Molecular Analysis of Eight American Type Culture Collection Gonococcal Strains by *Neisseria gonorrhoeae* Multiantigen Sequence Typing and *PorB* Sequence Typing

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Background: Molecular epidemiological typing of *Neisseria gonorrhoeae* is crucial for monitoring the spread of resistant strains. As reference strains can be used for laboratory internal quality control, we genetically characterised the American Type Culture Collection (ATCC) gonococcal strains by *Neisseria gonorrhoeae* multiantigen sequence typing (NG-MAST) and *porB* sequence typing using public multilocus sequence typing (PubMLST).

Methods: Eight ATCC gonococcal reference strains (ATCC 19424, ATCC 31426, ATCC 35541, ATCC 43069, ATCC 43070, ATCC 49226, ATCC 49926, and ATCC 49981) from Culti-Loops (Thermo Fisher Scientific, USA) were cultured. After DNA extraction, *porB* and *tbpB* were amplified and sequenced. Sequence types (STs) and allele numbers were each determined by NG-MAST (<http://www.ng-mast.net>) and *porB* sequence typing using PubMLST (<http://pubmlst.org/neisseria/porB/>).

Results: ATCC 19424 was identified as ST 266 by NG-MAST, and as Allele 946 by PubMLST. ATCC31426 was assigned a novel ST by NG-MAST, and was assigned Allele 958 with 1.2% mismatch by PubMLST. ATCC 35541 was identified as ST 12 by NG-MAST, and as Allele 624 by PubMLST. ATCC 43069 and ATCC 43070 were both identified as ST 681 by NG-MAST, and as Allele 984 by PubMLST. ATCC 49226 was identified as ST 1572 by NG-MAST, and as Allele 2110 by PubMLST. ATCC 49926 and ATCC 49981 were both identified as ST 16496 by NG-MAST, and as Allele 928 by PubMLST.

Conclusions: The ST data obtained for ATCC gonococcal reference strains by NG-MAST and *porB* sequence typing using PubMLST can be used for quality assurance of molecular epidemiological typing in clinical microbiological laboratories.

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Key Words: *Neisseria gonorrhoeae*, American Type Culture Collection, Reference, *porB*, *tbpB*, *Neisseria gonorrhoeae* multiantigen sequence typing, Public multilocus sequence typing

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INTRODUCTION

Neisseria gonorrhoeae is a species of Gram-negative diplococci that causes gonorrhoea. Gonorrhoea is the second most common bacterial sexually transmitted infection, which leads to substantial morbidity and

economic costs worldwide. There are an estimated 78 million new cases of gonorrhoea among adults aged 15 to 49 years worldwide [1-3]. Molecular epidemiological typing of *N. gonorrhoeae* is crucial for monitoring the spread of resistant strains, especially in light of the globally increasing antimicrobial resistance of this pathogen [4,5].

Among the various methodologies used in epidemiological studies, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) and public multilocus sequence typing (PubMLST) approaches have been widely adopted for molecular typing [6-17].

NG-MAST is an online public database based on the analysis of sequences of internal fragments of two hypervariable genes, *porB* and *tbpB*, which encode superficial gonococcus antigens [5]. The NG-MAST analysis results are presented in the form of discrete allele numbers and sequence types (STs) for each isolate. This approach has been used globally to study prevalent strain genotypes, their transmission in sexual networks and core groups, and to describe distributions of resistant strains [6-11]. PubMLST is also an online public database, which originated as part of the development of the multilocus sequence typing (MLST) approach to the characterisation of bacterial strains in 1998 [12]. Databases for *Neisseria* spp. are available for the MLST approach using seven genes (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, and *pgm*) and are also available for single gene approaches which target *porA*, *porB*, *fetA*, *fHbp*, *nhba*, and *nadA*. There have been many epidemiological studies to characterise *Neisseria* spp. using PubMLST [11,13-17].

Microbial reference strains are essential in diagnostic or research studies to verify the reliability of the test results and can be used as internal quality control for quality assurance. The American Type Culture Collection (ATCC) is one of the most commonly used microbial reference resources worldwide. In the current study, we genetically characterised and evaluated the stabilities of ATCC *N. gonorrhoeae* reference strains by NG-MAST and *porB* sequence typing using PubMLST.

