (PS) was calculated using logistic regression with respect to age, clinical departments, antibiotic usage, HPV infection. In addition, PS was adjusted by a standardized mortality ratio weighting (SMRW) method in which a weight of 1 was assigned for cases and a weight of \([PS (1 – Pt)]/[(1 – PS) Pt]\) for controls, respectively. The proportion of treatment (Pt) was calculated by the number of cases / the number of cases plus controls. Different clinical manifestations between \( C.\ trachomatis \) genotypes were then compared using the PS-adjusted pseudo-population created by the statistical procedures and presented as adjusted odds ratio (aOR).

Results

Performance of NGHTS for identifying mixed genotypes

The schematic diagram of the identification of \( C.\ trachomatis \) mixed-genotype infection using NGHTS was shown in Supplementary Figure S2. The nested-PCR to construct NGHTS library was capable of amplifying 10 copies per reaction of the recombinant plasmid DNAs for 8 \( C.\ trachomatis \) genotypes (Supplementary Figure S3). To further assess the sensitivity and accuracy of NGHTS in distinguishing mixed \( C.\ trachomatis \) genotypes, we prepared a series of plasmid DNA mixtures of two \( C.\ trachomatis \) genotypes at the ratio of 1–99, including genotype F and G, J and E as well as E and F. The proportion of different genotypes determined by NGHTS was excellently correlated with the ratio we prepared (Figure 1). For example, when we added 1% of the minor genotype into the mixture, the proportion of the minor genotype determined by NGHTS ranged from 1.11 to 4.69%, suggesting that NGHTS could detect at least 1% of the minor genotype in clinical samples with mixed-genotype infection.

Next, we designed genotype-specific primers to amplify the minor genotypes identified by NGHTS. We found that these genotype-specific primers could specifically amplify the minor genotypes when using plasmid DNAs as templates (Supplementary Figure S4A) and in the samples with mixed genotypes of \( C.\ trachomatis \) (Supplementary Figure S4B). We also used PCR products of 7 samples infected with two genotypes to transfect \( E.\ coli \) cells. Ten colonies per sample were randomly picked up and sequenced to determine their genotypes of \( C.\ trachomatis \). We found that 5 out of the 7 samples contained the same \( C.\ trachomatis \) genotypes as those determined by NGHTS although the proportion of the genotypes were slightly different from the data obtained by NGHTS (Supplementary Table S4). Taken together, our results confirmed the good performance of NGHTS in identifying the composition and proportion of \( C.\ trachomatis \) genotypes in the clinical samples.

Prevalence and genotype distribution of Chlamydia trachomatis infection

A total of 8,087 participants were tested for \( C.\ trachomatis \) Pgp2 gene and 311 (3.8, 95% confidence interval [CI] 3.4–4.2%) were found to be positive (Figure 2). The prevalence of \( C.\ trachomatis \) infection was 2.8, 3.7 and 4.8% for the participants from PEC, ART and GC, respectively (Figure 2). The \( ompA \) gene was successfully amplified and sequenced in 97.4% (303/311) \( C.\ trachomatis \) pgp2-positive samples, and was classified into 8 \( C.\ trachomatis \) genotypes and 3 genogroups proposed by Yuan et al. (1989). The most common three \( C.\ trachomatis \) genotypes were E (27.4%, 83/303), F (21.5%, 65/303) and J (18.2%, 55/303, Table 1). Two subjects (0.66%, 2/303) were infected with genotype B (Table 1). For the subjects infected with single \( C.\ trachomatis \) genotype, the genotyping results of NGHTS and Sanger sequencing were identical (Supplementary Table S5). For the 8 \( C.\ trachomatis \) pgp2-positive samples without genotyping results, both Sanger sequencing primers and NGHTS primers failed to amplify the target \( ompA \) gene.

Moreover, NGHTS identified 6.6% (20/303) \( C.\ trachomatis \) positive samples to be infected with two \((n = 18)\) or three \((n = 2)\) \( C.\ trachomatis \) genotypes (Table 2). The most frequent two genotypes observed in the mixed-genotype infections were F (55.0%, 11/20) and E (50.0%, 10/20), respectively. Co-infection of genotype F and G accounted for 20.0% (4/20) of the samples with mixed genotypes (Table 2). Of note, among the 20 samples infected with mixed \( C.\ trachomatis \) genotypes, 10 (50%) samples were dominated by one \( C.\ trachomatis \) genotype, i.e., the proportion of the major genotype >90% (Table 2). In addition, we measured the bacterial load of \( C.\ trachomatis \) using qPCR in 17 samples with mixed-genotype infections and 232 samples of single-genotype infection. Although the bacterial load was slightly higher in the mixed-genotype infections than in the single-genotype infections, i.e., 1.36 × 10⁷ (IQR: 6.00 × 10⁶, 4.37 × 10⁷) copies/mL vs. 0.85 × 10⁷ (IQR: 1.44 × 10⁷, 3.97 × 10⁷) copies/mL, the difference was not statistically significant (\( p = 0.476 \)).
Association between clinical manifestations and *Chlamydia trachomatis* genotypes

For the 303 genotype-defined subjects, no significant difference was observed in terms of age, clinical departments, antibiotic usage and HPV infection ($p > 0.2$, Supplementary Table S6). Similar results were obtained between the subjects with single or mixed-genotype infections ($p > 0.3$, Supplementary Table S6). In our study, 70.1% (218/303) of the participants reported 14 symptoms or signs including vaginosis (38.3%), PID (10.9%) and cervicitis (8.4%; Supplementary Table S7). Of note, only 29.9% (93/311) of subjects were asymptomatic *C. trachomatis* infection. However, the percentage of asymptomatic *C. trachomatis* infection was significantly higher (71.3%) in PEC compared to ART (23.7%) and GC (10.4%, $p < 0.001$, Table 3). PID was more often recorded in the patients from ART (33.9%) compared to the subjects from PEC (0%) or GC (8.5%, $p < 0.001$). In our study, PID was also more frequently observed in older subjects ($p = 0.017$) and those with antibiotic usage history ($p = 0.001$). Furthermore, vaginosis was more likely diagnosed in younger women, especially those under 25 years old ($p = 0.03$, Table 3).

