Have the same pathogenicity of Ureaplasma Parvum and Ureaplasma Urealyticum in non-specific cervicitis? -based on digital droplet PCR assay

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Research Article

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Have the same pathogenicity of *Ureaplasma Parvum* and *Ureaplasma Urealyticum* in non-specific cervicitis? -based on digital droplet PCR assay.

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Abstract:

Background: *Ureaplasma* spp. are association with a various of infectious diseases in female, but it still limited evidence for the pathogenicity in nonspecific cervicitis. The aim of this study was to develop and evaluate a digital droplet PCR (ddPCR) assay for quantified the load of *Ureaplasma* spp in cervical swabs.

Methods: A total of 293 non-specific cervicitis (NSC) patients and 211 asymptomatic female fulfilled the inclusion criteria. Cervical swabs were identified by qPCR and further absolutely quantified by ddPCR.

Results: The prevalence of *U.parvum* were 51.9% (152/293) and 46.9% (99/211); while *U.urealyticum* were 8.2% (24/293) and 8.1% (17/211) in the NSC and Control group, respectively. In addition, the average Ct value and median copy number of *U.parvum* were 31.33 (n=152) and 5.99 × 10^5 copies/ml (n=48) in the NSC group and 33.68 (n=99) and 1.74

10^4 copies/ml (n=33) in control group, respectively, suggest that the load of *U.parvum* of NSC group were significantly higher than the asymptomatic individual (P<0.001). But, the median load number of *U.urealyticum* were 1.26×10^3 copies/ml (n=22) and 5.35×10^3 (n=14) copies per microliter two groups, the difference was no statistical significance (P>0.05).

Conclusions: our study suggests that often carrying *U.parvum* at a high load but not *U.urealyticum* may have an important implications on the development and progression of cervicitis among female.

Keywords: *Ureaplasma parvum*, *Ureaplasma urealyticum*, qPCR, droplet digital PCR, Absolute Quantitation, cervicitis

Introduction:

Ureaplasma spp. has 14 known serotypes and subdivided into two biovars: *Ureaplasma urealyticum* (*U.urealyticum*) and *Ureaplasma parvum* (*U.parvum*). This pathogen is suspected to be the causative agents of non-gonococcal urethritis[1], bacterial vaginosis[2], cervicitis[3], and multiple adverse pregnancy outcomes that include spontaneous preterm birth[4], chorioamnionitis[5], preterm premature rupture of membranes (PPROM)[6]. Currently, the role of *Ureaplasma* spp. remains controversial[7, 8], largely due to high rates of isolation of these bacteria from the urogenital tract of a seemingly asymptomatic adult. Many pathogens such as HPV, *C. trachomatis*, and *N. gonorrhoeae* have been confirmed to be causative factors of cervicitis, but more work is required to gain a clear understanding of the cervicitis associated with *Ureaplasma* spp.

The European STI Guidelines Editorial Board states stated that there is insufficient evidence that *Ureaplasma* spp. can cause cervicitis and not recommended the routine testing of asymptomatic or symptomatic females for the presence of *Ureaplasma* spp. [9], of note, except in cases of high bacterial load[10, 11]. However, the quantified bacterial load at all previous studies have taken the qPCR technology that referred to external references. Several studies showed that ddPCR had much higher sensitivity and is more than resistant to PCR inhibition than qPCR [12, 13]. Moreover, a recent study suggested that the copy numbers obtained by qPCR were higher between 1 and 31 times compared to ddPCR (mean: 9.7; SD 7.7)[14]. Regarding digital droplet PCR (ddPCR), is a method of absolute nucleic acid quantification without external references and robustness to variations in PCR efficiency[15, 16].

Hence, after the screening the positive of *Ureaplasma* spp specimens by qPCR, we take a more precise ddPCR technology to accurate quantification of *Ureaplasma* spp. in partly suspicious positive cervical swabs. In this study, we investigated the prevalence and aimed to unequivocal quantified a load of *Ureaplasma* spp. in non-specific cervicitis patients and inquire into the pathogenicity of two *Ureaplasma* biovars.

