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Original Article

Molecular epidemiology of selected sexually transmitted infections

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Abstract: *Neisseria gonorrhoeae* (NG), *Chlamydia trachomatis* (CT), *Trichomonas vaginalis* (TV) and *Mycoplasma genitalium* (MG) are established pathogens for human genital tract. However, the role of *Ureaplasma urealyticum* (UU) and *Ureaplasma parvum* (UP) in genital pathology is poorly unerstood. A prospective study to investigate the prevalence of above infections was performed on a cohort of 1,718 consecutive patients attending a Genitourinary Medicine (GUM) clinic. A previously published in-house real-time PCR assay, for the detection of CT DNA in genital swabs, was modified for this study. Two amplification reactions detected the DNAs of TV, NG, MG, CT, UU and UP in genital swabs from 4 (0.2%), 11 (0.6%), 17 (1%), 129 (8%), 282 (16%) and 636 (37%) patients, respectively. 594 (70%) of 848 women and 333 (38%) of 870 men were infected with at least one type of microorganism. Among 594 infected females, 485 (82%) had a single infection, 97 (16%) had a double infection, and 12 (2%) had a triple infection. Of the 333 infected men, 304 (91%) had a single infection, 27 (8%) had a double infection, and 2 (1%) had a triple infection. The prevalence of infection in both genders decreased with increasing age. The prevalence proportion of UP was significantly higher in women (54%) compared with men (18%). The high prevalence of UU and UP suggests that these bacteria are commensals of genital tract.

Keywords: *Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis, Mycoplasma genitalium, Ureaplasma urealyticu, Ureaplasma parvum,* epidemiology, PCR

Introduction

According to the latest report of the World Health Organisation (WHO), the estimated number of new cases of gonorrhoea, chlamydia and trichomoniasis in the age group 15-49 years was 328 million globally in 1999 [1]. This estimate was based on epidemiological modelling rather than molecular data and hence may have the potential for gross errors. *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG), *Trichomonas vaginalis* (TV), *Mycoplasma genitalium* (MG), *Ureaplasma urealyticum* (UU) and *Ureaplasma parvum* (UP) are sexually transmitted infections (STIs). The role of CT, NG, TV and MG in genital disease is well established. CT and NG can cause serious sequelae such as pelvic inflammatory disease (PID) and infertility [2, 3]. Although MG has recently been reported not to be a major risk factor in PID, further studies are required to confirm this and also to clarify the role of MG in epididymitis [4]. No systemic complications have been reported due to genital TV infection but there is evidence that vaginal TV infection facilitates the spread of HIV infection [5]. Treatment is recommended for patients infected with any of the above four pathogens. However, this is not the case for *Ureaplasma* in spite of emerging evidence for their role in urethritis, infertility, postpartum endometritis, chorioamnionitis, spontaneous abortion, still-birth, premature birth, perinatal morbidity and mortality, pneumonia, bacteraemia, and chronic lung disease of prematurity [6]. Along with experimental investigations, large scale epidemiological and clinical studies are needed to determine the true pathogenic potential of UU and UP. At present, such studies are not possible because of the lack of an economical, sensitive and specific assay with clinically acceptable turnaround time (TAT).

In the UK, sexually active individuals aged less than 25 years are screened for genital chlamyd-

ial infection to prevent complications and cost to the National Health Service (NHS). Such screening as part of a national programme is not considered cost effective for other STIs. We used the existing infrastructure and care pathways for Chlamydia Screening Programme (CSP) to study the molecular epidemiology of the above STIs. We set out to achieve the following characteristics for this service; 1) the new assay should be economical and automated with high throughput and 2), delivery of results to clinicians for all six organisms should be within the agreed TAT for CSP (booking to authorisation ≤ 5 days). The new assay replaced the existing assay [7] for CSP for a trial period of three months. The development of this assay and results of the trial are presented in this report.