In addition, patients from GC were more likely to present with vaginosis (51.8%, $p < 0.001$) and vaginal cleanliness of degree IV (50.7%, $p < 0.001$) compared to the subjects from PEC (16.3% for vaginosis and 12.7% for degree IV) and ART (32.2% for vaginosis and 20.7% for degree IV, Table 3). PID was more often recorded in the patients from ART (33.9%) compared to the subjects from PEC (0%) or GC (8.5%, $p < 0.001$). In our study, PID was also more frequently observed in older subjects ($p = 0.017$) and those with antibiotic usage history ($p = 0.001$). Furthermore, vaginosis was more likely diagnosed in younger women, especially those under 25 years old ($p = 0.03$, Table 3).

The association between *C. trachomatis* genogroup or genotypes and clinical manifestations was analyzed by multivariate logistic regression among 283 subjects infected with single *C. trachomatis* genotype after controlling the parameters of age, clinical departments, antibiotic usage and HIV infection (Table 4). Compared to genotype E, subjects infected with genotype G were more often diagnosed as PID (27.8% vs. 9.6%, OR = 3.06, 95%CI, 1.29–8.28; $p = 0.023$, Table 4) and vaginal cleanliness of degree IV (40.0% vs. 27.3%, OR = 6.91, 95%CI, 1.25–38.1, $p = 0.026$, Table 4). Further analysis of propensity score
reweighting data using the SMRW method confirmed the association of *C. trachomatis* genotype G infection with PID (aOR = 3.61, 95% CI, 1.02–12.8, \( p = 0.046 \), Supplementary Table S8), but not vagina cleanliness of degree IV (aOR = 3.00, 95% CI, 0.65–13.9, \( p = 0.161 \), Supplementary Table S8) even though all other parameters were well balanced after weighting (Supplementary Table S9).

### Comparison of clinical manifestations between *Chlamydia trachomatis* single and mixed-genotype infections

Although the subjects with *C. trachomatis* single and mixed-genotype infections reported similar symptoms, mixed-genotype infections were more likely to result in worse vagina cleanliness of degree IV (OR = 8.61, 95% CI, 1.53–48.5; \( p = 0.015 \), Table 5) than single genotype infections. Further stratified analysis revealed that the occurrence of worse vagina cleanliness and cervical SIL (OR = 5.76, 95% CI, 1.06–31.20, \( p = 0.042 \)) was mainly observed in the mixed genotype-infected subjects whose minor *C. trachomatis* genotype was \( \geq \)10% (Table 5).

![Flow chart for detection and genotyping of *C. trachomatis* in an observational study.](image_url)
We then conducted propensity score reweighting analysis to balance the factors of age, clinical departments, antibiotic usage, HPV infection and major C. trachomatis genotypes (Supplementary Table S10). Further logistic regression analysis confirmed the association of worse vagina cleanliness (aOR = 5.17, 95%CI, 1.03–25.9, p = 0.046) and cervical SIL (aOR = 5.51, 95%CI, 1.17–26.01, p = 0.031) with mixed-genotype infection when compared to single-genotype infection in particular for the mixed-genotype infections in which minor C. trachomatis genotype was ≥10% (Table 5). These results indicated that mixed-genotype infections of C. trachomatis may be associated with worse vaginal inflammation and cervical squamous intraepithelial lesion.

**Discussion**

In this study, a prevalence of 3.8% of current C. trachomatis infection was documented in the subjects who visited Chenzhou Hospital of China for either annual physical examination, diagnosis or treatment of infertility or gynecological diseases. The predominant C. trachomatis genotypes were E, F and J, which are similar to our previous findings (Chen et al., 2020). In addition, genotype B, an ocular genotype to cause ocular infection, was detected in two subjects in our study. However, genotype B ocular strain may be a recombinant strain with a urogenital genomic backbone and ocular genotype B ompA insert. Similar findings have been reported in one subject in Zheng et al. study and four subjects in Lesiak-Markowicz et al. study, respectively (Zheng et al., 2007; Lesiak-Markowicz et al., 2019).

In our study, we adapted NGHTS technology to determine C. trachomatis genotypes in particular mixed-genotype infections. Our results indicated that both NGHTS and Sanger sequencing correctly identified C. trachomatis genotypes in 283 subjects who infected with single genotype of C. trachomatis. Moreover, NGHTS was able to identify the presence and proportion of mixed C. trachomatis genotypes in 6.6% of C. trachomatis positive samples. We further found that mixed-genotype infections were associated with worse vaginal cleanliness and cervical SIL. To the best of our knowledge, this is the first study to assess the feasibility of NGHTS to identify and quantify mixed-genotype infection of C. trachomatis in China. Our preliminary results support NGHTS as a simple and useful method for differentiating C. trachomatis genotypes and determining mixed-genotype infections.

There are several genotyping methods for C. trachomatis including high-resolution multilocus sequence typing (hr-MLST) of multiple genes, and one serovar may consist of several different sequence types (STs) of C. trachomatis (Versteeg et al., 2015). However, sequencing of the ompA gene is still widely used to determine the serovars or genotypes of C. trachomatis and the
TABLE 3 Clinical manifestations of C. trachomatis-infected women with respect to age, clinical departments, antibiotic usage and HPV infection.

