Correlation between Ureaplasma Subgroup 2 and Genitourinary Tract Disease Outcomes Revealed by an Expanded Multilocus Sequence Typing (eMLST) Scheme

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Abstract

The multilocus sequence typing (MLST) scheme of Ureaplasma based on four housekeeping genes (ftsH, rpL22, valS, and thrS) was described in our previous study; here we introduced an expanded MLST (eMLST) scheme with improved discriminatory power, which was developed by adding two putative virulence genes (ureG and mba-np1) to the original MLST scheme. To evaluate the discriminatory power of eMLST, a total of 14 reference strains of Ureaplasma serovars and 269 clinical strains (134 isolated from symptomatic patients and 135 obtained from asymptomatic persons) were investigated. Our study confirmed that all 14 serotype strains could successfully be differentiated into 14 eMLST STs (eSTs), while some of them could not even be differentiated by the MLST, and a total of 136 eSTs were identified among the clinical isolates we investigated. In addition, phylogenetic analysis indicated that two genetically significantly distant clusters (cluster I and II) were revealed and most clinical isolates were located in cluster I. These findings were in accordance with and further support the concept for two well-known genetic lineages (Ureaplasma parvum and Ureaplasma urealyticum) in our previous study. Interestingly, although both clusters were associated with clinical manifestation, the sub-group 2 of cluster II had pronounced and adverse effect on patients and might be a potential risk factor for clinical outcomes. In conclusion, the eMLST scheme offers investigators a highly discriminative typing tool that is capable for precise epidemiological investigations and clinical relevance of Ureaplasma.

Introduction

Ureaplasma is a member of the class Mollicutes and one of the smallest free-living organisms. It lacks a cell wall, displays limited biosynthetic abilities, requires cholesterol and hydrolyzes urea as a metabolic substrate to generate ATP [1]. To date, Ureaplasma is subtyped into 14 serovars that can be reclassified into two species. Ureaplasma parvum (UPA) includes 4 serovars (UPA1, UPA3, UPA6, and UPA14), while Ureaplasma urealyticum (UUR) comprises the remaining 10 serovars (UUR2, UUR4, UUR5, UUR7, UUR8, UUR9, UUR10, UUR11, UUR12, and UUR13) [2]. Genome sizes of UPA are between 0.75–0.78 Mbp and those of UUR are between 0.84–0.95 Mbp [1,3].

Ureaplasma is regarded as a commensal organism in the urogenital tract of sexually active adults and the colonization rate of Ureaplasma has been found between 40 to 80% in female [4]. It is always implicated in many diseases including inflammation, non-gonococcal urethritis, chorioamnionitis, adverse pregnancy outcomes, infertility, bronchopulmonary dysplasia in neonates, etc. [5–10].

Why Ureaplasma are commensal organisms in some instances and arouse clinical manifestation in others? Whether there are any associations of particular species or serovars to clinical manifestations? Although many attempts are tried, the pathogenesis of Ureaplasma induced reverse outcome is still not yet clear.

For investigating the epidemiology of Ureaplasma, several molecular subtyping methods have been developed, including traditional PCR for species or serovars determination, restriction fragment length polymorphism (RFLP), pulsed field gel electrophoresis (PFGE) and real-time PCR [2,11–13]. Recently, a multilocus sequence typing (MLST) scheme with four housekeeping genes has been established and verified by Zhang et al. [14]. Compared to any other molecular subtyping method, MLST is more sensitive, specific, and reproducible. Moreover, MLST is a well-accepted way for illustrating the diversity and population structure of different bacterial species [15,16]. Recent studies have

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All of the allele sequence files are available from the NCBI Genbank database under reference KM086576-KM086707.

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displayed that virulence genes may provide genetic markers additionally [17–21]. MLST with antigen gene sequences has been developed to investigate the isolates involved in meningococcal disease [17].