MATERIALS AND METHODS

1. Culture of *N. gonorrhoeae* reference strains

Eight ATCC gonococcal reference strains (ATCC 19424, ATCC 31426, ATCC 35541, ATCC 43069, ATCC 43070, ATCC 49226, ATCC 49926, and ATCC 49981) were analysed. ATCC 49226 is the strain designated by the Clinical and Laboratory Standards Institute-

designated strain for antimicrobial susceptibility testing of *N. gonorrhoeae* [18]. Culture was performed 5 times with a 1-month interval for each reference strain with five individually packaged Culti-Loops (Thermo Fisher Scientific, Waltham, MA, USA) from February to June 2018. Culti-Loops were stored in 4°C according to the instructions of the manufacturer. *N. gonorrhoeae* strains were cultured on chocolate agar plates (Shin Yang Chemical, Seoul, Korea) and incubated for 48 hours at 36°C under 5% CO₂. A single colony was subcultured once prior to DNA preparation.

2. DNA extraction

Bacterial suspensions (OD₅₄₀=1.0-2.0) were prepared in 0.17 mol/L phosphate-buffered saline. The bacteria were pelleted by centrifugation at 2,000×g for 5 minutes, washed once, resuspended in phosphate-buffered saline, and boiled for 5 minutes. The lysate was centrifuged at 2,000×g for 5 minutes, and the supernatant was used for DNA extraction. Bacterial DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) using a standard protocol. DNA was extracted individually from colonies isolated from each loop to confirm reproducibility.

3. Amplification and sequencing of *porB* and *tbpB*

Polymerase chain reaction (PCR) and sequencing were performed as previously described [5]. Primers for *porB* (5'-CAAGAAGACCTCGGCAA-3' [forward] and 5'-CCGACAACCACTTGGT-3' [reverse]) were designed using conserved sequences encoding pre-loop 2 and pre-loop 8 of the porin protein, to amplify a 737-bp fragment. Primers for *tbpB* (5'-CGTTGTCGGCAGCGCGAAAAC-3' [forward] and 5'-TTCATCGGTGCGCTCGCCTTG-3' [reverse]) were designed using conserved sequences to amplify a 589-bp fragment. The PCR reaction involved an initial 4-minute denaturation at 95°C, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C (*porB*) or 69°C (*tbpB*) for 30 seconds, and elongation at 72°C for 1 minute, with a final 10-minute extension at 72°C and cooling to 4°C. The size of the amplified products was confirmed by electrophoresis. DNA was extracted from the gel and then sequenced using a BigDye Terminator v3.1

Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3730xl Genetic Analyzer (Applied Biosystems).

4. Molecular analysis by NG-MAST and *PorB* sequence typing

For NG-MAST, an online public database (<http://www.ng-mast.net>) was used to assign allele numbers to the *porB* and *tbpB* genes and STs for each strain. For *porB* sequence typing, an online public database (<http://pubmlst.org/neisseria/porB/>) was used to type the *porB* gene and assign allele numbers to each strain.

RESULTS

1. Recovery of *N. gonorrhoeae* reference strains from Culti-Loops

ATCC 19424, ATCC 43069, ATCC 49226, and ATCC 49981 were recovered from all five cultures. ATCC 31426, ATCC 35541, and ATCC 43070 were recovered from four out of five cultures. ATCC 49926 was recovered from three

out of five cultures (Table 1). The overall recovery rate was 87.5%.

2. Sequence type and allele number determination by NG-MAST and PubMLST

The results of NG-MAST and *porB* sequence typing using PubMLST are presented in Table 1. ATCC 19424 was identified as ST 266 by NG-MAST, and as Allele 946 by PubMLST. ATCC 31426 was identified as a novel ST by NG-MAST and assigned Allele 958 with a 1.2% mismatch by PubMLST. ATCC 35541 was identified as ST 12 by NG-MAST, and as Allele 624 by PubMLST. ATCC 43069 and ATCC 43070 were both identified as ST 681 by NG-MAST, and as Allele 984 by PubMLST. ATCC 49226 was identified as ST 1572 by NG-MAST, and as Allele 2110 by PubMLST. ATCC 49926 and ATCC 49981 were both identified as ST 16496 by NG-MAST, and as Allele 928 by PubMLST.

