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Whole-Genome Sequencing for Characterization of Capsule Locus and Prediction of Serogroup of Invasive Meningococcal Isolates

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ABSTRACT Invasive meningococcal disease is mainly caused by *Neisseria meningitidis* serogroups A, B, C, X, W, and Y. The serogroup is typically determined by slide agglutination serogrouping (SASG) and real-time PCR (RT-PCR). We describe a whole-genome sequencing (WGS)-based method to characterize the capsule polysaccharide synthesis (*cps*) locus, classify *N. meningitidis* serogroups, and identify mechanisms for nongroupability using 453 isolates from a global strain collection. We identified novel genomic organizations within functional *cps* loci, consisting of insertion sequence (IS) elements in unique positions that did not disrupt the coding sequence. Genetic mutations (partial gene deletion, missing genes, IS insertion, internal stop, and phase-variable off) that led to nongroupability were identified. The results of WGS and SASG were in 91% to 100% agreement for all serogroups, while the results of WGS and RT-PCR showed 99% to 100% agreement. Among isolates determined to be nongroupable by WGS (31 of 453), the results of all three methods agreed 100% for those without a capsule polymerase gene. However, 61% (WGS versus SASG) and 36% (WGS versus RT-PCR) agreements were observed for the isolates, particularly those with phase variations or internal stops in *cps* loci, which warrant further characterization by additional tests. Our WGS-based serogrouping method provides comprehensive characterization of the *N. meningitidis* capsule, which is critical for meningococcal surveillance and outbreak investigations.

KEYWORDS capsule, global isolates, invasive isolates, meningitis, meningococcal, *Neisseria meningitidis*, whole-genome sequencing

Neisseria meningitidis is a leading cause of serious bacterial infections, with clinical manifestations including meningitis and septicemia (1). Among the 12 defined *N. meningitidis* serogroups, invasive meningococcal disease (IMD) cases are mainly associated with serogroups A, B, C, W, X, and Y (2, 3). Meningococcal strains that do not produce surface capsule are considered nongroupable. The serogroup distribution varies geographically (4). Serogroups B, C, and Y cause the majority of meningococcal cases in North America (1, 5). Serogroups B and C have been the most common in Europe (6); however, several European countries have reported an increasing incidence of serogroup W isolates belonging to clonal complex 11 (7–9). Meningitis epidemics in African countries, historically due to serogroup A, are now more frequently associated with serogroups X, W, and, increasingly, C (10, 11). Massive vaccination campaigns have resulted in a significant reduction in serogroup A disease (12). The *N. meningitidis* polysaccharide capsule is a major meningococcal virulence factor and vaccine target (2, 3). Rapid identification and characterization of *N. meningitidis* strains and their capsule

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type are key for assessing the burden of serogroup-specific disease, outbreak investigation, and vaccination recommendations.

The capsule polysaccharide biosynthesis (*cps*) locus is clustered into six regions arranged in the order of D-A-C-E-D′-B. Regions A, B, and C are responsible for capsule biosynthesis, translocation, and transport, respectively (2). Different methods are used to classify *N. meningitidis* isolates into serogroups based on the chemical composition of capsule polysaccharides. Conventionally, the *N. meningitidis* serogroup is determined using phenotypic (e.g., slide agglutination serogrouping [SASG]) and/or genotypic (e.g., real-time PCR [RT-PCR]) assays. SASG is sensitive and easy to perform but requires multiple tests with serogroup-specific antisera (13) and can be prone to subjective interpretation of agglutination results, occasionally producing conflicting outcomes. PCR-based assays have been used to determine the capsular genogroup of meningococcal isolates and resolve the inconsistent reproducibility of SASG results between laboratories (14). In contrast to SASG, RT-PCR detects target genes within the *cps* locus, which informs the capsular genotype. However, RT-PCR focuses only on a small region (50 to 170 bp) of a single *cps* gene, without providing information on other *cps* and regulatory genes responsible for capsule polysaccharide biosynthesis. Therefore, RT-PCR does not determine capsule expression.