| Manifestations          | Age (year) | p* value | Clinical departmentsb | p value | Antibiotic usage (previous 3 months) | p value | HPV infection | p value |
|-------------------------|------------|----------|-----------------------|---------|-------------------------------------|---------|---------------|---------|
|                         | ≤25        | 25–35    | 35–45                 | >45     | PEC                                 | n = 80  | n = 59        | n = 164 |
| Asymptomatic (%)        | Yes        | 20.6     | 27.9                  | 32.8    | 0.15                                | 0.15    | >0.001        | 0.012   |
|                         | No         | 79.4     | 71.2                  | 67.2    | 59.5                                | 0.03    | <0.001        | 7.1     |
| Vaginosis (%)           | Yes        | 52.4     | 39.3                  | 29.3    | 28.6                                | 0.03    | <0.001        | 57.1    |
|                         | No         | 47.6     | 60.7                  | 70.7    | 71.4                                | 0.03    | <0.001        | 42.9    |
| Pelvic inflammatory disease (%) | Yes | 6.3 | 14.3 | 17.2 | 0.0 | 0.017 | 0.0 | 33.9 | 8.5 | 0.001 | 42.9 | 9.7 | 0.001 | 8.5 | 12.1 | 0.398 |
|                         | No         | 93.7     | 85.7                  | 82.8    | 100.0                               | 0.03    | <0.001        | 57.1    |
| Cervicitis (%)          | Yes        | 6.3      | 7.9                   | 10.3    | 11.9                                | 0.726   | 12.5          | 10.2    | 6.1 | 0.218 | 0.0 | 9.0 | 0.493 | 8.5 | 8.6 | 0.964 |
|                         | No         | 93.7     | 92.1                  | 89.7    | 88.1                                | 0.03    | <0.001        | 57.1    |
| Vagina cleanliness (%)  | I/II       | 31.6     | 35.3                  | 41.7    | 40.6                                | 0.97    | 12.5          | 10.2    | 6.1 | 0.218 | 0.0 | 9.0 | 0.493 | 8.5 | 8.6 | 0.964 |
|                         | III        | 36.8     | 37.3                  | 33.3    | 31.3                                | 0.03    | 12.5          | 10.2    | 6.1 | 0.218 | 0.0 | 9.0 | 0.493 | 8.5 | 8.6 | 0.964 |
|                         | IV         | 31.6     | 27.5                  | 25.0    | 28.1                                | 0.03    | 12.5          | 10.2    | 6.1 | 0.218 | 0.0 | 9.0 | 0.493 | 8.5 | 8.6 | 0.964 |
| Cervical abnormalities (%) | Benign | 76.6     | 82.8                  | 83.3    | 86.1                                | 0.792   | 88.6          | 83.9    | 78.1 | 0.371 | 87.5 | 82.0 | 0.78 | 62.7 | 88.3 | <0.001 |
|                         | ASC-US     | 10.6     | 5.2                   | 4.2     | 2.8                                 | 0.03    | 12.5          | 10.2    | 6.1 | 0.218 | 0.0 | 9.0 | 0.493 | 8.5 | 8.6 | 0.964 |
|                         | SIL        | 12.8     | 12.1                  | 12.5    | 11.1                                | 0.03    | 12.5          | 10.2    | 6.1 | 0.218 | 0.0 | 9.0 | 0.493 | 8.5 | 8.6 | 0.964 |

*p values were calculated using Chi-square tests. Bold p value indicate statistically significant, i.e., p < 0.05.

bPEC: physical examination center; ART: assisted reproductive technology; GC: gynecology clinics.

ASC-US, atypical squamous cells of undetermined significance; SIL, squamous intraepithelial lesion.

results are highly associated with the serotyping methods using a large panel of monoclonal antibodies (Mukherjee et al., 2011). We would like to emphasize that the purpose of our study is to identify mixed infections of C. trachomatis and their pathological impact, not to accurately determine the sequence types of C. trachomatis in the clinical samples.

Sanger sequencing is a powerful tool for genotyping and has been used to determine C. trachomatis genotypes. However, it may not be suitable for identifying mixed-genotype infections of C. trachomatis since Sanger sequencing only produces one consensus sequence according to the alignment results of multiple sequencing data and the intensity of sequencing signals at each point; hence, only the dominant genotype sequence could be identified (Gharsallah et al., 2018). Although the mixed-genotype infections can be identified by PCR-RFLP, hybridization methods and DNA microarray assay, these assays are not sensitive enough to identify all the mixed-genotype infections and cannot quantify genotype proportions in mixed-genotype infections. In addition, these assays cannot detect novel mutations or genovariants (Gharsallah et al., 2012). NGHTS has opened a new frontier to characterize the composition of complex populations of microbes and to uncover novel sequences or mutations, and it overcomes the constraints of Sanger sequencing and can achieve greater than 10,000 base pair coverage per sample (Shendure and Ji, 2008). Meanwhile, NGHTS is capable of obtaining tens or even hundreds of thousands of reads simultaneously, and the sensitivity of detecting low-frequency mutation sites and minor sequences has been dramatically enhanced. For example, Quer et al. (2015) have reported that NGHTS is a reliable method for genotyping hepatitis C virus (HCV) and identifying mixed-genotype infections of HCV through a phylogenetic classification of sequencing reads of HCV NS5B gene. In our study, we found that NGHTS could readily determine different proportions of C. trachomatis genotypes D-K at the ratio of 1:1.1–1:92.7 in clinical samples. In contrast, the detection limit of microarray assay was about 1:5, which lags far behind the detection performance of NGHTS. Although NGHTS methodology is not simple and needs support of research laboratories, we have assessed the possibility of NGHTS in large-scale implementation by labeling primers with different barcode sequences and mixing 10 samples in one NGHTS reaction. Therefore, NGHTS will dramatically improve the efficiency and greatly reduce the cost for identifying C. trachomatis genotypes. Additionally, the potential quantitation bias caused by nested-PCR should be taken into account, thus the NGHTS is only a relatively quantitative method.
It has been reported that the prevalence of C. trachomatis mixed-genotype infection varied from 2 to 19% (Stothard, 2001; Molano et al., 2004; Xiong et al., 2006; Zhang et al., 2012). It remains to elucidate if mixed-genotype infections represent co-infection or superinfection of different C. trachomatis genotypes. Hsu et al. (2006) proposed that two separate episodes of C. trachomatis infection and the lack of immunological cross protection between C. trachomatis genotypes may result in mixed-genotype infections. It has been reported that several C. trachomatis genotypes such as F, G and K may result in more severe clinical manifestations (Molano et al., 2004; Millman et al., 2006; Gao et al., 2007). In the current study, we also observed that C. trachomatis genotype G infection was more prone to PID (Table 4). Our results are consistent with the findings of Gao et al. (2007). Till now, there are few studies to compare the difference of clinical manifestations between single and mixed-genotype infections of C. trachomatis. Our preliminary results indicated that mixed-genotype infections may result in worse vaginal cleanliness and cervical SIL (Table 5) while we found no significant difference of vaginal cleanliness among different single-genotype C. trachomatis infections, suggesting that co- or super-infection