DISCUSSION

All the analysed ATCC reference strains, except for ATCC 31426, were identified as previously known types by both NG-MAST and PubMLST. For ATCC 31426, which was the only β -lactamase – positive strain among the eight strains in this study, the *porB* sequence was assigned Allele 958 with 98.8% similarity by PubMLST, and the combination of Allele 8442 for *porB* and Allele 362 for *tbpB* resulted in it being identified as a novel ST by NG-MAST.

ATCC 43069 and ATCC 43070 were identified as the same type by both NG-MAST and PubMLST. According to information provided by the ATCC website (<https://www.atcc.org>), ATCC 43069 and ATCC 43070 were deposited by the same individual. There is a possibility that the two strains share the same origin. ATCC 49926 and ATCC 49981 were also identified as the same type by both NG-MAST and PubMLST; however, in this case, the information provided by the ATCC was insufficient to infer whether they have the same origin or not. The phylogenetic analysis of *porB* and *tbpB* was also in agreement with the NG-MAST and PubMLST results for

Table 1. Molecular characteristics of ATCC *Neisseria gonorrhoeae* reference strains (n=8)

ATCC strain	Recovery*	NG-MAST			Pub-MLST: Allele (<i>porB</i>)
		Allele		ST	
		<i>porB</i>	<i>tbpB</i>		
19424	5/5	201	90	266	946
31426	4/5	8,442 [†]	362	New	958 [‡]
35541	4/5	8	5	12	624
43069	5/5	103	196	681	984
43070	4/5	103	196	681	984
49226	5/5	1,022	6	1,572	2110
49926	3/5	90	350	16,496	928
49981	5/5	90	350	16,496	928

Abbreviations: ATCC, the American Type Culture Collection; NG-MAST, *Neisseria gonorrhoeae* multiantigen sequence typing; PubMLST, public multilocus sequence typing; ST, sequence type.

*Total number of successful/attempted cultures. DNA extraction and any subsequent procedures were individually performed for all the isolated colonies from each loop to confirm reproducibility.

[†]The sequence was not found in the database; however, it shared 99% similarity with Allele 8442. [‡]There was a 1.2% mismatch of *porB* sequence with Allele 958.

Fig. 1. Phylogenetic analysis of the *porB* and *tbpB* gene sequences. Abbreviation: ATCC, the American Type Culture Collection.

ATCC 43069 and ATCC 43070, and for ATCC 49926 and ATCC 49981 (Fig. 1).

We used five individually packaged Culti-Loops (Thermo Fisher Scientific) for each reference strain to verify the reproducibility of the test results. Although colonies were not obtained from some cultures, we confirmed that the *porB* and *tbpB* sequences, and the resulting NG-MAST and PubMLST type identifications, were the same during the repeated analyses. Culti-Loops are disposable bacteriological loops containing stabilised, viable microorganisms in a gel matrix, and have complete traceability, with ATCC licence [19]. Although the loops were stored and cultured as instructed by the manufacturer, the recovery rate was 87.5%. Increasing the recovery rate of Culti-Loops should be addressed to facilitate their applicability in clinical microbiological laboratories.

Continuous monitoring of gonococcal strains by molecular sequencing analysis is crucial for determining trends in epidemiological change and bacterial resistance. Reference strains are especially useful for laboratory method quality assurance in epidemiological surveillance. Since NG-MAST and PubMLST are online public databases being adopted for molecular typing globally,

in the current study, we genetically characterised eight ATCC gonococcal strains using these databases. To the best of our knowledge, analysis of these ATCC gonococcal reference strains by NG-MAST and PubMLST had not been reported before. The data obtained in this study can be used for quality assurance of molecular epidemiological typing in clinical microbiological laboratories.

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