Methods

Study population

The study population comprised 1,718 consecutive individual patients attending the Genitourinary Medicine (GUM) clinic at Addenbrooke's Hospital, Cambridge from October to December 2008. This cohort included 848 (49%) females, median age 24 years (range: 12-75) and 870 (51%) males, median age 27 years (range: 15-87). Eighty seven of 1,718 patients visited the GUM clinic on more than one occasion and provided further 94 samples. Microbiology data from only the first visit of each patient is presented in this paper. Genital swabs were collected according to the existing routine for CSP. Two swabs (endocervical and urethral) were obtained from female patients, placed in a single tube and treated as a single sample. A single urethral swab was analysed from each male patient. Swabs for chlamydia testing were collected after taking swabs for microscopy and NG culture. The IDEIA chlamydia collection kit, S600730, was used for the collection of genital swabs as described previously [8]. PCRs for NG, MG, TV, UP and UU were performed on samples collected for CT. No additional sample was collected for this study. The Chair of the Local Research & Ethics Committee deemed this project as service development and hence exempted it from the formal committee approval. The data generated by our assay for all six microorganisms were reported to GU physicians in real-time as a single report for each patient. In spite of the lack of published evidence, it was decided to treat ureaplasma positive patients who met all of the following criteria: infection with UP alone with PCR Ct value of < 20 cycles or infection with UU alone with PCR Ct value of < 20 cycles or infection with both UP or UU with PCR Ct value of < 20 cycles for one of the organisms, and negative routine diagnostic tests including PCR for CT, NG, TV & MG, and presence of unexplained genital symptoms.

DNA extraction

DNA from 200 µl of each sample was extracted using an XTR1-1 kit, (Sigma-Aldrich, Steinheim, Germany), and the CAS-1820 X-Tractor Gene™ according to the manufacturer's protocol. DNA was concentrated during extraction and was eluted in 100 µl of elution buffer. All liquid handling, except for a single step in aliquoting clinical samples and preparing the master mixture for PCR, was performed using CAS-1200™ Precision Liquid Handling System (Corbett Robotics, QLD 4113 Australia).

Polymerase chain reaction

Clinical samples were grouped in batches of 94 each. Two separate amplification reactions were performed on each batch. Each reaction was designed to detect four different targets. Reaction no. 1 (PCR-1) targeted cryptic plasmid and *omp*A gene of CT, *por*A gene of NG, and an internal control [7, 9]. Reaction no. 2 (PCR-2) targeted *MgPa* adhesion gene of MG, urease gene of UP and UU, and a repeated DNA sequence of TV [10-12]. There was a single pair of primers and a probe for each of the above targets except UP and UU. Due to homology in the urease gene, a single pair of primers and two specific probes were used for UP and UU as described previously [12]. Amplification conditions and ingredients of reactions were the same for both PCRs except for the combinations of primers and probes. One positive and one negative control were included for both PCRs. A mixture of DNA extracted from bacterial cultures of CT and NG was used as positive control for PCR-1. A mixture of DNA extracted from bacterial cultures of MG, TV, UU and UP was used as a positive control for PCR-2. Nuclease-free water was used as a negative control in both PCRs. The sequences of primers and probes and formats of PCR-1 and PCR-2 are shown in **Table 1** [7, 9-12]. The real-time