Whole-genome sequencing (WGS) is a high-resolution method that provides information on variations in genes that may affect their function and better elucidates genetic mutations in the *cps* locus that could affect capsule expression. The comprehensiveness of WGS analysis potentially makes it a reliable tool to predict a bacterial phenotype, such as capsule expression. A small study has previously been done to evaluate the practicality of WGS for capsule typing over other phenotypic and genotypic approaches (15). In this study, we developed an automated, WGS-based method to perform in-depth sequence analysis of the *cps* locus of invasive meningococcal isolates and to predict their serogroup. We analyzed a comprehensive data set of a convenience collection of isolates from diverse geographical locations worldwide comprising all invasive serogroups. We found novel genomic organizations within region A (capsule biosynthesis) of the *cps* locus in some serogroups. Furthermore, this WGS-based method has enabled us to identify the molecular mechanisms resulting in the nongroupability of an isolate. Lastly, we evaluated the agreement level between WGS and conventional SASG and RT-PCR serogrouping approaches.

MATERIALS AND METHODS

Bacterial strain collection. This study included a convenience collection of 453 *N. meningitidis* isolates from 12 different countries and 24 U.S. states obtained between 1962 and 2015, including isolates collected from 2009 to 2014 from Active Bacterial Core surveillance (ABCs) (16) (see Table S1 in the supplemental material). These isolates were collected from invasive meningococcal disease cases and represented all six invasive serogroups (serogroups A, B, C, W, X, and Y) and nongroupable *N. meningitidis*.

Slide agglutination. SASG was performed as previously described (13). Briefly, *N. meningitidis* isolates were grown on Trypticase soy agar II (TSA II) plates supplemented with 5% sheep blood (BD, Sparks, MD) for 18 to 24 h at 37°C in 5% CO_2. A 5% formalinized saline solution was used to suspend the bacterial cells. Serogroup (A, B, C, E, W, X, Y, and Z)-specific antisera (Remel, Lenexa, KS) were used to assess the agglutination of the bacterial suspension. The intensity of the agglutination reaction was rated according to the WHO laboratory manual (13).

RT-PCR for capsular serogroup determination. *N. meningitidis* isolates were grown as described above. To prepare crude bacterial lysates, several colonies from the agar plates were suspended in 1 ml of 10 mM Tris-HCl buffer (pH 8.0). Cell suspensions were lysed by incubating at 100°C for 10 min and then stored at −20°C for RT-PCR analysis (14, 17). All isolates were initially tested for the presence of *sodC* for species identification (13, 18), followed by detection of serogroup-specific *cps* gene targets. The serogroup-specific (polymerase) gene targets for the RT-PCR assay included *csaB*, *csb*, *csc*, *csw*, and *csy* for serogroups A, B, C, W, and Y, respectively (14, 17). The RT-PCR target gene for serogroup X is *csxB*, located downstream from its capsule polymerase, *csxA* (3).

Whole-genome sequencing. The genomes of *N. meningitidis* isolates used in this study were sequenced using either the Illumina (HiSeq2500 or MiSeq) or Pacific BioSciences (PacBio) technology, as described previously (19). Reads were trimmed using Cutadapt (version 1.8.1) software (20) to remove adapter sequences and bases with a quality score of $<$Q20 and assembled with the SPAdes (version 3.7) algorithm (21). Genomes were required to have an average coverage depth of $\geq$25 times, and contigs were excluded as spurious if they had $\leq$10% of the genome-wide coverage depth.

TABLE 1 Capsule genes included in WGS-based serogrouping analysis

Serogroup	Gene(s) in the following *cps* locus region[a]: A (capsule biosynthesis)	C (capsule transport)	E	B (capsule translocation)	Total no. of genes
A	*csaA*, ***csaB***, *csaC*, *csaD*	*ctrA*, *ctrB*, *ctrC*, *ctrD*	*tex*	*ctrE*, *ctrF*	11
B	*cssA*, *cssB*, *cssC*, ***csb***	*ctrA*, *ctrB*, *ctrC*, *ctrD*	*tex*	*ctrE*, *ctrF*	11
C	*cssA*, *cssB*, *cssC*, ***csc***	*ctrA*, *ctrB*, *ctrC*, *ctrD*	*tex*	*ctrE*, *ctrF*	11
W	*cssA*, *cssB*, *cssC*, ***csw***	*ctrA*, *ctrB*, *ctrC*, *ctrD*	*tex*	*ctrE*, *ctrF*	11
X	***csxA***, *csxB*, *csxC*	*ctrA*, *ctrB*, *ctrC*, *ctrD*	*tex*	*ctrE*, *ctrF*	10
Y	*cssA*, *cssB*, *cssC*, ***csy***	*ctrA*, *ctrB*, *ctrC*, *ctrD*	*tex*	*ctrE*, *ctrF*	11
Capsule null			*tex*		1

[a]Polymerase genes are shown in boldface.