Materials and methods

Study population

From July 2019 to November 2020, patients attending the gynecology outpatient or health clinic in Minhang Hospital, Fudan University were recruited. All of the women were aged ≥18 years, has exhibited symptoms of cervical discharge (purulent or mucopurulent endocervical exudate) or cervical bleeding[17], had not been received the gynecologic intervention and antibiotics treatment in the preceding 3 months, had not used intrauterine contraceptive device, had been sexually activated in last 3 months and were not currently pregnant or menstruating. A total of 293 non-chlamydial non-gonococcal females were cases group (mean age 41.0± SD 11.3), and 211 asymptomatic females were the control group (mean age 43.7± SD 8.4).

Specimen collection and DNA extracting

Cervical swab samples were obtained according to protocols performed by experienced clinicians. *T. vaginalis* and Fungal were examined under a microscope. Total DNA from 200 μl fresh cervical swabs were extracted from the supernatant using MiniBest Universal Genomic DNA Extraction Kit (Takara) following the manufacturer's instruction. Extracts were resuspended in 100 μl of Nuclease-Free Water. DNA samples were stored at -20℃ or -80℃ until use. The concentration of nucleic acids was determined with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). Human papillomavirus (HPV), *C.trachomatis*, or *N.gonorrhoeae* were confirmed by the commercial kits based on the TaqMan probes (Kehua, Shanghai, China) according to the manufacturer's instructions. *U.parvum* serovar 1 (ATCC 27813) and *U.urealyticum* serovar 4 (ATCC 27816) were used as standard strains.

Primers and probes

The nucleotide sequence of 14 servers of *Ureaplasma* spp. was download from the GenBank and aligned using Mega X software [18]. The primers and TaqMan probes were designed by Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) based on the parC gene conserved regions of *U.parvum* and *U.urealyticum*, respectively (Table no.2 and Appendix 1). The TaqMan probes of target genes and house-keeping genes were marked with FAM and VIC at 5' terminal, respectively (Table no. 1). The same primers and probes were used in both qPCR and ddPCR reactions.

Real-time quantitative (qPCR)

The qPCR amplification was performed in a total volume of 20 μl, including the 10 μl of Probe qPCR Mix (with UNG) (Takara), 20X target/reference primers/probes mix (the 500 nM target/reference primers as well as 250 nM target/reference probe), as well as 9 μl of each template DNA. The qPCR condition was as follows: 25˚C for 10min, followed by 45 cycles of 15 s at 95˚C and 34 s at 60˚C. Fluorescent accumulation data were analyzed using the ABI 7500 Software Version 2.3 (Applied Biosystems). The threshold cycle (CT) values of < 40 were defined as a suspicious positive result for the *U.parvum* and *U.urealyticum*. Besides, PCR extraction was monitored by amplifying human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each cervical swab. The CT values of >37 suggest the failure of DNA extraction or sampling unqualified. Standard curves were generated by plotting the CT of the qPCR performed on ten-fold dilution series of purified DNA from *U.parvum*, *U.urealyticum*.

Digital droplet PCR (ddPCR)

The ddPCR was performed using a QX200 Droplet Digital PCR system (Bio-Rad). The Mastermix for ddPCR included 1× ddPCR Supermix for Probes (no dUTP), 20X target/ reference primers/probes mix (including the 900 nM target and reference primers as well as 250 nM target probe (FAM-labelled) and reference probe (VIC-labelled)) together with 9 μl sample DNA. The generation of droplets was performed by the QX200 Droplet Generator according to the manufacturer's protocols. PCR amplification was carried out on an Applied Biosystems Veriti 96-Well Thermal Cycler using the following PCR conditions: 95˚C for 10 min followed by 40 cycles of denaturation at 94˚C for 30 s, 60˚C for 1 min and the enzyme was deactivated at 98˚C for 10 min. The plate was stored at 4˚C until droplets were analyzed by the QX200 Droplet Reader and QuantaSoft software version 1.7.4 (Bio-Rad). In each run a non-template control were included. Threshold between positive and negative droplet populations were set manually using per-plate positive and no-template controls as a guide. Usually, droplets with an amplitude above 6,000 were considered positive for the *U.parvum* assay, above 2,000 were considered positive for the *U.urealyticum* assay. Standardized copies mean that copy number of *U.parvum* or *U.urealyticum* divided by copy number of GAPDH gene and multiply by a thousand.