TABLE 4 Relationships of clinical manifestations to C. trachomatis genotypes in 283 women infected with single-genotype of C. trachomatis.

| Manifestations                        | C. trachomatis genotype (n = 261) | C. trachomatis genogroup (n = 283) |
|---------------------------------------|-----------------------------------|-----------------------------------|
|                                       | E n = 83                          | D n = 40                          |
|                                       | F n = 65                          | G n = 18                          | J n = 55                          |
|                                       |                                   |                                   |                                   |
| Asymptomatic                          |                                    |                                   |                                   |
| Yes (n, %)                            | 22 (26.5)                         | 15 (37.5)                         | 20 (30.8)                         | 6 (33.3)                          | 13 (23.6)                          |
| OR (95% CI)                           |                                    |                                   |                                   |                                   |                                   |
| Vaginosis                             |                                    |                                   |                                   |                                   |                                   |
| Yes (n, %)                            | 33 (39.8)                         | 14 (35.0)                         | 20 (30.8)                         | 5 (27.8)                          | 28 (50.9)                          |
| OR (95% CI)                           |                                    |                                   |                                   |                                   |                                   |
| Pelvic inflammatory disease           |                                    |                                   |                                   |                                   |                                   |
| Yes (n, %)                            | 8 (9.6)                           | 2 (5.0)                           | 7 (10.8)                          | 5 (27.8)                          | 9 (16.4)                           |
| OR (95% CI)                           |                                    |                                   |                                   |                                   |                                   |
| Cervicitis                            |                                    |                                   |                                   |                                   |                                   |
| Yes (n, %)                            | 5 (6.0)                           | 4 (10.0)                          | 8 (12.3)                          | 2 (11.1)                          | 3 (5.5)                            |
| OR (95% CI)                           |                                    |                                   |                                   |                                   |                                   |
| Vagina cleanliness (vs. I/II)         |                                    |                                   |                                   |                                   |                                   |
| III (n, %)                            | 16 (29.1)                         | 9 (36.0)                          | 17 (37.8)                         | 6 (40.0)                          | 18 (45.0)                          |
| OR (95% CI)                           |                                    |                                   |                                   |                                   |                                   |
| IV (n, %)                             | 15 (27.3)                         | 7 (28.0)                          | 9 (20.0)                          | 6 (40.0)                          | 10 (25.0)                          |
| OR (95% CI)                           |                                    |                                   |                                   |                                   |                                   |
| Cervical abnormalities (vs. Benign)   |                                    |                                   |                                   |                                   |                                   |
| ASC-US (n, %)                         | 4 (6.0)                           | 2 (5.7)                           | 1 (1.8)                           | 0                                | 3 (7.7)                            |
| OR (95% CI)                           |                                    |                                   |                                   |                                   |                                   |
| SIL (n, %)                            | 4 (6.0)                           | 6 (17.1)                          | 9 (16.4)                          | 6 (40.0)                          | 10 (25.0)                          |
| OR (95% CI)                           |                                    |                                   |                                   |                                   |                                   |

Note: Genotypes of < 5% of the total patients were analyzed only in their corresponding genogroups.

aOdds ratio (OR) was calculated in multivariate logistic regression analysis in which the parameters of age, clinical departments, antibiotic usage and HPV infection were under control. Bold OR and aOR indicate statistically significant, i.e., p < 0.05.

Genotype E and B-complex genogroup were set as reference group, respectively.

ASC-US, atypical squamous cells of undetermined significance; SIL, squamous intraepithelial lesion.
and the interaction of different C. trachomatis strains may contribute to stronger inflammatory reactions and worse vaginal or cervical damage (Gharsallah et al., 2012). It has been found that cervical SIL and cervical cancer are related to C. trachomatis infection (Smith et al., 2004; Castellsagué et al., 2014) or C. trachomatis/HPV co-infection (de Paula et al., 2007; da Silva Barros et al., 2012; de Abreu et al., 2014; Magalhães et al., 2015). Our further analysis indicated that C. trachomatis mixed-genotype infection was associated with cervical SIL compared with single-genotype infection even controlling for HPV infection (Table 5). Furthermore, Madeleine et al. (2007) reported that C. trachomatis genotypes B, D, E, G, I, and J were associated with squamous cell cancer although this association was not supported by other studies (Smith et al., 2004). We also did not observe the association of different C. trachomatis genotypes with SIL (Table 4). Previous studies indicated that C. trachomatis could inhibit apoptosis of C. trachomatis-infected cells, trigger host DNA damage, and induce cell proliferation (Chumduri et al., 2013). These results support C. trachomatis infection as a potential cause of SIL (Kun et al., 2013). Therefore, it is reasonable to speculate that mixed-genotype infection may facilitate C. trachomatis-associated effects on the development of cervical SIL due to the synergistic effect of multiple genotype infections. Similar synergistic impact has been observed in HPV mixed-genotype infections in which cervical SIL was significantly more frequent in mixed-genotype HPV infection than in single genotype HPV infection (Bachtiary et al., 2002; Bruno et al., 2020; Oyervides-Muñoz et al., 2020; Kim et al., 2021). Further research did show that tumors caused by mixed-genotype infection of HPV had a higher PD-1 expression in tumor-infiltrating lymphocytes (TILs), which could help cancer cells evade the immune response and progress more quickly (Mendoza et al., 2021). Actually, inflammation per se is a risk factor for cell malignant transformation (Greten and Grivennikov, 2019). More severe inflammation caused by mixed-genotype infection of C. trachomatis may also play a role in the process of cervical SIL during C. trachomatis infection.