WGS-based method for *N. meningitidis* serogroup prediction. Genome sequences were searched with the BLAST program (22) using alleles in the PubMLST *Neisseria* sequence database (23) and insertion sequence (IS) elements from the ISFinder database (24). All BLAST hits with >90% identity were pooled, and overlapping hits were compared. From these overlapping hits, the best hit for each region was determined using the formula *I*·AL/AEL, where *I* is percent identity, AL is alignment length (in bp), and AEL is allele length (in bp). This formula gives preference to shorter, exact matches rather than longer, inexact matches.

The alleles identified in each assembly were scanned against a list of genes in regions A (capsule synthesis), B (capsule translocation), C (capsule transport), and E of the *cps* locus for each serogroup. A total of 11 capsule genes present in regions A, B, C, and E for serogroups A, B, C, W, and Y and 10 capsule genes present in these regions for serogroup X were evaluated to characterize the *cps* locus in *N. meningitidis* isolates (Table 1). The capsule genogroup was determined based on identification of at least one serogroup-specific gene. If no serogroup-specific capsule genes were identified in a given isolate (e.g., all of the capsule genes identified were found in multiple serogroups), the genogroup was reported as "unclear." If multiple serogroup-specific genes were identified in a single isolate (e.g., the polymerase gene for both serogroup C [*csc*] and serogroup B [*csb*] was identified in the same isolate), the isolate was flagged for further examination, as this indicates potential contamination.

Once all capsule genes were identified and a serogroup was assigned, we predicted capsule expression by searching for genetic variations that might impact expression in the capsule genes, such as premature internal stops in open reading frames (ORFs), alleles in the phase-variable off state, and gene deletion/insertion (partial gene deletion, missing genes, and IS). Internal stops were identified by translating each identified allele *in silico* using the BioPython Seq module (25). The phase-variable state of the identified alleles was obtained by querying the allele sequence against PubMLST through the REST API (26). These phase-variable off alleles contain a frameshift mutation that introduces several premature internal stops throughout the ORF. Partial genes were identified by checking each gene for <95% alignment coverage against its closest reference; disrupted genes were identified as partial genes adjacent to an IS element. The capsule characterization code is available at https://github.com/ntopaz/characterize_neisseria_capsule. This repository contains the Python code and instructions needed to run the software.

Statistical analysis. The level of agreement between WGS and SASG or RT-PCR was determined; the denominator was the total number of samples tested for the paired methods. Exact 95% confidence intervals (CIs) for concordance rates were calculated using the Clopper and Pearson method (27). Statistical analysis was performed using SAS (version 9.4) software (SAS Institute Inc., Cary, NC).

Accession number(s). The sequence reads for the data used in this project were submitted to the Sequence Read Archive (SRA) or NCBI under accession numbers RQJL00000000, RQJM00000000, SRR8200119 to SRR8200492, SRX123123, SRX1986030 to SRX1986032, SRX1986037, SRX1986041, SRX1986048, SRX1986049, SRX1986053, SRX1986061, SRX1986069, SRX1986073, SRX1986083, SRX1986089, SRX1986094, SRX1986100, SRX1986110 to SRX1986113, SRX1986116, SRX1986117, SRX1986119, SRX1986231, SRX1986233 to SRX1986235, SRX1986243, SRX1986245, SRX1986246, SRX1986248, SRX1986249, SRX2013348, SRX2013349, SRX2013468, SRX2013480 to SRX2013492, SRX2013503, SRX2013524, SRX2013557, SRX3141623, SRX3141624, SRX3141626 to SRX3141632, SRX3141649 to SRX3141655, SRX3411061, SRX3411113, SRX4034967, SRX4034976, SRX4035073, SRX4035075, SRX4035102, SRX4035179, SRX4035211, SRX4035253, SRX4035255, SRX4035257.