Statistics

Continuous variables are expressed as mean±standard deviation. The data were calculated in Stata 13.0 (StataCorp, TX, USA) and visualized using GraphPad Prism 8 Software, Inc (La Jolla, CA, USA). T-test were used to compare the differences between the two groups. Differences were considered statistically significant for p values <0.05.

Results

Firstly, we confirmed the diagnosis performance of primers and probe by qPCR. As Supplement Figure no. 1 shown, the primers of *U.parvum* and *U.urealyticum* identified the great specificity by melt curve. Besides, the primers targeted *U.parvum* can amplify the four serotypes (Up1, Up3, Up6, Up14) from national standard strains, similarly, the primers targeted *U.urealyticum* can amplify the ten serotypes (Uu2, Uu4-5, Uu7-13).

After the clinical test for HPV infection by experienced medical staff, the HPV-negative cervical swab was further examined under a microscope to rule out fungal and trichomonas infection. 538 specimens performed the *C.trachomatis*, *N.gonorrheae*, *U.parvum*, and *U.urealyticum* test by qPCR in quadruplicate. Finally, a total of 504 samples enrolled in this study, including 211 asymptomatic persons and 293 non-specific cervicitis patients (table no. no. 1).

According to qPCR, The prevalence of *U.parvum* was 51.9% (152/293) and 46.9% (99/211); *U.urealyticum* was 8.2% (24/293) and 8.1% (17/211)); mixed infection was 3.8% (11/293) and 2.4% (5/211) in NSC and control group, respectively (Table no. 3). There was no significant difference between any two groups ($P > 0.05$). Moreover, the mean Ct of *U.urealyticum* was 33.16 (n=24) and 34.97 (n=17) in two groups, there is also not statistics significantly ($P=0.196$) (Figure no.2 and Table no.3). However, the mean Ct of *U.parvum* in the NSC group was Ct is 31.33 (n=152), compared with the mean of Ct value of *U.parvum* in

the control group is 33.68 (n=99), there is a significant difference in two groups (P<0.0001) (Figure no. 2 and Table no. 3).

The diagnostic performances of ddPCR were excellent (Supplement Figure no.3). A total of 95 *U.parvum* positive specimens according to the digital random method (Mean Ct valuve: 31.89±4.03) and all 40 *U.urealyticum* positives specimens (Mean ct valuve: 34.01±4.46) performed the ddPCR. Of note, a total of 14 specimens with the average Ct of *U.parvum* was 37.24±1.73 were not detected of *U.parvum* by ddPCR. Similarly, the copies of 3 cases out of 40 positive specimens for *U.urealyticum* were zero copy by ddPCR, in which the average Ct was 38.00±0.35 (data not shown). Moreover, both methods showed a high degree of linearity (*U.parvum*-qPCR: R^2=0.998 and *U.parvum*-ddPCR: R^2=0.998, supplement Figure no.2; *U.urealyticum*-qPCR: R^2=1 and *U.urealyticum*-ddPCR: R^2=1, Figure no.1) but ddPCR has a smaller dynamic range and higher precision in the low copy sample (Figure no. 1). As Figure no. 3 showed, there was a strong correlation between the absolute copy number of ddPCR and Ct value of qPCR about *U.parvum* (n=81,R^2=0.80), while the correlation with *U.urealyticum* was relatively poor (n=37, R^2=0.50).

In ddPCR, the median bacteria load for *U.parvum* was 5.99×10^5copies/ml in the NSC group (n=48) and 1.74×10^4copies/ml in the control group (n=33), respectively. However, the median load for *U.urealyticum* was 1.26×10^3 copies/ml in the NSC group (n=22) and 5.35×10^3copies/ml in the control group (n=14), respectively (Table no. 4). *U.parvum* has significant differences between the NSC group and the control group in terms of Ct value of qPCR, copy number, and standardized copy number (copy number of *U.parvum* divided by

copy number of GAPDH gene) by ddPCR (P<0.0001). On the contrary, *U.urealyticum* has no significant differences no matter one of three aspects in two groups (P>0.05) (Figure no. 3).