In addition, previous studies have revealed high recombination rates among C. trachomatis strains, indicating their important role in the evolutionary pathways (Gomes et al., 2007). For example, the recombination event between the MOMP genes of genotype I and H may generate the composite MOMP of Ia variant (Madeleine et al., 2007). Therefore, it is reasonable to speculate that mixed-genotype infection may facilitate C. trachomatis-associated

| Manifestations                      | Single genotype event/N (%) | C. trachomatis mixed-genotype infection* | Without dominant genotype (N = 10) | With dominant genotype (N = 10) |
|-------------------------------------|-------------------------------|-----------------------------------------|------------------------------------|---------------------------------|
|                                     | Event/N (%)                  | OR (95% CI)                             | OR (95% CI)                        | OR (95% CI)                     |
| Asymptomatic                        | 83/283 (29.3)                | 0.83 (0.24–2.86)                        | 1.07 (0.08–5.64)                   | 0.80 (0.19–4.15)                |
| Vaginosis                           | 108/283 (38.2)               | 1.52 (0.57–4.03)                        | 1.91 (0.51–7.16)                   | 1.19 (0.27–5.24)                |
| Pelvic inflammatory disease         | 32/283 (11.3)                | 0.6 (0.11–3.21)                         | 0 NA                               | 2/10 (0.18–9.19)                |
| Cervicitis                          | 24/283 (8.5)                 | 1.1 (0.23–5.16)                         | 1.5 (0.17–12.92)                   | 1.09 (0.11–8.13)                |
| Vagina cleanliness (vs. I/II)       | 70/195 (35.9)                | 2.46 (0.43–14.24)                       | 0 NA                               | 4/30 (0.46–15.78)               |
| IV                                  | 51/195 (26.2)                | 8.61 (1.53–48.53)                       | 5.76 (1.06–31.22)                  | 3.44 (0.43–27.33)               |
| ASC-US                              | 13/230 (5.7)                 | 1.18 (0.12–11.35)                       | 0 NA                               | 1.21 (0.11–10.25)               |
| SIL                                 | 26/230 (11.3)                | 2.8 (0.75–10.5)                         | 3 (1.06–31.22)                     | 1.06 (0.11–7.64)                |

*Mixed-genotype infections were divided into two groups with or without dominant genotype in which the proportion of minor genotypes was <10% or ≥10%, respectively.

Odds ratio (OR) was calculated in multivariate logistic regression analysis in which the parameters of age, clinical departments, antibiotic usage and HPV infection were under control. Bold and aOR indicate statistically significant, i.e., p < 0.05.

NA, not applicable.

ASC-US, atypical squamous cells of undetermined significance; SIL, squamous intraepithelial lesion.
be resistant to tetracycline (Jones et al., 1990), macrolides (Misyurina et al., 2004), even multiple antibiotics (Somani et al., 2000). In an in vitro study (Suchland et al., 2009), a co-infection model was used to successfully generate tetracycline-resistant C. trachomatis L strain from co-infection of tetracycline-resistant C. suis R19 and a tetracycline-sensitive L1 strain. The ompA gene of C. trachomatis can be inserted into different genomic backbones through potential gene recombination. However, the recombination of ompA may happen most likely between L strain and I/J/K strains or between ocular and urethral branches or between L2b and D-Da strains (Somboonna et al., 2011; Harris et al., 2012; Mattičić et al., 2016; Borges et al., 2021). Hadfield et al. (2017) reported that in their comprehensive global genome dynamic analysis of C. trachomatis, only serovar D and J appeared in both monophyletic lineage T1 and T2. In addition, recombination did not disrupt the ompA gene of the most prevalent genotype E. Furthermore, the ompA gene of C. trachomatis is a single copy gene and may have a lower probability of switching compared to the multi-copy genes, e.g., cryptic plasmid (Joseph et al., 2011). Dalevi et al. (2002) found that the major factors influencing the structure of the C. trachomatis genomes are nucleotide substitutions and deletions, and the frequency of horizontal gene transfer events was low. Although the recombination of the ompA gene was mainly reported in case reports, the mixed-genotype infection identified in our study was just based on the ompA typing system and did not address the potential recombination of the ompA gene. WGS or hr-MLST needs to be performed to address the mixed genotype identified in our study and to identify any potential recombinants arising as a result of mixed infection. Our preliminary results prove the importance of identifying mixed-genotype infections of C. trachomatis by using NGHTS as a screening assay. Furthermore, qPCR is more sensitive than conventional PCR in detecting C. trachomatis infection. It is necessary to use qPCR for simultaneous detection to ensure accuracy when NGHTS is used to detect C. trachomatis mixed-genotype infections in clinical samples.

Our study has some limitations. (1) The cross-sectional study cannot determine the cause-effect of mixed-genotype infection of C. trachomatis and cervical damage. Our findings should be explained with caution. (2) The number of C. trachomatis positive samples is small due to the low prevalence of C. trachomatis infection and mixed-genotype infections of C. trachomatis although a total of 8,087 clinical samples were screened in our study. However, the sample size of 20 mixed-genotype infections was relatively large compared with previous studies. We also used the propensity scores weighting analysis to ensure the accuracy and reliability of the results. (3) All the subjects were from one hospital and were consecutively recruited from the selected clinics. The selection of the participants may limit the generalizability of the findings. (4) Conventional PCR detection rather than qPCR was used in our study and may underestimate C. trachomatis prevalence. The ompA target genotyping did not cover the entire ompA gene due to the read length limitation of NGHTS. (5) Antibiotic resistance of C. trachomatis is an ongoing concern although Hadfield, et al. reported that the comprehensive global genome dynamics of C. trachomatis did not show evidence of circulating genomic resistance in C. trachomatis (Hadfield et al., 2017). In our study, we did not investigate antibiotic resistance since the purpose of our study was to genotype C. trachomatis in the samples analyzed. We confirmed NGHTS as a useful tool for sensitive and accurate identification of C. trachomatis genotypes in particular mixed-genotype infections. Our results revealed the feasibility of NGHTS in characterizing mixed C. trachomatis genotypes and its clinical value. Mixed infections of C. trachomatis may associate with worse vaginal cleanliness and cervical SIL. Therefore, NGHTS should be further assessed and implemented as a routine screening assay for diagnosis and genotyping of C. trachomatis infections.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA786082.