RESULTS

A schematic work flow of the WGS-based serogrouping method is shown in Fig. 1. Using this method, we placed each sequence assembly into one of three categories: (i) complete *cps* locus; (ii) incomplete *cps* locus due to phase-variable off, internal stops, missing genes, partial gene deletion, or genes disrupted by IS elements; and (iii) capsule null locus (*cnl*). Isolates in category (i) are considered groupable, and their serogroup is determined based on serogroup-specific genes. The presence of genetic mutations, deletions, and insertions impacting expression of the capsule genes resulted in a nongroupable designation. Isolates that lacked all genes in *cps* locus regions A, B, and C were nongroupable and designated *cnl*.

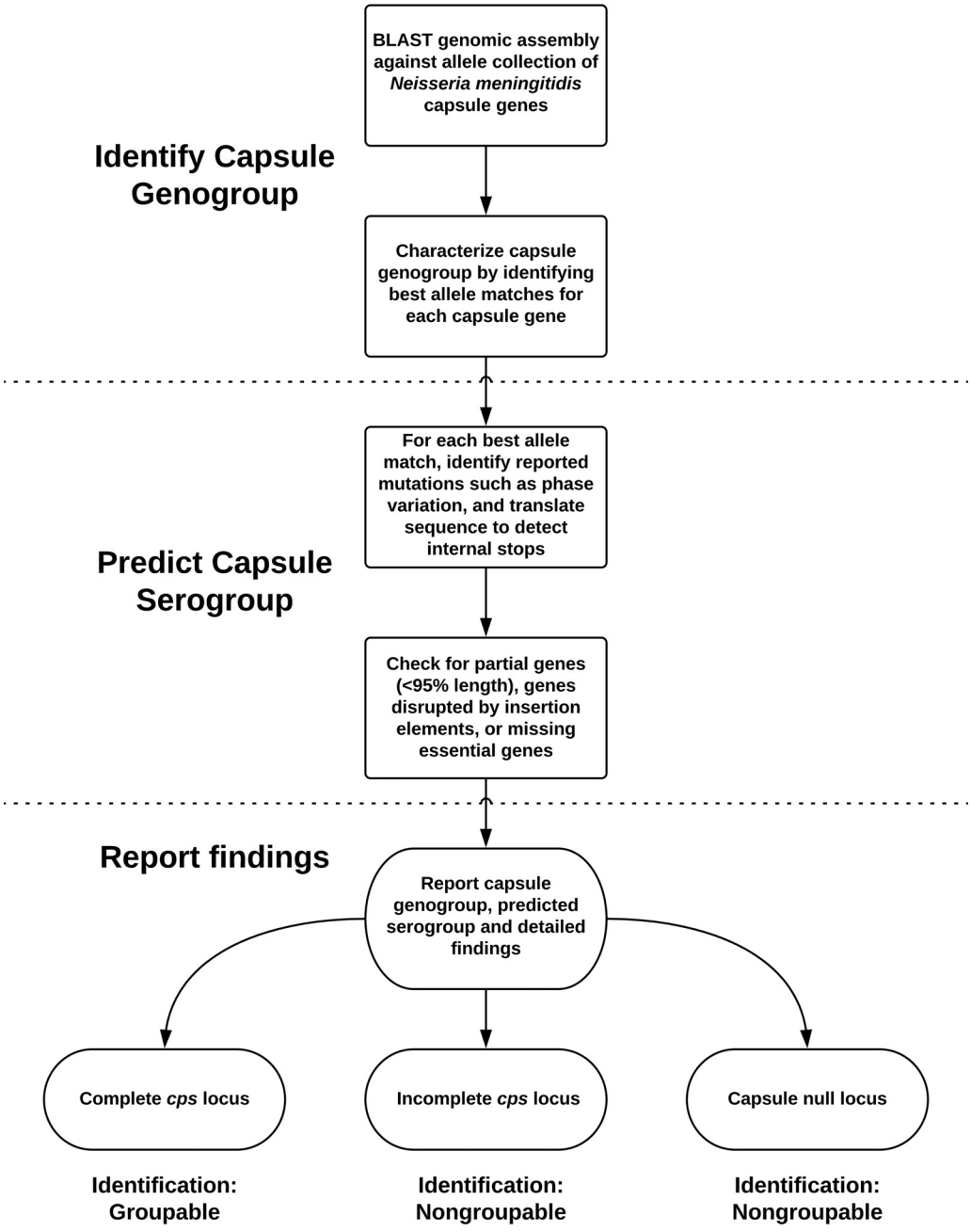

FIG 1 Schematic presentation of WGS-based *N. meningitidis* serogroup prediction method.