Table 1. Nucleotide sequence of primers and probes

Code of primers & probes	Sequences of primers & probes	Fluorophores & quenchers for probes	Reference
	PCR-1		
C. trachomatis			[7]
CT-Plasmid-FP	5´-AACCAAGGTCGATGTGATAG-3´		
CT-Plasmid-RP	5´-TCAGATAATTGGCGATTCTT-3´		
CT-Plasmid Probe	5´-CGAACTCATCGGCG-MGB-3´	FAM/NFQ	
CT-MOMP-FP	5´-GACTTTGTTTTCGACCGTGTT-3´		
CT-MOMP-RP	5´-ACARAATACATCAAARCGATCCCA-3´		
CT-MOMP-Probe	5´-ATGTTTACVAAYGCYGCTT-MGB-3´	VIC/NFQ	
N. gonorrhoeae			[9]
NG-FP	5´-CCGGAACTGGTTTCATCTGATT-3´		
NG-RP	5´-GTTTCAGCGGCAGCATTCA-3´		
NG-Probe	5´-CGTGAAAGTAGCAGGCGTATAGGCGGACTT-3´	ROX/BHQ-2	
Internal control (sequence in **Table 3** of reference no. 8)			[8]
IC-FP	5´-GTGCTCACACCAGTTGCCGC-3´		
IC-RP	5´-GCTTGGCAGCTCGCATCTCG-3´		
IC-Probe	5´-ATTGTGTGGGTGTGGTGTGGGTGTGTGC-3´	CY5/BHQ3	
	PCR-2		
M. genitalium			[10]
MG-FP	5´-GAGAARTACCTTGATGGTCAGCAA-3´		
MG-RP	5´-GTTAATATCATATAAAGCTCTACCGTTGTTATC-3´		
MG-Probe	5´-ACTTTGCAATCAGAAGGT-MGB-3´	FAM/NFQ	
T. vaginalis			[11]
TV-FP	5´-CATTGACCACACGGACAAAAAG-3´		
TV-RP	5´-CGAAGTGCTCGAATGCGA-3´		
TV-Probe	5´-TCATTTCGGATGGTCAAGCAGCCA-3´	ROX/BHQ-2	
U. parvum & U. urealyticum (a single set of primers for both biovars and a specific probes for each biovar)			[12]
UP-UU-FP	5´-AAGGTCAAGGTATGGAAGATCCAA-3´		
UP-UU-RP	5´-TTCCTGTTGCCCCTCAGTCT-3´		
UP-Probe	5´-TCCACAAGCTCCAGCAGCAATTTG-3´	CY5/BHQ-2	
UU-Probe	5´-ACCACAAGCACCTGCTACGATTTGTTC-3´	JOE/BHQ-1	

Abbreviations: FP: Forward primer; RP: Reverse primer; IC: Internal control; FAM: 6-carboxyfluorescein; ROX: 6-carboxy-X-rhodamine; JOE: 6-carboxy-4´, 5´-dichloro-2´, 7´-dimethoxy-fluorescein; VIC: proprietary product (Applied Biosystems, UK); CY5: a sulfoindocyanine dye; BHQ: Black-hole quencher; NFQ: Non-fluorescent quencher.

PCR was performed using Rotorgene™ 6000, Corbett Robotics, QLD 4113 Australia. Primers and probes were synthesised by *metabion* international AGi Germany and Applied Biosystems, Cheshire, UK. PCR was performed in a 26 µl volume, containing 12 µl of DNA from a clinical sample or positive control, 3 mM $MgCl_2$, 12.5 µl of Platinum® Quantitative PCR SuperMix-UDG, Invitrogen™ life technologies, Paisley, UK, 6.25 pmol of each primer and 2.5 pmol of each probe. Internal control DNA was added into the master mixture for PCR-1 as described previously [7]. Amplification reaction profile included heating at 50°C for 2 minutes and 95°C for 2 minutes followed by 45 cycles of 95°C for 1 second and 60°C for 60 seconds. The acquisition of signal was performed at 60°C during each cycle as described previously [7].

Quality control

PCR-1 & -2 used primers and probes from five previously published assays. The sensitivity, specificity and comparative performance with a relevant gold standard for all these assays had been thoroughly investigated and reported in previous publications [7, 9-12]. Hence, these aspects of the new assay were not investigated extensively. The analytical sensitivity of PCR-1 & -2 was measured on 10 fold serial dilutions (range: 4 x 10^6 to 4 copies/reaction) of two synthetic clones that contained targets of amplification. Genscript synthesised the targets of amplification and then cloned them in pUC57 vector (http://www.genscript.com/gene). The genetic maps of these clones and the accession number of each target are shown in **Table 2**. The effect of multiplexing on sensitivity was investigated by the comparison between uniplex and quadruplex PCRs. The analytical specificity of PCR-1 & -2 was tested on DNA from a limited number of available microorganisms; *Streptococcus mutans, Streptococcus canis*, *Streptococcus sanguis*, *Staphylococcus aureus*, *Staphylococcus epidermidis, Neisseria meningitidis, Neisseria sicca, Neisseria subflava, Neisseria mucosa, Escherichia coli, Corynebacterium ulcerans, Pasteurella haemolytica, Fusobacterium necrophorum, Yersinia enterocolitica*, *Corynebacterium diptheriae*, *Arcanobacterium haemolyticum*, *Prevotella buccae*, *Veillonella parvula*, *Fusobacterium ulcerans*, *Prevotella melaninogenica*, *Haemopuilus actinomycetemcomitans*, *Actinomyces israelii*, *Clostridium difficile*, *Acholeplasma laidlawii*, *Mycoplasma fermentans*, *Mycoplasma hyorhinis*, *Mycoplasma orale and Mycoplasma hominis*. Before the start of trial, the validation of new assay was performed by testing 282 random anonymised leftover specimens in parallel by uniplex and quadruplex PCRs. In accordance with the Centres for Disease Control and Prevention guidelines [13], confirmatory testing was done only for those infections that had the low prevalence, i.e. TV, NG and MG. Confirmatory testing was done by repeating either the PCR-1 or -2 on re-extracted DNA from original samples [13]. The results of NG culture were also available for the final analysis.