Ethics statement

The studies involving human participants were reviewed and approved by Ethical Committee of Chenzhou No. 1 People’s Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JZ and JS: analysis and interpretation of data, conduction of experiment, and drafting of manuscript. LL: acquisition of data and conduction of experiment. CA, HL, and LW: performed experiment, and drafting of manuscript. ST and HC: conception, design, and finalizing manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1041789/full#supplementary-material

References

Bachtiary, B., Obermair, A., Dreier, B., Birner, P., Breitenecker, G., Knochle, T., et al. (2002). Impact of multiple HPV infection on response to treatment and survival in patients receiving radical radiotherapy for cervical cancer. Int. J. Cancer 102, 237–243. doi: 10.1002/ijc.10708
Bao, Y., Hu, M., Gao, G., Huang, J., and Zhang, Z. (2015). Multivariate analysis for pelvic floor dysfunction. Zhong Nan Da Xue Xue Bao Yi Xue Ban 40, 1229–1233. doi: 10.1087/zs13-16507
Batteiger, B., Lennington, W., Newhall, W., Katz, B., Morrison, H., and Jones, R. (1989). Correlation of infecting serovar and local inflammation in genital chlamydial infections. J. Infect. Dis. 160, 332–336. doi: 10.1093/infdis/160.2.332
Bax, C., Quint, K., Peters, R., Ouburg, S., Ostvogy, P., Mutsaers, J., et al. (2011). Analyses of multiple-site and concurrent Chlamydia trachomatis serovar infections, and serovar tissue tropism for urogenital versus rectal specimens in male and female patients. Sex. Transm. Infect. 87, 503–507. doi: 10.1136/sti.2010.048173
Borges, V., Isidro, J., Correa, C., Cordeiro, D., Viera, L., Lodhia, Z., et al. (2021). Transcontinental dissemination of the L2b/D-Da recombinant Chlamydia trachomatis lymphogranuloma venerum (LGV) strain: need of broad multi-country molecular surveillance. Clin. Infect. Dis. 73, e1084–e1007. doi: 10.1093/cid/ciaa067
Bourén, J., Bentley, J., Bosse, P., Girard, F., Haefner, H., Menton, M., et al. (2012). 2011 colposcopic terminology of the International Federation for Cervical Pathology and Colposcopy. Obstet. Gynecol. 120, 166–172. doi: 10.1097/AOG.0b013e318252459c
Brasilien, D. M., Borges Bdo, N., and Ferreira, W. A. (2016). Genotyping and prevalence of Chlamydia trachomatis infection among women in Belem, Para, northern Brazil. J. Infect. Dev. Cities. 10, 134–137. doi: 10.3855/jidc.6474
Brown, A. C., and Christiansen, M. T. (2019). Whole-genome sequencing of Chlamydia trachomatis directly from human samples. Methods Mol. Biol. 2042, 45–67. doi: 10.1007/978-1-4939-6964-0_6
Bruno, M., Scala, G., Cassar, N., and Boemi, S. (2020). Multiple HPV 16 infection with two strains: a possible marker of neoplastic progression. BMC Cancer 20:444. doi: 10.1186/s12885-020-06946-7
Burnett, L., Lunn, G., and Coico, R. (2009). Biosafety: guidelines for working with pathogenic and infectious microorganisms. Curr. Protoc. Microbiol. 13, 111–1114. doi: 10.1002/9780471729259.mc11a143
Carr, P., Felsentein, D., and Friedman, R. (1998). Evaluation and management of vaginitis. J. Gen. Intern. Med. 13, 335–346. doi: 10.1046/j.1525-1497.1998.00101.x
Castellsague, X., Pawlita, M., Roura, E., Margall, N., Waterboer, T., Bosch, F., et al. (2007). Molecular detection of Chlamydia trachomatis infections in cervical samples with normal and abnormal histopathological findings. Diagn. Cytopathol. 35, 198–202. doi: 10.1002/dc.20621
Dean, D., Oudens, E., Bolan, G., Padian, N., and Schachter, J. (1995). Major outer membrane protein variants of Chlamydia trachomatis are associated with severe upper genital tract infections and histopathology in San Francisco. J. Infect. Dis. 172, 1013–1022. doi: 10.1093/infdis/172.4.1013
Foschi, C., Nardini, P., Banzola, N., D’Antuono, A., Compri, M., Cevenini, R., et al. (2016). Chlamydia trachomatis infection prevalence and serovar distribution in a high-density urban area in the north of Italy. J. Med. Microbiol. 65, 510–520. doi: 10.1099/jmm.0.000261
Fu, L., Sun, Y., Han, M., Wang, B., Xiao, F., Zhou, Y., et al. (2022). Incidence trends of five common sexually transmitted infections excluding HIV from 1990 to 2019 at the global, regional, and National Levels: results from the global burden of disease study 2019. Front. Med. (Lausanne) 9:851635. doi: 10.3389/fmed.2022.851635
Galão Vautel, L., Entrocassi, C., Portu, A. I., Castro, E., Di Bartolomeo, S., Ruettger, A., et al. (2016). High frequency of Chlamydia trachomatis mixed infections detected by micorray assay in south American samples. PLoS One 11:e0153511. doi: 10.1371/journal.pone.0153511
Gao, X., Chen, S. Y., Yin, Y. P., Zhong, M. Y., Shi, M. Q., Wei, W. H., et al. (2007). Distribution study of Chlamydia trachomatis serovars among high-risk women in China performed using PCR-restriction fragment length polymorphism genotyping. J. Clin. Microbiol. 45, 1185–1189. doi: 10.1128/jcm.02076-06
Geisler, W. M., Suchland, R. J., Whittington, W. L., and Stamm, W. E. (2003). The relationship of serovar to clinical manifestations of urogenital Chlamydia trachomatis infection. Sex. Transm. Dis. 30, 160–165. doi: 10.1093/stid/30.2.160
Gharsallah, H., Frihda-Gargouri, O., Besbes, F., Sellami, H., and Hamzami, A. (2012). Development and application of an in-house reverse hybridization method for Chlamydia trachomatis genotyping. J. Appl. Microbiol. 113, 846–855. doi: 10.1111/j.1365-2672.2012.05375.x
Gharsallah, H., Frihda-Gargouri, O., Borm, R. J., Hamzami, A., and Bruisten, S. M. (2018). Comparison of reverse hybridization and ompA sequencing methods.
cervical cancer screening program in northeastern from Brazil. *Arch. Gynecol. Obstet.* 291, 1095–1102. doi:10.1007/s00404-014-3514-z