Capsule characterization and serogroup assignment using WGS. To provide a proof of principle for the benefits of WGS, we analyzed the *cps* locus in 453 global isolates, in which 145 *N. meningitidis* serogroup C (32.0%) isolates were identified, followed by *N. meningitidis* serogroup A ($n = 88$ isolates; 19.4%), *N. meningitidis* serogroup B ($n = 74$; 16.3%), *N. meningitidis* serogroup W ($n = 63$; 13.9%), *N. meningitidis* serogroup Y ($n = 33$; 7.3%), and *N. meningitidis* serogroup X ($n = 19$; 4.2%). Each isolate had intact polymerase and other capsule genes required for serogroup and genogroup determination. Conversely, 31 isolates (6.8%) were classified as nongroupable due to an incomplete *cps* locus. Four (12.9%) of these 31 nongroupable isolates had a capsule null locus, and 2 isolates (6.5%) had indistinguishable genogroups due to missing capsule polymerase genes. The remaining nongroupable isolates mainly comprised 11 (35.5%) genogroup B, 6 (19.4%) genogroup C, 3 (9.7%) genogroup E, and 2 (6.5%) genogroup

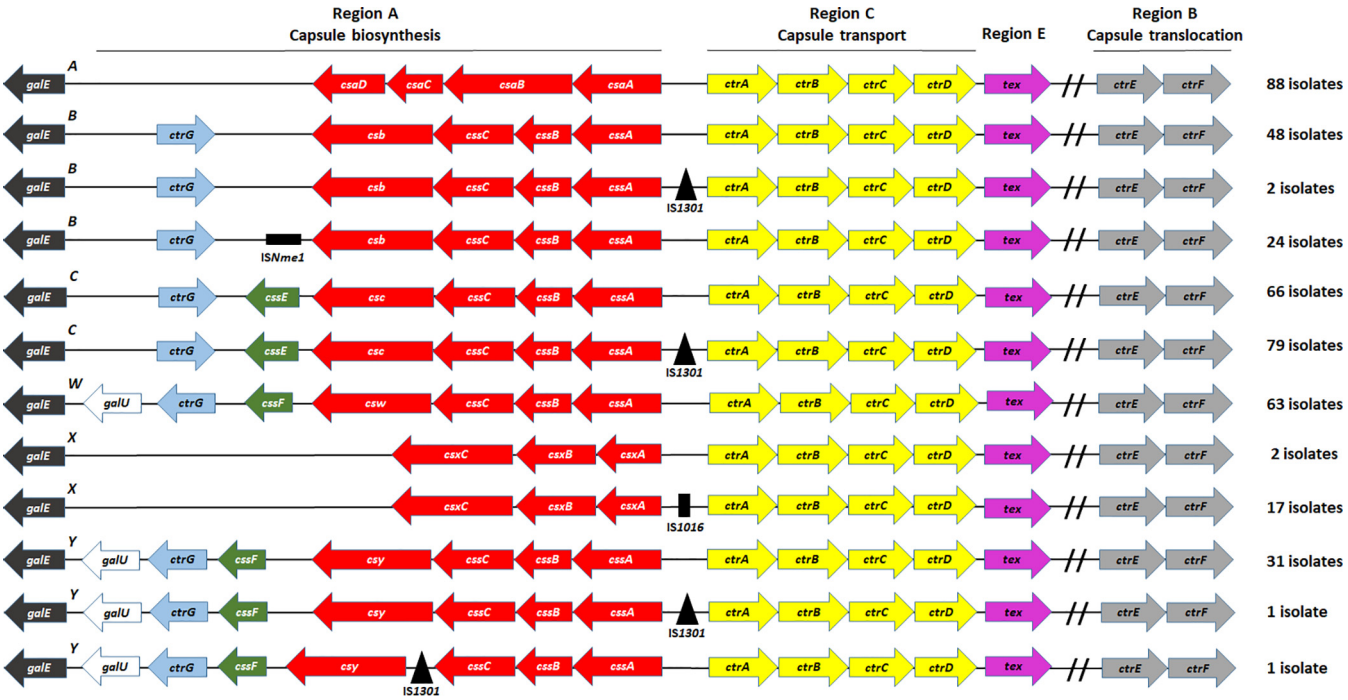

FIG 2 Genomic organization of intact *cps* loci for *N. meningitidis* global isolates. Arrows indicate the transcriptional orientation of each gene. Insertion sequence (IS) elements are depicted as black triangles (IS*1301*), horizontal rectangles (IS*Nme1*), and vertical rectangles (IS*1016*). IS elements are found in serogroup B, C, X, and Y isolates ($n = 124$ of a total of 422 isolates serogroupable by WGS).

