Presence of Chlamydia trachomatis and Mycoplasma spp., but not Neisseria gonorrhoeae and Treponema pallidum, in women undergoing an infertility evaluation: high prevalence of tetracycline resistance gene tet(M)

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Abstract

Chlamydia trachomatis, Mycoplasma spp., Neisseria gonorrhoeae and Treponema pallidum are sexually transmitted pathogens that threaten reproductive health worldwide. In this study, vaginal swabs obtained from women (n = 133) that attended an infertility clinic in China were tested with qPCRs for C. trachomatis, Mycoplasma spp., N. gonorrhoeae, T. pallidum and tetracycline resistance genes. While none of vaginal swabs were positive for N. gonorrhoeae and T. pallidum, 18.8% (25/133) of the swabs were positive for Chlamydia spp. and 17.3% of the swabs (23/133) were positive for Mycoplasma species. All swabs tested were positive for tetracycline resistance gene tet(M) which is the most effective antibiotic for bacterial sexually transmitted infections. The qPCRs determined that the gene copy number per swab for tet(M) was 7.6 times as high as that of C. trachomatis 23S rRNA, and 14.7 times of Mycoplasma spp. 16S rRNA. In China, most hospitals do not detect C. trachomatis and Mycoplasma spp. in women with sexually transmitted infections and fertility problems. This study strongly suggests that C. trachomatis and Mycoplasma spp. should be routinely tested in women with sexually transmitted infections and infertility in China, and that antimicrobial resistance of these organisms should be monitored. Further studies are warranted to determine the prevalences in different regions and associated risk factors.

Keywords: Chlamydia trachomatis, Mycoplasma spp., tet(M), Neisseria gonorrhoeae, Treponema pallidum

Introduction

Female infertility, including vaginal multi-pathogen infection induced infertility, is a major public health concern worldwide. Chlamydia trachomatis, Mycoplasma spp., Neisseria gonorrhoeae and Treponema pallidum have been extensively shown to be associated with infertility, particularly because of endometrial and tubal inflammation.

Chlamydia trachomatis is an obligate intracellular bacterial pathogen which remains the leading cause of bacterial sexually transmitted disease worldwide. Infections in the lower genital tract are frequently asymptomatic and, if untreated, can ascend to the upper genital tract, potentially leading to complications such as tubal factor infertility, and subfertility (Tang et al. 2015; Karinen et al. 2004). The organism is often undiagnosed in routine examinations performed in infertility clinical in China (Zheng et al. 2017).

Mycoplasmas are frequently isolated from the genital tract with M. genitalium and M. hominis considered...
responsible for genital diseases, infertility, and obstetric complications (Haggerty and Ness 2007; Taylor-Robinson and Lamont 2011; Kataoka et al. 2006). *M. hominis* has been found in about two-thirds of women with bacterial vaginosis and in 10% of women with salpingitis and endometritis (Judlin 2003).

*Neisseria gonorrhoeae* is the etiological agent of gonorrhea, the second most frequently reported sexually transmitted infection (STI) in the world. *N. gonorrhoeae* is a cause of pelvic inflammatory disease in women, which can lead to serious reproductive complications including tubal infertility, ectopic pregnancy, and chronic pelvic pain (Costa Lourenço et al. 2017; Kirkcaldy et al. 2016). The World Health Organization reported over 78 million new cases of gonococcal infection in people aged 15–49 worldwide during 2012 (World Health Organization 2016). Gonorrhea is usually symptomatic in men. However, there can be asymptomatic gonococcal cervicitis and the complicated gonorrhea may cause infertility in women (Costa Lourenço et al. 2017; Ison 2011).

*Treponema pallidum* can lead to the complex and systemic disease, syphilis which reduces the clinical pregnancy rate after in vitro fertilization/intra-cytoplasmic sperm injection (Wang et al. 2015). Furthermore, *T. pallidum* infection with varied clinical presentations can cause neurological, cardiovascular and other multisystem damage, leading to a long time course and serious, even life-threatening consequences.

Tetracycline is the antimicrobial of choice against bacterial STIs including *C. trachomatis, Mycoplasmas* and *Ureaplasma*. There is a high-level of resistance to tetracyclines in genital bacteria including lactobacilli which is due mainly to the presence of the *tet*(M) gene that mediates resistance to tetracyclines (de Barbeyrac et al. 1996; Dégrange et al. 2008; Mardassi et al. 2012).