Discussion

So far, there is only limited convincing evidence that *U.parvum* and *U.urealyticum* are the causative agents of non-specific cervicitis infections, partly because of lack of consensus regarding cervicitis case definition and standardized, reproducible assay to detect associated sexually transmitted pathogens used in studies[3, 19]. The cervicitis patients and asymptomatic individuals enrolled in this study were strictly selected via medical history screening, light microbiology observation, and laboratory tests to exclude the other Sexually transmitted pathogenic, including the fungi, *T.trichomonas*, HPV*, C.trachomatis,* and *N. gonorrhea.*

Our study suggested that the prevalence of *U.parvum* and *U.urealyticum* were 51.9% Vs 46.9% , 8.2% Vs 8.1% in the two groups, respectively. The difference in the prevalence of those two pathogens between the two groups was not statistically significant. But obviously, the prevalence of *U.urealyticum* was significantly lower than the *U.parvum*. Currently, this difference was not completely understood.

By qPCR assay, the mean of Ct of *U. urealyticum* in two groups is very close, suggesting that *U. urealyticum* may not have a causal role in cervicitis. Due to the almost Ct of qPCR were large than 30, the results of qPCR perhaps not very accurate (Figure no.2). Hence, we taken a

newest promising PCR technology, droplet digital PCR (ddPCR), that enables the absolute quantitation of nucleic acids and provided higher precision, accuracy and repeatability[15].

Our data suggested that the correlation of *U.parvum* is higher (R^2=0.80, Figure no.3a) but of *U.urealyticum* is relatively poor (R^2=0.50, Figure no.3e) between two methods. But conclusions are on the whole the same of two methods. As expected, there were significance differences of the copy number of *U.parvum* in two groups (P<0.0001) and the absolutely copy number in non-specific cervicitis is higher than 10 times compared with the control group (Table no. 4). By contrast, the difference in the copy number of *U.urealyticum* between two groups was not statistically significant (P>0.05, Table no. 4).

Recently, several studies have shifted to focus on the bacteria load. A study suggested that the amount of *U.parvum* copy number was significant correlation with histologic chorioamnionitis [20]. Similarly, a study was made by Contini C et al. [21] using qPCR to quantify the bacteria load of *U.parvum* were 1.3×10^{-1} copy/cell in spontaneous abortion (SA, n=14) and 2.8×10^{-3} copy/cell in the female who underwent voluntary interruption of pregnancy (VI, n=15), contrarily, the bacteria load of *U.urealyticum* were 3.3×10^{-3} and 1.6×10^{-3} copy/cell in SA and VI, respectively.

In the current study, we quantified, for the first time, the precise bacteria load of *U.parvum* and *U.urealyticum* through ddPCR technology. It is generally considered that *Ureaplasma* spp. load $\geq10^4$ CCU/ml is the signal of active infection and may require treatment with antibiotics.

The establishment of this reference value is based on the liquid culture. However, traditional liquid culture is only a qualitative method with relatively poor sensitivity, exists of false-positives and false-negative, and indistinguishable *Ureaplasma* spp. serovars[22]. At present, real-time PCR has become a common technique in molecular diagnosis and overcomes the above-mentioned shortcomings of liquid culture, and can perform relative quantified or absolute quantification through the standard curve. However, the construction of the standard curve is complicated and time-consuming. Moreover, the quantitative accuracy of qPCR is easily affected by amplification efficiency and PCR inhibitors.