During recent years, some studies reported that the putative pathogenic genes or proteins of *Ureaplasma* may be responsible for clinical outcomes [1,3]. It contains multiple banded antigen (MBA) and its paralogous proteins, urease, phospholipase C, A1, and A2 (PLC, PLA1, PLA2), immunoglobulin-a (IgA) protease, nucleases, putative O-sialoglycoprotein peptidase, macrophage infection mutant protein (MimD), resisting hostile environment, etc. Compared to housekeeping genes, virulence genes diverse more rapidly and can be included for phylogeny studies of *Ureaplasma*. Thus, combination of housekeeping genes and virulence genes may be more suitable for illustrating the rationale of *Ureaplasma* being associated with clinical outcomes.

In our previous study, a total of 99 sequence types (STs) were identified from 14 reference strains and 269 clinical isolates according to the MLST data analysis. In addition, clonal complex (CC) 2 was found to be more frequently associated with symptomatic patients of diseases. Although it had the discriminating capacity to differentiate two biovars of *Ureaplasma*, the main drawback was it could not successfully differentiating all the serovars from each other and the specific clinical relevance of CC1 consisted the vast majority of clinical isolates. Moreover, we further confirmed the biotypes of clinical isolates, and found that isolates of CC1 were UPA and those of CC2 were UUR [14].

| Gene               | Annotation                     | Size (bp) of fragments Analyzed | Coverage of complete CDS (%) | PCR and sequence primers (5'-3') (F/R)* | Reference |
|--------------------|--------------------------------|-------------------------------|-----------------------------|----------------------------------------|-----------|
| ftsH               | Cell division protein FtsH     | 463                           | 21.38                       | TAAAAAAAGACGACTTAACCTCACC (F) AAAAAAGTGGCGCTTGTGGTGTG (R) | 14        |
| rplL22             | 50S ribosomal protein L22      | 456                           | 48.71                       | TTCCAAACTGAAAGAGACACT (F) TTTTTTTTCAGTATAGAAGTCATC (R) | 14        |
| valS               | Valyl-tRNA synthetase          | 335                           | 12.76                       | GTCTCAAGAATGATGACCTTTAGCGGCCT (F) GCCAACAATGATGAGTCATC (R) | 14        |
| thrS               | Threonyl-tRNA synthetase       | 598                           | 34.31                       | TGATCTGTATTCAGCTCTATA (F) AGCGGTTAAATACCTTTAGTTT (R) | 14        |
| ureG               | Urease complex component       | 482                           | 77.62                       | TTAATTGCGTTAGGTCGACCTG (F) TCAATTCAATCGACCAAGAT (R) | This study |
| mba-np1            | MBA N-terminal paralog         | 480                           | 22.66                       | TAGCGGATTATTCGTTGGAACACTA (F) TAGCTTACACGCACCAACCATC (R) | This study |

*F* indicates forward primer and *R* indicates reverse primer.

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**Table 1.** Primer sequences, size of fragment, and percent coverage of complete coding sequence for six loci in this study.
Table 2. Loci characteristics and discriminatory power of eMLST schemes based on four housekeeping genes and two putative virulence genes.

| Gene   | No. alleles | No. polymorphic sites | % polymorphic sites | Average number of nucleotide differences per site (d) | No. discriminatory index n (D.I.) | Tajima’s D test* | tajima’s D test value |
|--------|-------------|-----------------------|---------------------|-----------------------------------------------------|-------------------------------|----------------|----------------------|
| ftsH   | 39          | 138                   | 29.81%              | 0.289 ± 0.021                                      | 0.07219                       | 0.07219        | 0.2898 ± 0.126       |
| rpL22  | 21          | 51                    | 15.22%              | 0.085 ± 0.063                                      | 0.05578                       | 0.05578        | 0.1919 ± 0.213       |
| valS   | 17          | 49                    | 13.37%              | 0.089 ± 0.039                                      | 0.04809                       | 0.04809        | 0.5367 ± 0.437       |
| thrS   | 10          | 39                    | 8.09%               | 0.072 ± 0.024                                      | 0.030 ± 0.024                 | 0.030 ± 0.024 | 0.0126 ± 0.012       |
| ureG   | 35          | 100                   | 20.83%              | 0.456 ± 0.024                                      | 0.191345                      | 0.191345       | 1.016 ± 0.284        |