The aim of the present work was to evaluate the presence of *Mycoplasma* in the vaginal swabs, a set of primers and probes were designed using Vector NTI to amplify a 174-bp fragment of the *Mycoplasma* spp. FRET-qPCR. To investigate the presence of *Mycoplasma* in the vaginal swabs, a set of primers and probes were designed using Vector NTI to amplify a 174-bp fragment of the *Mycoplasma* spp. 16S rRNA gene (Table 1). PCR amplification was performed in a LightCycler 480-II real-time PCR platform using a high-stringency 18-cycle step-down temperature protocol: 6 × 1 s @ 95 °C, 12 s @ 64 °C, 8 s @ 72 °C; 9 × 1 s @ 95 °C, 12 s @ 62 °C, 8 s @ 72 °C; 3 × 1 s @ 95 °C, 12 s @ 60 °C, 8 s @ 72 °C; followed by 30 low-stringency cycles: 30 × 1 s @ 95 °C, 12 s @ 57 °C, 30 s @ 67 °C, and 10 s @ 72 °C. Twenty μl PCR reactions were prepared containing 10.0 μl DNA template, 0.2 μl forward primer (100 μM), 0.2 μl reverse primer (100 μM), 4.0 μl 5× PCR buffer, 0.4 μl 10 μM dNTP, 0.3 μl 5 U/μl Taq DNA polymerase and 4.9 μl ultrapure H2O. The PCR products were further verified, purified, and sequenced as mentioned above. A standard PCR with a long amplicon (703–713 bp) of the 16S rRNA gene were performed on positive samples based on FRET-qPCR to determine the *Mycoplasma* species (Yoshida et al. 2002).

For use as quantitative standards, the products of the *Mycoplasma* FRET-qPCR on vaginal swabs were gel purified using the QIAquick Gel Extraction Kit (Qiagen, Hong Kong, China), purification using the QIAquick PCR Purification Kit (Qiagen), and sequencing with forward and reverse primers (BGI, Shanghai, China).

**Mycoplasma FRET-qPCR**

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After using the molecular mass of the 16S rRNA gene to calculate the molarity of the solution, dilutions were made to give solutions containing 10,000, 1000, 100, 10, and 1 gene copies per reaction. These were amplified by *Mycoplasma* FRET-PCR in triplicate to determine the detection limit of the PCR.

The specificity of this PCR was further verified with the amplification of DNAs from *Salmonella Typhimurium* (ATCC 14028), *Escherichia coli* (ATCC25922), *C. trachomatis* (ATCC VR-571B), *Ehrlichia canis*, *Anaplasma phagocytophilum* and *Rickettsia felis*. *Neisseria gonorrhoeae* and *T. pallidum* qPCR

Two sets of primers were used to detect *N. gonorrhoeae* and *T. pallidum* by the *porA* pseudogene and the *polA* gene respectively as described (Whiley et al. 2004; Heymans et al. 2010).

**RPPs gene qPCR**

A qPCR was established to quantify a class of tetracycline resistance genes encoding for ribosomal protection proteins (RPPs) in vaginal swabs in this study. The *tet*(M), *tet*(S), *tet*(O), *tet*(Q), *tet*(T), *tet*(36), *tet*(44) sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov). Using the Clustal Multiple Alignment Algorithm, we identified a highly conserved 245-bp PCR target on the above seven tetracycline resistance genes (Table 1). The thermal protocol was identical to what described above for the *Mycoplasma*-qPCR. The specificity of the primers was verified by BLASTn and DNA sequencing of obtained PCR products. The sensitivity of the *tet*(M)-qPCR was determined as mentioned above.