At this time, ddPCR, an emerging technology, partitioned the PCR reaction into about 20,000 nanometer-sized droplets. Then, the relative concentration of targets, primers and probes are higher, while the inhibitor is relatively lower, which perfectly overcoming the difficulties of conventional qPCR[13]. At present, despite the role of *Ureaplasma* spp. is constant controversial, its pathogenicity is gradually accepted with increasingly clinical observation studies[23, 24]. In addition, several in vitro studies demonstrate *Ureaplasma* spp. may modulate cytokine[25, 26] and proinflammatory responses were clear dose-dependent[27]. Interestingly, a study indicated that a high dose of *U.parvum* (10^7 cfu/ml) can significantly increase prothrombin/thrombin mRNA expression to promote the onset of PPROM, while a lower dose of *U.parvum*(10^5 cfu/ml) has shown no significant effect[6]. It indicates that there may exists a threshold effect in various the association diseases of *U.parvum* infection. Therefore, it is essential for the establishment of a reference value to discriminate the infection or colonization derived of *Ureaplasma* spp. through precisely quantitative technology.

Our study has several limitations. On the one hand, the standard curves of qPCR had a wider linear range of 6 log units (Figure no. 1), but ddPCR has smaller dynamic range, lower throughput, and higher cost due ro specialized instrumentation and consumables (supplement), which to a large extent limited the application in clinical. On the other hand, the prevalence of *U.urealyticum* is relatively lower, the number of population recruited in the study on a whole was relative smaller. Moreover, this was a single-center cross-sectional study. Multicenter Prospective studies with a large population in future will provide more definitive evidence.

This study is the first to report on the absolute quantitative bacterial load of *Urealyticum* spp. in non-specific cervical swabs. The ddPCR assay can quantify the burden of asymptomatic and symptomatic individuals, and provide clinicians with important information on diagnosis and treatment effects. In conclusion, our research on non-specific cervicitis and asymptomatic individuals indicates that the *U.parvum* rather than *U.urealyticum* may play a potential role in the development and progression of cervicitis among female. But the pathogenicity of *U.parvum* might relatively low, only at high dose may induce the inflammation. More research is needed to elucidate the difference in the role of *Ureaplasma* serotypes and pathogenicity of the relevant clinical diseases of *Ureaplasma* spp. infection.

Abbreviations

ddPCR: digital droplet PCR

U.parvum: *Urealyticum parvum*

U.urealyticum: *Urealyticum urealyticum*

NSC: non-specific cervicitis

PPROM: preterm premature rupture of membranes

HPV: Human papillomavirus

CT: threshold cycle

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Declarations

Ethics approval and consent to participate

The study was approved by the Ethical committee of the Minhang University, Fudan University. The study abides by the ethical norms and principles for research as established by the Declaration of Helsinki. Informed consent was obtained from all subjects. All patients gave permission for the collection of their cervical swabs and for their medical records to be accessed and recorded.

Consent for publication

No.

Availability of data and materials

The data generated during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have declared that no conflict of interest exists. Both authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

Funding

No funding.

Authors' contributions

Yanfang Huang designed the experiments, collected clinical specimens, performed the experiments and analyzed the data, drafted the manuscript.

Xiaoqin Xu, Panpan Lv collected clinical specimens and performed the experiments.

Zhen Zhao designed the experiments, analyzed the data, obtained funding and supervised the study, drafted the manuscript.

All authors participated in revising of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Figures