Results

Performance of PCR-1 and -2

Although the analytical sensitivity of previous five assays on which this assay was based ranged from < 0.2 to 10 Geq/PCR, the sensitivity of this assay was set at a slightly higher level to achieve high specificity. PCR-1 and -2 were optimized to detect 40 copies per reaction of clone-1 and -2, respectively. The comparison of uniplex and quadruplex PCRs demonstrated no loss of sensitivity due to multiplexing. As these reactions were based on TaqMan real-time PCR, they provided semi-quantitative results (the Ct-value was inversely related to the concentration of DNA). A series of optimization experiments showed no cross reactivity between different primers and probes in the multiplex format. Both PCRs detected their targets in a specific manner and no amplicons were generated from the bacteria included in the specificity panel. The pre-trial validation detected the DNAs of TV, NG, MG, CT, UU and UP in genital swabs from 3 (1%), 2 (0.7%), 6 (2%), 20 (7%), 39 (14%) and 111 (39%) patients, respectively. There was 100% concordance between results generated by uniplex and quadruplex PCRs.

Trial cohort

Nine hundred and twenty seven (54%) of 1,718 consecutive patients were infected with at least one of six microorganisms investigated in this study. By gender, 594 (70%) of 848 women and 333 (38%) of 870 men were infected. Among 594 infected females, 485 (82%) had a single infection, 97 (16%) had a double infec-

Table 2. Genetic map of PCR-1 & -2 synthetic clones

PCR-1 Clone: *C. trachomatis omp*A gene (AF202457), *C. trachomatis* cryptic plasmid (X0606) *N. gonorrhoeae por*A pseudogene (AJ223447) and internal control (synthetic)
5´-atggtgactttgttttcgaccgtgttttgaaaacagatgtgaataaagaatttcagatgggagcggcgcctactaccagcgatgtagcaggcttacaaaac-gatccaacaacaaatgttgctcgtccaaatcccgct tatggcaaacacatgcaagatgctgaaatgtttacgaacgctgcttacatggcattaaatatctgggatcgttttgatgtattttgtacattCTGCAGttaaaaac-caaggtcgatgtgatagggaaagtatgtggaatgtcgaactcatcggcgataagggtgttggatcaatttcttccttcatctagaaacaaagacgttagaga-aacgatagataagtctgattcagagaagaatcgccaattatctgatttctCTGCAGccggaactggtttcatctgattactttccagcgtgaaagtagcaggc-gtataggcggacttgctgttttgactcggaacaaattgaatgctgccgctgaaacCTGCAGgtgctcacaccagttgccgcggaaagtatgtggaatgttaa-cacacccacaccacacccacacacgtgttggatcaatttcgagatgcgagctgccaagc-3´
PCR-2 Clone: *M. genitalium MgPa* gene (M31431), a repeated sequence in *T. vaginalis* (L23861) and *U. parvum* urease gene (AF085733) and *U. urealyticum* gene (AF085729)
5´-aatgctgttgagaaataccttgatggtcagcaaaactttgcaatcagaaggtatgataacaacggtagagctttatatgatattaacttaCTGCAGa-caaacattgaccacacggacaaaaagtgtcatttcgg atggtcaagcagccaatcgcattcgagcacttcgaagaaCTGCAGagctaaaggtcaaggtatggaagatccaatttttgagcaaattgctgctggagctt-gtggacttaaaatccatgaagactgaggggcaacaggaaatgcgCTGCAGagctaaaggtcaaggtatggaagatccaatctttgaacaaatcgtag-caggtgcttgtggtcttaagattcacgaagactgaggggcaacaggaaacgct-3´