**Table 1 Oligonucleotide primers used in this study**

| PCR                                      | Target     | Primer/probe Sequence (5’–3’)           | Size (bp) | Ref          |
|-----------------------------------------|------------|-----------------------------------------|-----------|--------------|
| **Generic for 11 Chlamydia species**    | 23S rRNA   | UP1 GGCGTTTAGGTGCAGGAGAAGCT            | 168       | Guo et al. (2016) |
|                                         |            | UP2 GGCGTTTAGGGTGCAGGAGAAGCT          |           |              |
|                                         |            | DN GAGAAGGGGAGAAGAAGCT                |           |              |
|                                         |            | FLU1 AGCGAAAGAAGAAGAAGCT             |           |              |
|                                         |            | FLU2 AGCGAAAGAAGAAGAAGCT             |           |              |
|                                         |            | LCRed GCAGTGACAGACGACGACGACG         | 700       | Yoshida et al. (2002) |
| **Generic for Mycoplasma species**      | 16S rRNA   | UP CTCCTGAGGTAGATYTATCGGA             | 174       | This study   |
|                                         |            | DN TGCCACATGTACCGACGACGACG         |           |              |
|                                         |            | FLU AGCGAAAGAAGAAGAAGCT             |           |              |
|                                         |            | LCRed GCAGTGACAGACGACGACG         |           |              |
| **Mycoplasma species for sequencing**   | 16S rRNA   | UP ACTCTACGGGGAGAGGACGAGTA          | 700       | Yoshiida et al. (2002) |
|                                         |            | DN TGCCACATGTACCGACGACGACG         |           |              |
| **N. gonorrhoeae**                      | porA pseudogene | UP CGGTTTCCGGTGGTACGTA | 132       | Whiley et al. (2004) |
|                                         |            | DN AACTGGTTTCTGTTACGACGACG     |           |              |
| **T. pallidum**                         | polA       | UP GGTAGAAGGGAGGGCTAGTA           | 104       | Heymans et al. (2010) |
|                                         |            | DN CTAAGATCTCTTTTCTGATGTTAGG    |           |              |
|                                         |            | TaqMan probe FAM-ACACAGCAGCCTGCTGG |           |              |
| **RPPs gene**                           |            | UP CCACCGAAATCTGCTGGGTT          | 245       | This study   |
|                                         |            | DN ATCCTGAAATCTGCTGGGTT       |           |              |

After using the molecular mass of the 16S rRNA gene to calculate the molarity of the solution, dilutions were made to give solutions containing 10,000, 1000, 100, 10, and 1 gene copies per reaction. These were amplified by *Mycoplasma* FRET-PCR in triplicate to determine the detection limit of the PCR. The specificity of this PCR was further verified with the amplification of DNAs from *Salmonella Typhimurium* (ATCC 14028), *Escherichia coli* (ATCC25922), *C. trachomatis* (ATCC VR-571B), *Ehrlichia canis*, *Anaplasma phagocytophilum* and *Rickettsia felis*. *Neisseria gonorrhoeae* and *T. pallidum* qPCR

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**Statistical analysis**

All statistical analyses were performed with the Statistica 7.0 software package (StatSoft, Inc., Oklahoma, USA). Positivity of pathogens in different age groups was compared using the Chi squared Test. Differences of copy numbers of pathogens and *tet*(M) gene in different age groups were logarithmically transformed and analyzed with the two-tailed Tukey honest significant difference (HSD) test in one-way ANOVA. Differences at $P \leq 0.05$ were considered significant.
Results
Establishment of the Mycoplasma FRET qPCR and tet(M) qPCR
The Mycoplasma FRET qPCR established in this study detected the target Mycoplasma 16S rRNA with a detection limit of one copy 16S rRNA per reaction. It did not detect Salmonella Typhimurium, Escherichia coli, C. trachomatis, Ehrlichia canis, Anaplasma phagocytophilum and Rickettsia felis. The RPPs gene-qPCR we established had a detection limit of one gene copy per reaction while its specificity was verified by sequencing.

Prevalence and copy numbers of C. trachomatis, Mycoplasma spp., N. gonorrhoeae, T. pallidum and tet(M) in vaginal swabs from infertile women
While none of the swabs were positive for N. gonorrhoeae and T. pallidum, 31.6% of the swabs (42/133) were positive for C. trachomatis and/or Mycoplasmas infection. In six women (4.5%) coinfection with both C. trachomatis and Mycoplasma spp. was observed. All the swabs were positive for the tet(M) gene.

C. trachomatis was found to be the only chlamydial species in all vaginal swabs with a positivity of 18.8% (25/133). The average gene copy number of C. trachomatis was 27,855 (± 21,301 SEM) per swab.

The FRET-qPCR followed by standard PCR that generates a longer amplicon of the 16S rRNA gene determined that 17.3% (23/133) of swabs were positive for C. trachomatis and/or Mycoplasmas infection. In six women (4.5%) coinfection with both C. trachomatis and Mycoplasma spp. was observed. All the swabs were positive for the tet(M) gene.

The average gene copy number for C. trachomatis was 27,855 (± 21,301 SEM) per swab.

Sequence analysis of 22 RPPs PCR products revealed eight different tet(M) sequences (MF769608–MF769615) (Fig. 2). The sequences of MF769608 and MF769620 in this study were identical to the tet(M) sequences (KU545550 and MF422120) in GenBank.

All the vaginal swabs were positive for tet(M) with the average gene copy number per swabs being 212,878 (± 39,025 SEM) which was 7.6 times that for C. trachomatis 23S rRNA and 14.7 times that for Mycoplasma spp. 16S rRNA.