*Average number of nucleotide differences per site (d), Average number of nucleotide differences per site (v), Discrimination index (D.I.), Tajima’s D test, and values. Discrimination index (D.I.) values were determined using DnaSpv5 software [28], which is based on the differences between the 0 and π values. Discrimination index (D.I.) values were calculated on the basis of numbers of allelic types [j], numbers of strains belonging to each type [ni], and total numbers of strains analyzed [N] with the following equation [29]. Higher D.I. value indicates higher discriminatory power.
Codon-based analyses of positive selection
Evidence for recombination breakpoints was assessed using the genetic algorithm detection (GARD) method and individual codons were analyzed for positive selection using the following methods: Single Likelihood Ancestor Counting (SLAC), the Fixed Effect Likelihood (FEL), the Random Effect Likelihood (REL), and the Mixed Effects Model of Evolution (MEME) of HyPhy software, which were implemented in the Datamonkey web server and applied REV and HKY85 models of nucleotide substitution. To avoid a high false-positive rate, sites with p values < 0.1 for SLAC, FEL and MEME models, and Bayes Factor > 50 for REL model were accepted as candidates for selection.

Phylogenetic analysis
A Neighbor-Joining tree of *Ureaplasma* isolates was constructed by MEGA 5.0 using the number of nucleotide differences in the concatenated sequences (total of 2814 bp) of six loci, with 1,000 bootstrap tests. To visualize the large data better, we selectively compressed subtrees with genetically similar isolates using the Compress/Expand function in MEGA 5.0.
An Expanded Multilocus Sequence Typing (eMLST) Scheme of Ureaplasma
Results

Levels of genetic diversity between eMLST loci

On the basis of four loci selected in the MLST for Ureaplasma, we added two putative virulence loci (ureG and mba-np1) to develop an eMLST scheme. The sizes of ureG and mba-np1 partial sequences analyzed in this study were 482 bp and 480 bp, respectively. While the ftsH and mba-np1 genes had the highest number of polymorphic sites due to greater quantity of alleles. The number of alleles per locus ranged from 10 (number of polymorphic sites due to greater quantity of alleles. The gene displayed 30 alleles and 95 polymorphic sites. Overall, the p did not deviate significantly from zero (respectively). While the partial sequences analyzed in this study were 482 bp and 480 bp, for eMLST analysis (Table 2).

We also analyzed the ureG and mba-np1 gene sequences using Fixed Effect Likelihood (FEL) and Random Effect Likelihood (REL) tests of selection. With both FEL and REL algorithms, there were also no clear evidence of sites under episodic diversifying selection which could be identified for either gene using both HKY85 and REV models of substitution (p<0.1, threshold). These analyses provided limited evidence for any positive selection to the two putative virulence genes. Although MEME algorithm highlighted six mba-np1 gene sites that may be under episodic diversifying selection, the results were still inconsistent with many other tests of selection. Besides, we also created the generate phylogenetic trees, which based on the two putative virulence gene and four housekeeping gene sequences, and indicated that they were congruent with those based on housekeeping genes (Figure S1). On this basis, it is reasonable to believe that these genes were not under diversifying selection and have co-evolved with the housekeeping genes.

Phylogenetic analyses

As the ureG and mba-np1 genes were under purifying selection and containing phylogenetically valuable information, they could be utilized in combination with the housekeeping genes for investigating the phylogenetic relationships within this species. To investigate the genetic relationship of isolates of Ureaplasma, a Neighbor-Joining tree, with 1,000 bootstrap replications, was constructed on the basis of the concatenated sequences of six gene fragments (Fig. 1). All the 283 isolates could be divided into two genetically significantly distant clusters. To visualize the large data set better, we selectively compressed subtrees with genetically similar isolates.