Genetic map of PCR-1 & -2 synthetic clones showing DNA sequence (from 5´ to 3´direction) and the order of targets of amplification with spacer regions (CTGCAG). The accession number of each target is shown in brackets.

tion, and 12 (2%) had a triple infection. Of the 333 infected men, 304 (91%) had a single infection, 27 (8%) had a double infection, and 2 (1%) had a triple infection. Infection with UP was diagnosed in 636 (37%) of 1,718 patients. The prevalence proportion was significantly higher in women (482 of 848 (54%) compared with men 154 of 870 (18%)).

To determine the association between age and the prevalence of infection, the age of participants was categorised into four groups (≤ 20 years, 21-30, 31-40 and > 41 years), in keeping with previously published studies [14, 15]. The prevalence of infection in each age group of women (78%, 72%, 59% and 55% respectively) and men (48%, 44%, 27% and 24% respectively) decreased with increasing age. There were only six patients in our cohort (four women & two men) who were positive for UP with PCR Ct value of < 20 cycles. All six patients were asymptomatic hence no one was treated for ureaplasma infection. The results of PCR-1 and -2 are summarised in **Table 3**.

Confirmatory testing, extra cost and turn around time

Confirmatory testing was needed for 32 positive samples; NG (11), TV (4) and MG (17). Since there was insufficient material to do this for 2 MG positive samples, confirmatory testing was done only on the remaining 30. Repeat testing on original samples confirmed the results for all of 30 positive samples, thus demonstrating 100% reproducibility. NG culture was negative for 2 of 11 PCR positive samples. The only extra cost for this service was that of consumables for an additional single amplification reaction (~£2). Turn around time for 94.3% of samples was ≤ 5 days.

Discussion

A large number of microorganisms infect the genital tract but only a few are considered as established pathogens. The impact of the majority of microorganisms on the health of the genital tract is unknown. Furthermore it is not clear how different microorganisms interact with each other in the disease process. An array of sophisticated molecular technologies has now become available in research settings to identify simultaneously a large number of microorganisms in any clinical sample. Although at present, such techniques are largely beyond the reach of diagnostic microbiology laboratories, most such laboratories use PCR for the diagnosis of infection. We used the opportunity provided by CSP to develop an economical assay that generated additional data on the incidence of five other microorganisms in addition to the detection of CT DNA in genital swabs. The existing assay [7] for CSP was modified to develop the new assay which detected DNA from six microorganisms in two quadruplex amplification reactions in a sensitive, specific and semi-quantitative manner. Although there was an additional consumables cost of £2 per sample for the second amplification reaction