Prevalence and copy numbers of C. trachomatis, Mycoplasma and tet(M) in different age groups
The average age of the 128 women for whom we had data was 29.35 years (range 21–44 years). For analysis we divided the women into four groups: 21–25, 26–31, 32–37, and 38–44 years.

The positivity for C. trachomatis in the 38–44 age group (42.9%) was significantly higher (P < 0.01) than in the younger age groups (32–37 years, 25.0%; 21–25 age group (21.4%) and 26–31 age group (15.7%) (Fig. 3). Similarly, the positivity for Mycoplasma in the 21–25 age group (7.1%) was significant lower (P < 0.01) than in the older age groups (26–31 age group, 20.5%; 32–37 age group, 16.7%; 38–44 age group, 14.3%) (Fig. 3).

The average gene copy numbers of C. trachomatis, Mycoplasma and tet(M) did not differ significantly between the four age groups (Fig. 3) although the total gene copy number per swab was significantly higher for tet(M) (P < 10^-4) than for C. trachomatis 23S rRNA and Mycoplasma spp. 16S rRNA.

Discussion
This study demonstrated that 31.6% of women attending an infertility clinic in China tested positive for C. trachomatis and/or Mycoplasma spp. whereas these two pathogens are usually not detected in women with STIs and fertility problems in most Chinese hospitals (Peng et al. 2017; Bao et al. 2016). In contrast, we failed to identify the two pathogens (N. gonorrhoeae and T. pallidum) that are at the top of the list for STI surveillance in China. Surprisingly, all the swabs tested in this study were positive for tet(M), the resistance gene against tetracycline which is the most commonly used and most effective antimicrobial for bacterial STIs. Our study strongly suggests that C. trachomatis and Mycoplasma spp. should be routinely tested for in women with STIs and infertility in China, and that the antimicrobial resistance of these organisms should be monitored.

The Chinese government launched a massive campaign to eliminate STIs in the 1950s, and STIs were thought to be extremely uncommon by the 1960s in China (Cohen et al. 1997). However, since the early 1980s, STIs have reemerged with the introduction of the open door policy and economic liberalization and are now recognized as a major public-health problem in China as they have
spread from the high risk group to the general population (Chen et al. 2007).

In China, HIV/AIDS, syphilis, and gonorrhea are reportable STIs according to the Law of the People’s Republic of China on Prevention and Treatment of Infectious Diseases. The reported incidence of primary and secondary syphilis was 11.7 cases per 100,000 residents in 2009, an increase of 2.1-fold since 2005 (Chen et al. 2011). However, the reported incidence of gonorrhea has decreased by about 30% (Chen et al. 2011) with the overall prevalence of \( N. gonorrhoeae \) infection in the general population being 0.08% for women and 0.02% for men (Parish et al. 2003). The overall trend is that the prevalence of \( N. gonorrhoeae \) and \( T. pallidum \) has dropped significantly in China (Chen et al. 2011; Adachi et al. 2016), congruent to the findings as none of the 133 vaginal swabs were tested positive for \( N. gonorrhoeae \) and \( T. pallidum \) in this study.

\( C. trachomatis \) infections of the lower female genital tract are frequently asymptomatic. However, if infections do not resolve or persist untreated, organisms can ascend and pathology in the upper genital tract, potentially causing salpingitis and functional damage to the fallopian tubes and tubal factor infertility (Hafner 2015). The incidence of genital \( C. trachomatis \) infections increased by nearly 40% from 37.20 per 100,000 persons in 2010 and 51.3 per 100,000 in 2014 in Guangdong Province of China (Wong et al. 2017). Based on a Chinese Health and Family Life Survey, the prevalence of \( C. trachomatis \) was 2.6% (95% CI 1.6–4.1%) in Chinese women (Conejero et al. 2017).
et al. 2013; Parish et al. 2003). This is similar to the 2.7% (n = 1717) positivity for women reported in the Netherlands (Morré et al. 1999). While the international standards recommend annual screening for *C. trachomatis* in sexually active women (Conejero et al. 2013), most hospitals in China do not detect *C. trachomatis* in women during regular checks or in women with STIs and fertility problems. The high prevalence of *C. trachomatis* demonstrated in this study strongly suggests that hospitals in China should follow the international recommendation and national guideline for routine surveillance of *C. trachomatis*.