Among the 14 type strains of the Ureaplasma serovars, UPA (UPA1, UPA3, UPA6 and UPA14) existed in cluster I and UUR (UUR2, UUR4, UUR5, UUR7, UUR8, UUR9, UUR10, UUR11, UUR12 and UUR13) were present in cluster II. These results were highly congruent with previously identified genetic lineages. Among the 269 clinical isolates, cluster I constituted the overwhelming majority of 245 isolates and cluster II included only 24 isolates.

In the major cluster I, five sub-groups were observed and the member of each sub-group was high genetic similarity. Sub-group A contained 114 isolates (48 eSTs), sub-group B included 78 isolates (26 eSTs), sub-group C comprised 30 isolates (17 eSTs), sub-group D incorporated 10 isolates (10 eSTs), and sub-group E contained 12 isolates (11 eSTs). In the cluster II, sub-group 1 and 2 comprised 12 (11 eSTs) and 11 (11 eSTs) isolates, respectively.

Comparison between MLST and eMLST

In our previous study, the 283 isolates were classified into 99 STs by using the MLST scheme [14], while a total of 146 eSTs were revealed in this study. According to the eMLST scheme, all 14 Ureaplasma serovars could successfully be differentiated into each other and were assigned to an unique eST, while some serovars (UUR3 and UUR5, UUR9 and UUR12) were not able to be differentiated using the MLST scheme. To better understand the discriminatory power of eMLST and MLST, we compared the eSTs and STs received form the corresponding scheme (Fig. 2), and 13 STs could be sub-divided into two or more eSTs. It was noteworthy that a total of 11 and 15 eSTs were revealed in the ST1 and ST22, which were the predominant STs. In addition, we also calculated the discriminatory index (95% confidence intervals)

Statistical analysis

IBM SPSS Statistics 19.0 was used to analyze the association between sub-groups and diseases, based on the Chi-square test. The p values<0.05 were considered statistically significant.
between two methods in terms of the 283 isolates analysed. Our data also indicated that results from discriminatory index value of MLST and eMLST methods were 86.9% (95% CI 83.9–89.9) and 93.9% (95% CI 92.0–95.9), respectively (p<0.001). While the eMLST scheme appears to demonstrate higher levels of resolution overall when compared to our previous MLST scheme.

Association with symptomatic and asymptomatic infection

Five sub-groups and one singleton in cluster I and two sub-groups and one singleton in cluster II were selected for studying the association with symptoms (Table 3). We calculated the number and proportion of symptomatic infection and asymptomatic infection in all sub-groups and singletons. In the 134 clinical specimens obtained from symptomatic patients, there were 55 (41.04%), 38 (28.36%), 12 (8.96%), 4 (2.99%), and 7 (5.22%) samples clustered in sub-group A, B, C, D, and E of cluster I; while 7 (5.22%) and 10 (7.46%) isolates cases belonged to sub-group 1 and 2, respectively. Obviously, sub-group A and B comprised of the bulk of strains analyzed. Compared to the asymptomatic infection, the rate of sub-group 2 was relatively higher in symptomatic infection (p = 0.005).

Discussion

As amplification and sequencing technology become increasing automated and available, MLST and eMLST will become more convenient and prompt for studying the epidemiology of Ureaplasma. Moreover, all the information can be submitted to internet-based databases for effortless comparison, thus a global epidemiological records will be generated. MLST scheme represents basic clonal assignments based on the variation in several housekeeping genes [15,16,33], whereas virulence genes can be adopted to “zoom in” on clones and differentiate very closed strains. Obviously, eMLST could provide a higher level of discrimination than MLST, based on the combination of housekeeping genes and virulence genes of this species, and therefore may be more appropriate for studying the epidemiology. For example, an eMLST scheme designed for Propionibacterium acnes is capable to differentiate pathogenic from non-pathogenic (commensal) strains and provides improved high resolution typing (91 eSTs from 285 isolates) to have important therapeutic and diagnostic implications [34].