Table 3. A summary of PCR results for 1,718 consecutive individual patients

		Sex									
		Male					Female				
		Age Group (years)									
Name of Bacteria	No	≤ 20	21-30	31-40	≥ 41	Total No. (%)	≤ 20	21-30	31-40	≥ 41	Total No. (%)
Single Infection											
NG	2	0	1	1	0	2	0	0	0	0	0
TV	2	0	0	1	0	1	0	1	0	0	1
MG	10	0	6	1	0	7	0	2	0	1	3
CT	59	8	35	6	2	51	2	4	1	1	8
UU	195	21	61	13	10	105	31	41	11	7	90
UP	521	28	75	19	16	138	102	200	53	28	383
Infected patients no. (%)	789 (85)	57 (43)	178 (41)	41 (24)	28 (21)	304 (35)	135 (59)	248 (58)	65 (53)	37 (52)	485 (57)
Double Infection											
CT+NG	3	1	1	0	0	2	0	1	0	0	1
CT+MG	1	0	1	0	0	1	0	0	0	0	0
NG+UP	1	0	0	0	0	0	1	0	0	0	1
NG+UU	1	0	1	0	0	1	0	0	0	0	0
MG+UU	2	0	0	0	1	1	0	1	0	0	1
MG+UP	2	0	0	0	0	0	0	1	0	1	2
TV+UP	2	0	0	0	0	0	1	1	0	0	2
CT+UU	15	1	2	3	1	7	3	3	2	0	8
CT+UP	40	1	8	0	0	9	13	17	1	0	31
UU+UP	57	2	1	2	1	6	18	27	5	1	51
Infected patients no. (%)	124 (13)	5 (4)	14 (3)	5 (3)	3 (2)	27 (3)	36 (16)	51 (12)	8 (7)	2 (3)	97 (11)
Triple Infection											
CT+NG+UP	2	1	0	0	0	1	1	0	0	0	1
CT+NG+UU	1	1	0	0	0	1	0	0	0	0	0
CT+UU+UP	8	0	0	0	0	0	4	4	0	0	8
NG+UU+UP	1	0	0	0	0	0	0	1	0	0	1
MG+UU+UP	2	0	0	0	0	0	0	2	0	0	2
Infected patients no. (%)	14 (2)	2 (1)	0	0	0	2 (0.2)	5 (2)	7 (2)	0	0	12 (1)
Infected patients no. (%)	927 (54)	64 (48)	192 (44)	46 (27)	31 (23)	333 (38)	176 (78)	306 (72)	73 (59)	39 (55)	594 (70)
All patients	1,718	134	433	172	131	870	227	427	123	71	848

(PCR-2), this extra work did not adversely influence the agreed TAT for CSP.

The report published by WHO states, "Regional prevalence estimates for trichomoniasis in women was calculated as being two times chlamydia prevalence. For men it was calculated as one tenth of the prevalence in women" [1]. The molecular data generated by our work cast doubt on this assumption. According to the data published by the Health Protection Agency about all new sexually transmitted infection episodes seen at GUM clinics in the UK from 1998 to 2007, the incidence of CT was 8 to 21 times higher than that of TV (www.hpa.org.uk). The weakness of our study is that it comes from a single geographical area with low prevalence of STIs. Larger, multi-centres studies in different geographical areas are needed to test the WHO's epidemiologic model more rigorously.

In accordance with the Centres for Disease Control and Prevention guidelines [13], confirmatory testing was done only for those infections which had a low prevalence i.e. NG, TV and MG. The possible explanation for the two NG-negative cultures, but PCR-positive samples, could be either increased sensitivity of PCR or the loss of viability of organisms during transportation. The molecular diagnosis of NG is fraught with false positive and false negative results. Hence, we used culture to back up the molecular investigations for NG. We did not find any recently reported culture positive, PCR negative isolate in our cohort [16, 17]. The GUM clinic in Cambridge manages a wide range of sexual health problems which may explain the higher age of cohort patients (median age for men = 27 years; women = 24 years) compared with those in clinics managing predominantly patients with sexually transmitted infection. This may also explain the lower CT and TV prevalence compared with some other clinics. Although the prevalence of STIs is greater among women (70%) as compared to men (38%), this is primarily explained by the higher prevalence of infection with UP in women. A high prevalence of UP among women in our cohort was in line with other published molecular studies. The prevalence of UP among 216 women attending GUM clinics in Sydney, Australia was reported to be 48% [18]. UP infection was detected among 117 of 280 (42%) healthy women attending their first prenatal visit in Sapporo, Japan [19]. A study from Brazil reported the prevalence of ureaplasma species among 224 women attending public health clinics to be 38% [20]. There is a scarcity of unbiased prospective prevalence data for a number of STIs especially UU and UP. We were able to generate this data by using existing pathway for CSP. The high prevalence of UU and UP suggests that these bacteria are members of normal genital flora. The data regarding the association between the above six infections and clinical features, particularly with reference to the role of UU and UP in disease, is presented in a separate paper [21].

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Disclosure of conflict of interest

No conflict of interest.

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