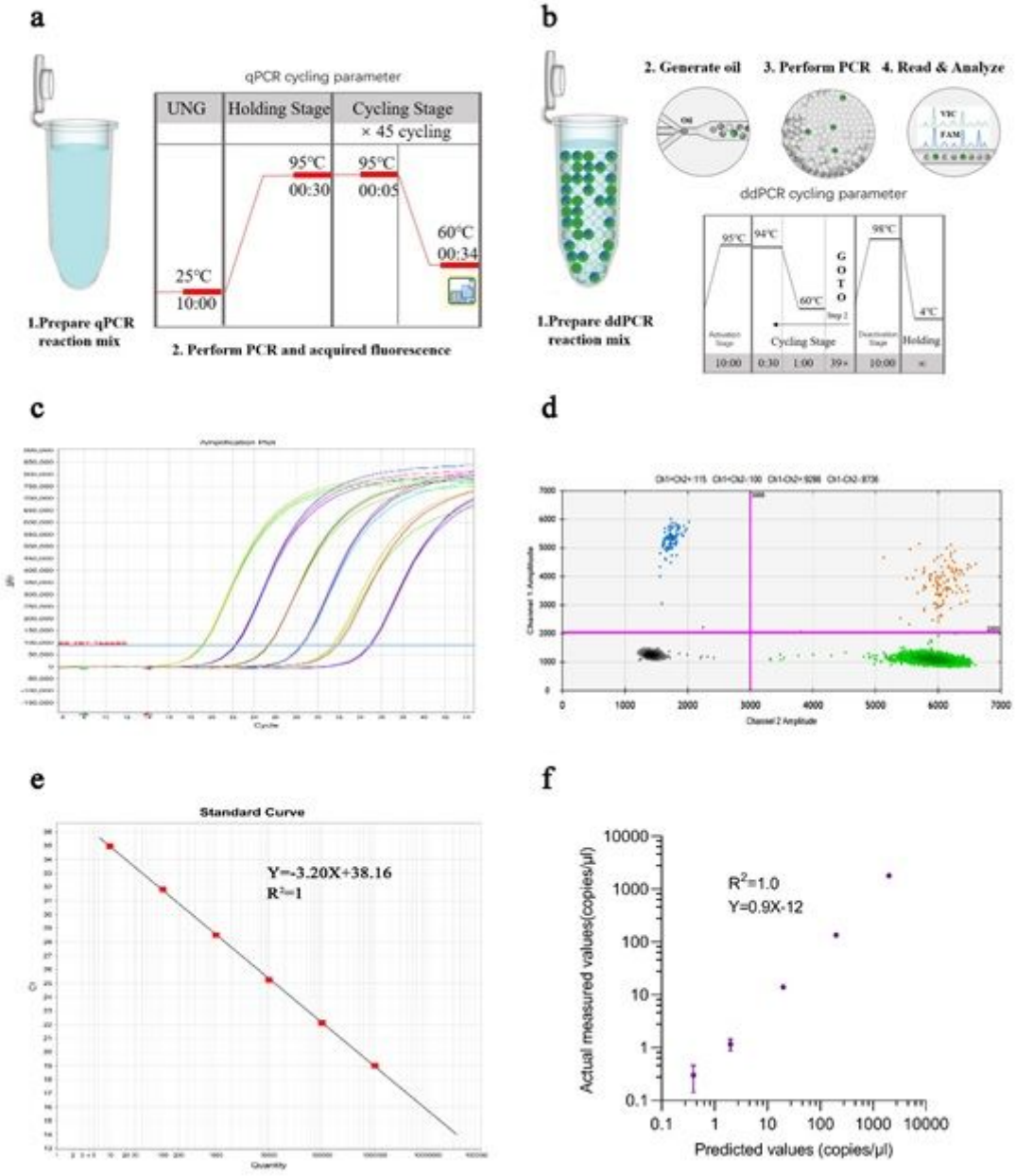

Figure 1

Schematic diagram illustrates the protocols and key difference between qPCR and droplet digital PCR (ddPCR). (a) The protocol of qPCR. (b) The protocol of ddPCR including the partitioned into 20,000 discrete droplets of a single PCR sample, generated droplet, performed PCR amplification, read droplet and analyzed ddPCR data. (c) Using ten-fold standard dilutions of U.urealyticum to illustrate qPCR linearity. (d) 2-D plot of droplet fluorescence. Set thresholds manually to obtain an accurate quantification of the target. (e) The standard curve of ten-fold standard dilutions of U.urealyticum in qPCR. (f) The standard curve of ten-fold standard dilutions of U.urealyticum in ddPCR.