In our previous study, we developed a MLST scheme that comprised four housekeeping genes for Ureaplasma [14]. Although it had highly discriminating capacity to differentiate the two biovars of Ureaplasma, it was inadequate to separate the 14 serovars and associate STs to clinical outcomes. To improve the resolution of this scheme, we increased the number of loci analysed. Due to the genome size of Ureaplasma (0.75–0.95 Mbp; approximately 600 genes in the genome), the number of candidate loci for an expanded MLST scheme, especially housekeeping loci, was very limited. As a result, we introduced two putative virulence genes (ureG and mba-np1) to the scheme as such genes are being increasing utilised in MLST schemes as they may be under positive selection, which can result in enhanced diversity and discriminatory power, and can also provide information on the evolution of virulence. In our study, the highly polymorphic sites and the low dN/dS ratio of ureG and MBA-NP1 gene indicated that they were suitable for genetic analysis. Moreover, we also confirmed that these genes were not under diversifying selection and have co-evolved with housekeeping genes, and on this basis, we would consider them as a part of the core genome of Ureaplasma.
Herein, 146 eSTs were revealed in the 283 isolates investigated. A Neighbor-Joining tree was constructed with the purpose of understanding the genetic relationship better, and two genetically significantly distant clusters (cluster I and cluster II) were shown with very high internal bootstrap values in the 283 isolates. As expected, this assortment showed exact congruence with the two well-known genetic lineages (UPA and UUR) analyzed in our previous study [14]. Most Ureaplasma strains existed in the major cluster I and only a small portion was present in the cluster II. Among the cluster I and II, five and two sub-groups were found, respectively, and the members always owed high genetic similarity. It was noteworthy that UU244 (eST121) and UU266 (eST143) were found as singletons and the same results were received through the MLST scheme by presenting as ST75 and ST96, which might be due to the relatively higher variation in the selected loci.

In our present study, the higher discriminating capacity of eMLST scheme was revealed as the isolates with the same STs could be further divided into several eSTs. Two of the most striking cases were ST1 (68 isolates) and ST22 (70 isolates) that represented as 11 eSTs and 15 eSTs, respectively. Another significant and prominent instance was that the present eMLST scheme provided a clear discrimination of the 14 serotype strains, in which the 14 reference strains of Ureaplasma serovars were able to be separated into 14 eSTs and presented in the cluster I and cluster II accurately by simultaneously targeting two virulence and four housekeeping genes.

Up to now, there is a great controversy regarding the virulence of Ureaplasma and the associations between species or serovars and diseases. With regard to relationship between species and bronchopulmonary dysplasia (BPD), different consequences received by researchers [5–7,10]. In recent study, no significant difference was found in the incidence of Ureaplasma species regarding symptoms [35]. Additionally, our previous study found persons colonized with CC2 were prone to associate symptom against a large panel of Ureaplasma isolates by adding two putative pathogenicity genes. Particularly, it enabled a more accurate differentiation scheme of all 14 reference serotypes and improved separation of 269 clinical isolates. Our data suggested that sub-group 2 were more likely to associate with clinical manifestations and further investigation was required to confirm it’s clinically meaningful.

**Supporting Information**

**Figure S1 Neighbor-Joining phylogenetic trees for four housekeeping genes fsH (A), rplL22 (B), valS (C) and thrS (D); two virulence genes ureG (E), and mba-mp1 (F).** All trees were essentially concordant with that previously obtained using four housekeeping loci, with the major divisions (Cluster I and II) forming the similar clades. (TIF)

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**Author Contributions**

Conceived and designed the experiments: XYX, JZ, YSY. Performed the experiments: YYX, JZ, YSY. Analyzed the data: ZR, YJ. Contributed reagents/materials/analysis tools: ZR, YJ. Contributed to the writing of the manuscript: JZ, YYK, ZR.

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