Maglo, T., and Salzberg, S. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi:10.1093/bioinformatics/btt507

Marcone, V., Recine, N., Gallinelli, C., Nicosia, R., Lichtenher, M., Degener, A. M., et al. (2012). Epidemiology of *Chlamydia trachomatis* endocervical infection in a previously unscreened population in Rome, Italy, 2000 to 2009. *Euro Surveill.* 17:20203. doi:10.2353/eus.2017.20203-e

Martinez, M. A., Ovalle, A., Camponovo, R., and Vidal, R. (2015). *Chlamydia trachomatis* genovars causing urogenital infections in Santiago. *Chile. Infect Dis. (Lond)* 47, 156–160. doi:10.3153/jmens.2014.0365548.2014.977341

Matić, M., Klavš, I., Vidićnek Zorman, J., Vidmar Vovko, D., Kogoj, R., and Keš, D. (2016). Confirmed inguinal lymphogranuloma venereum genovar L2 in a man who had sex with men, Slovenia. *Euro Surveill.* 21, 2–5. doi:10.2807/1560-7817.ES.2016.21.5.30129

Mendoza, R., Haidary, T., Gabunat, E., Zhou, Y., Bukhari, Z., Connelly, C., et al. (2021). Mixed and nonvaccine high risk HPV types are associated with higher mortality in Black women with cervical cancer. *Sci. Rep.* 11:14064. doi:10.1038/s41598-021-49485-1

Millman, K., Black, C. M., Stamm, W. E., Jones, R. B., Hook, E. W., Martin, D. H., et al. (2006). Population-based genetic epidemiologic analysis of *Chlamydia trachomatis* serotypes and lack of association between ompA polymorphisms and clinical phenotypes. *Microbes Infect.* 8, 604–611. doi:10.1016/j.micinf.2005.08.012

Miyazawa, Y., Chiyotani, D., Nakanishi, T., Inada, T., and Akamatsu, K. (2004). Mutations in a 23S rRNA gene of *Chlamydia trachomatis* associated with resistance to macrolides. *Antimicrob. Agents Chemother.* 48, 1347–1349. doi:10.1128/AAC.48.4.1347-1349.2004

Molano, M., Meije, C. J. L. M., Morre, S. A., Pol, R., and Van den Brule, A. J. C. (2004). Combination of PCR targeting the VD2 of omp1 and reverse line blot analysis for typing of urogenital *Chlamydia trachomatis* Serovars in cervical scrape specimens. *J. Clin. Microbiol.* 42, 2935–2939. doi:10.1128/JCM.42.7.2935-2939.2004

Mukherjee, J., Sood, S., Bala, M., Satpathy, G., Mahajan, N., Kapil, A., et al. (2011). The role of a commercial enzyme immuno assay antigen detection system for diagnosis of C. trachomatis in genital swab samples. *Indian J. Med. Microbiol.* 29, 411–413. doi:10.1007/s12027-005-091180

Ngandjio, A., Clerc, M., Fonkoua, M. C., Thonnon, J., Njock, F., Pouillot, R., et al. (2003). Screening of volunteer students in Yaoundé (Cameroon, Central Africa) for *Chlamydia trachomatis* infection and genotyping of isolated C. trachomatis strains. *J. Clin. Microbiol.* 41, 4404–4407. doi:10.1128/JCM.41.7.4404-4407.2003

Nunes, A., Borrego, M. J., Nunes, B., Florindo, C., and Gomes, J. P. (2009). Evolutionary dynamics of ompA, the gene encoding the *Chlamydia trachomatis* major antigen. *J. Bacteriol.* 191, 7182–7192. doi:10.1128/JB.00895-09

Oyarvides-Munoz, M., Perez–Maya, A., Sanchez-Dominguez, C., Berlanga-Garza, A., Antonius-Macedo, M., Valdez-Chapa, L., et al. (2020). Multiple HPV infections and viral load association in Persistent Cervical Lesions in Mexican women. *Viruses* 12:40380. doi:10.3390/v121040380

Petrovay, F., Nemeth, I., Balazs, A., and Balla, E. (2015). *Chlamydia conjunctivitis*: prevalence and serovar distribution of *Chlamydia trachomatis* in adults. *J. Med. Microbiol.* 64, 967–970. doi:10.1099/jmm.0.000115

Quer, J., Gregori, J., Rodriguez-Frias, F., Buti, M., Madejon, A., Perez-del Pulgar, S., et al. (2015). High-resolution hepatitis C virus subtype using NS5B deep sequencing and phylogeny, an alternative to current methods. *J. Clin. Microbiol.* 53, 219–226. doi:10.1128/JCM.02093-14

Quint, K. D., van Doorn, I. J., Keter, B., de Koning, M. N., van den Mynckhof, H. A., Morre, S. A., et al. (2007). A highly sensitive, multiplex broad-spectrum PCR-DNA-enzyme immunoassay and reverse hybridization assay for rapid detection and identification of *Chlamydia trachomatis* serovars. *J. Med. Diagn.* 9, 631–638. doi:10.2353/jmdx.2007.070011