*Mycoplasma* species have been reported to be associated with perinatal morbidity and mortality while *M. hominis* has been mostly associated with chorioamnionitis and thought to be an etiological agent (Taylor-Robinson and Lamont 2011). The overall prevalence of urogenital *Mycoplasma* infections varies in different countries while international reports suggest an increase in infections due to *Mycoplasma* over the last decade (Díaz et al. 2013). The global prevalence of *M. genitalium* among symptomatic and asymptomatic sexually active women ranges between 1 and 6.4% (Pereboom et al. 2014). This variability in prevalence rates reported in different countries is perhaps due to differences in detection methods, types of samples studied, sample sizes, hygiene issues, socioeconomic status, age of participants, and absence of regular screening, treatment, and control.

![Fig. 3](imageURL) Prevalence and copy numbers of *C. trachomatis*, *Mycoplasma* and tet(M) gene in vaginal samples in four age groups. **a** The *C. trachomatis* positivity in 21–25 age group, 26–31 age group, 32–37 age group and 38–44 age group detected in this study were 21.4, 15.7, 25.0 and 42.9% respectively. The *Mycoplasma* positivity in above four age groups detected in this study were 7.1, 20.5, 16.7 and 14.3% respectively. The tet(M) gene positivity in above four age groups were all 100%. **b** The average gene copy numbers for *C. trachomatis* 23S rRNA, *Mycoplasma* spp. 16S rRNA and tet(M) gene were $10^{2.53} \pm 1.05$ SEM, $10^{2.81} \pm 1.24$ SEM, $10^{4.28} \pm 1.34$ SEM (being equivalent to $27,855 \pm 21,301$ SEM, $14,433 \pm 7872$ SEM, $212,878 \pm 39,025$ SEM), respectively. The gene copy number for four age groups did not differ significantly for *C. trachomatis*, *Mycoplasma* spp. and tet(M). However, the tet(M) copy number is significantly higher than *C. trachomatis* (averagely 7.6 times), and *Mycoplasma* spp. (averagely 14.7 times).
programs (Ahmadi et al. 2016). This study identified 22/23 of Mycoplasmas sequences being hominis taxa, and only one sequence was identified as Ca. M. girerdii.

For decades, tetracyclines, as broad-spectrum antibiotics, have been used extensively for treating bacteria-induced STIs. Resistance to tetracyclines is now increasingly prevalent in STIs, and the drug’s usefulness in treating urogenital infections is decreasing because of the presence of the tet(M). Surprisingly, in our study 100% of the swabs we tested were positive for tet(M) gene which was present in very high copy numbers, 7.6 times that of C. trachomatis 23S rRNA and 14.7 times that of Mycoplasma spp. 16S rRNA. This indicates the C. trachomatis and Mycoplasma spp. carried multiple copies of the tet(M) or that the gene was also present in other vaginal microbes such as lactobacilli. The antimicrobial resistance features of vaginal bacteria should be regularly monitored to provide the judicious treatment of STIs. The phylogenetic analysis based on eight sequences identified in this study and 12 species RPPs gene in GenBank revealed that all eight sequences belong to tet(M) taxa, which is one of the most prevalent class of RPPs gene conferring antibiotic resistance.

Traditionally, the diagnosis of genitourinary pathogens has been based on bacterial culture. Bacterial isolation, however, is cumbersome, costly, and time-consuming. It is also selective in that samples must be appropriately collected, transported and stored to maximize the number of viable bacteria. It is particularly difficult to grow obligate intracellular bacterial such as C. trachomatis while cell lines or chicken embryos are required for their growth. In this study, sensitive and specific qPCRs were applied for direct testing of samples for pathogens and resistant genes. This approach avoids the limitations of bacterial isolation and the resultant underestimation of pathogen prevalence.

In conclusion, we found a high prevalence of C. trachomatis, Mycoplasma spp. and tet(M) in vaginal swabs from women with fertility problems in China, whilst N. gonorrhoeae or T. pallidum were not detected. While more studies are warranted to investigate the prevalences of these organisms and tet(M) in larger populations from different regions, our data should alert health workers in China that C. trachomatis and Mycoplasma spp. might be the main pathogens in women with STIs and infertility problem.

Abbreviations
tet: tetracycline resistance gene; FRET-qPCR: fluorescence resonance energy transfer quantitative PCR; STI: sexually transmitted infection; RPPs: ribosomal protection proteins.

Authors’ contributions
CW conceived of the study. ML, XZ, KH, HQ, JZ and YK performed experiment, computational analysis. CW and ML wrote the paper. All authors read and approved the final manuscript.

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Not applicable.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
Sequences are deposited in GenBank with Accession Numbers MF769608–MF769620.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The study was approved by the Institutional Review Board of Northern Jiangsu People’s Hospital of China. All patients provided written consent prior to sample collection.

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