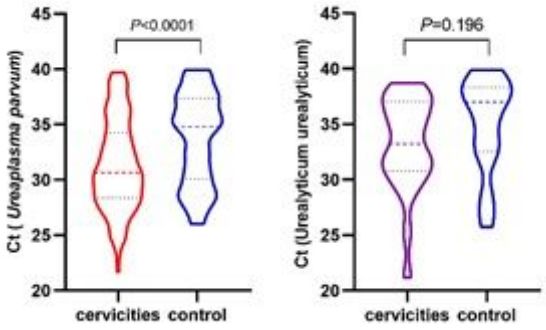

Figure 2

Violin map for Ureaplasma biovars in cervical swabs form non-specific cervicitis (NSC) patients and controls in qPCR. (a) qPCR results for Ureaplasma parvum; The mean Ct value of U.parvum in NSC group is 31.33±4.07 (n=152), the mean Ct value of U.parvum in control group is 33.68±4.10 (n=99). (b) qPCR results for Ureaplasma urealyticum. The mean Ct value of U.urealyticum in NSC group is 33.16±4.31 (n=24), the mean Ct value of U.urealyticum in control group is 34.97±4.40 (n=17). Data are representative of three independent experiments with 3 replicates for each concentration (means ± SD).

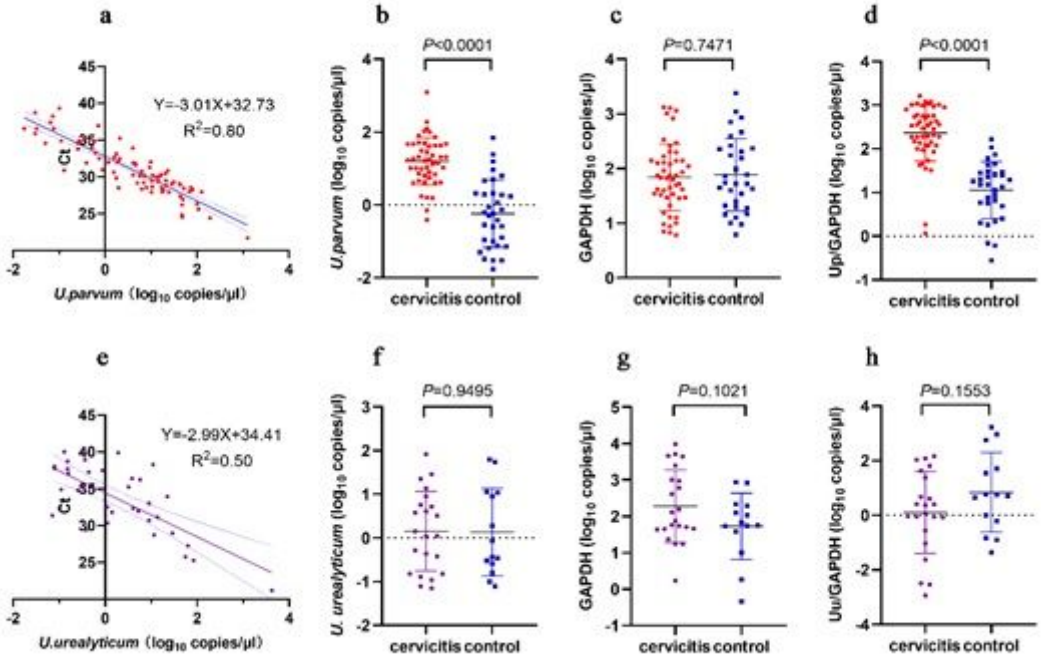

Figure 3

Ureaplasma biovars in cervical swabs form non-specific cervicitis (NSC) patients and controls in ddPCR. (a) The correlation of U.parvum between the qPCR and ddPCR (n=81). (b) the absolute copy number of U.parvum in NSC (n=48) and control group (n=33) by ddPCR. (c) The absolute copy number of GAPDH in NSC (n=48) and control group by ddPCR (n=33). (d) The standard copy number of U.parvum in NSC (n=48) and control group (n=33) by ddPCR. (e) The correlation of U.urealyticum between the qPCR and

ddPCR (n=37). (f) the absolute copy number of U.urealyticum in NSC (n=22) and control group (n=14) by ddPCR. (g) The standard copy number of U.urealyticum in NSC (n=22) and control group (n=14) by ddPCR. NSC: Non-specific cervicitis.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.docx
- Table2.docx
- Table2.docx
- Table3.docx
- Table4.docx
- appendix1.docx
- supplementFigure1.pdf
- supplementFigure2.pdf
- SupplementFigure3.pdf