Ruettger, A., Feige, J., Slickers, P., Schubert, E., Morre, S. A., Pannekoek, Y., et al. (2011). Genotyping of *Chlamydia trachomatis* strains from culture and clinical samples using an ompA-based DNA microarray assay. *Mol. Cell. Probes* 25, 19–27. doi:10.1016/j.mcp.2010.09.004

Shendure, J., and Ji, H. (2008). Next generation DNA sequencing. *Nat. Biotechnol.* 26, 1135–1145. doi:10.1038/nbt1486

Smith, J., Bosetti, C., Muñoz, N., Herrero, R., Bosch, F., Ehrlich-Neto, J., et al. (2006). *Chlamydia trachomatis* and invasive cervical cancer: a pooled analysis of the IARC multicentric case-control study. *Int. J. Cancer* 111, 431–439. doi:10.1002/ijc.20257

Somani, J., Bhullar, V., Worskow, K., Farshy, C., and Black, C. (2000). Multiple drug-resistant *Chlamydia trachomatis* associated with clinical treatment failure. *J. Infect. Dis.* 181, 1421–1427. doi:10.1086/315372
Somboonna, N., Wan, R., Ociua, D. M., Pettengill, M. A., Joseph, S. J., Chang, A., et al. (2011). Hypervirulent *Chlamydia trachomatis* clinical strain is a recombinant between lymphogranuloma venereum (L2) and D lineages. *mBio* 2:e00045-11. doi: 10.1128/mBio.00045-11

Spaargaren, J., Fennema, H. S., Morré, S. A., de Vries, H. J., and Coutinho, R. A. (2005). New lymphogranuloma venereum *Chlamydia trachomatis* variant, Amsterdam. *Emerg. Infect. Dis.* 11, 1090–1092. doi: 10.3201/eid1107.040883

Stothard, D. R. (2001). Use of a reverse dot blot procedure to identify the presence of multiple Serovars in *Chlamydia trachomatis* urogenital infection. *J. Clin. Microbiol.* 39, 2655–2659. doi: 10.1128/JCM.39.7.2655-2659.2001

Suchland, R., Sandoz, K., Jeffrey, B., Stamms, W., and Rockey, D. (2009). Horizontal transfer of tetracycline resistance among chlamydia spp. in vitro. *Antimicrob. Agents Chemother.* 53, 4604–4611. doi: 10.1128/AAC.00477-09

Versteeg, B., Dubbink, J. H., Bruisten, S. M., McIntyre, J. A., Morré, S. A., and Peters, R. P. (2015). High-resolution multilocus sequence typing reveals novel urogenital *Chlamydia trachomatis* strains in women in Mopani district, South Africa. *Sex Transm Infect* 91, 510–512. doi: 10.1136/sextrans-2014-051998

Woodhall, S., Gorwitz, R., Mighelsen, S., Gottlieb, S., Horner, P., Geisler, W., et al. (2018). Advancing the public health applications of *Chlamydia trachomatis* serology. *Lancet Infect. Dis.* 18, e399–e407. doi: 10.1016/s1473-3099(18)30159-2

Workowski, K., Stevens, C., Suchland, R., Holmes, K., Eschenbach, D., Pettinger, M., et al. (1994). Clinical manifestations of genital infection due to *Chlamydia trachomatis* in women: differences related to serovar. *Clin. Infect. Dis.* 19, 756–760. doi: 10.1093/clinids/19.4.756

Xiong, L., Kong, F., Zhou, H., and Gilbert, G. L. (2006). Use of PCR and reverse line blot hybridization assay for rapid simultaneous detection and Serovar identification of *Chlamydia trachomatis*. *J. Clin. Microbiol.* 44, 1413–1418. doi: 10.1128/JCM.44.4.1413-1418.2006

Yan, Y., Yuanfang, Z., Yon, X., Li-kuan, X., Jun, W., Yan, Z., et al. (2018). Genotype analysis of *Chlamydia trachomatis* infection in pregnant women and neonates. *Chin J Obstet Gynecol* 53, 319–322.

Yang, B., Zheng, H., Feng, Z., Xue, Y., Wu, X., Huang, J., et al. (2010). The prevalence and distribution of *Chlamydia trachomatis* genotypes among sexually transmitted disease clinic patients in Guangzhou, China, 2005–2008. *Ipn J Infect. Dis.* 63, 342–345.

Yu, F., Tang, Y. T., Hu, Z. Q., and Lin, X. N. (2018). Analysis of the vaginal microbiological status and genital tract infection characteristics of 751 pregnant women. *Med. Sci. Monit.* 24, 5338–5345. doi: 10.12659/msm.909051

Yuan, Y., Zhang, Y., Watkins, N., and Caldwell, H. (1989). Nucleotide and deduced amino acid sequences for the four variable domains of the major outer membrane proteins of the 15 *Chlamydia trachomatis* serovars. *Infect. Immun.* 57, 1040–1049. doi: 10.1128/iai.57.4.1040-1049.1989

Yue, X. A., Chen, P., Tang, Y., Wu, X., and Hu, Z. (2015). The dynamic changes of vaginal microecosystem in patients with recurrent vulvovaginal candidiasis: a retrospective study of 800 patients. *Arch. Gynecol. Obstet.* 292, 1285–1294. doi: 10.1007/s00404-015-3774-2

Zhang, J. J., Zhao, G. L., Wang, F., Hong, F. C., Luo, Z. Z., Lan, L. N., et al. (2012). Molecular epidemiology of genital *Chlamydia trachomatis* infection in Shenzhen, China. *Sex Transm Infect* 88, 272–277. doi: 10.1136/sextrans-2011-050163

Zheng, H., Jiang, L., Fang, D., Xue, Y., Wu, Y., Huang, J., et al. (2007). Application of an oligonucleotide array assay for rapid detecting and genotyping of *Chlamydia trachomatis* from urogenital specimens. *Diagn. Microbiol. Infect. Dis.* 57, 1–6. doi: 10.1016/j.diagmicrobio.2006.05.007