Y isolates and 1 isolate each (3.2%) of genogroups A, X, and Z. These isolates had either phase-variable off, partial genes (21 to 62% coverage), missing genes, IS elements, and/or internal stop codons in polymerase and/or other genes in *cps* locus region A and/or region C. An IS*1301* insertion was found in *csb*, *csc*, and *cszD* of genogroup B, C, and Z isolates, respectively, and in *cseC* and *ctrB* and in *cseB* and *ctrA* of two genogroup E isolates, resulting in disruption of the affected genes.

Identification of novel genomic organization of cps locus. To discover potential novel genomic organizations of the *cps* locus among our global collection, we examined all serogroupable invasive isolates ($n = 422$) and compared the result of our analysis with known *cps* locus conventions (2, 3). Our result showed that serogroups A and W each had one genomic organization structure for the *cps* locus, while serogroups C and X had two different structures and B and Y had three (Fig. 2). We identified three types of IS elements in intergenic regions (IGRs) of the capsule region A. All isolates with IS elements ($n = 124$) had a complete *cps* locus without disruption in the coding sequence of the adjacent genes, and capsule expression was confirmed by SASG. These isolates comprised 26 of 74 *N. meningitidis* serogroup B (35.1%), 79 of 145 *N. meningitidis* serogroup C (54.5%), 17 of 19 *N. meningitidis* serogroup X (89.5%), and 2 of 33 *N. meningitidis* serogroup Y (6.1%) isolates. IS elements were not found in the *cps* locus of serogroup A or W isolates within our collection. Of 124 isolates with IS elements, 2 *N. meningitidis* serogroup B isolates, 79 *N. meningitidis* serogroup C isolates, and 1 *N. meningitidis* serogroup Y isolate harbored an IS*1301* insertion within the highly conserved IGR between *cssA* and *ctrA*; 17 *N. meningitidis* serogroup X isolates harbored an IS*1016* insertion in the IGR between *csxA* and *ctrA* (Fig. 2). Furthermore, 24 other *N. meningitidis* serogroup B isolates had an IS*Nme1* insertion downstream of *csb*, and another *N. meningitidis* serogroup Y isolate had an IS*1301* insertion upstream of *csy*; neither IS position in the *cps* locus of the *N. meningitidis* serogroup B and Y isolates has previously been reported.

Phylogenetic clustering of *N. meningitidis* invasive isolates. Phylogenetic analysis was conducted for isolates from the global collection containing all six invasive

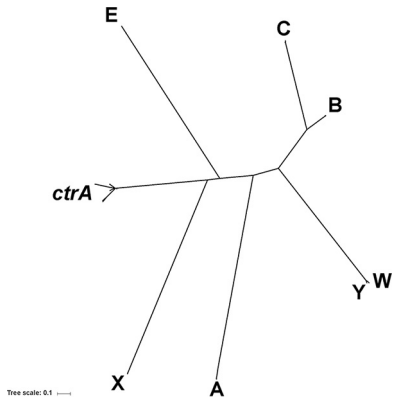

FIG 3 Phylogenetic clustering of capsule polymerase genes and *ctrA*. Clustering of 103 unique polymerase genes and *ctrA* gene alleles is shown in the phylogeny. Multiple-sequence alignment for the phylogeny was generated using the Muscle (v3.8.31) program (43) and used as the input for the creation of the maximum-likelihood tree using PhyML (v3.1) software (44). The trees were visualized using Interactive Tree of Life (iTOL) software (45).

serogroups to confirm that there is sufficient diversity between the serogroup-defining genes for differentiating serogroups via WGS. Of 453 isolates, 447 had intact capsule polymerase and *ctrA* genes, which were used to construct a phylogenetic tree (Fig. 3). A total of 103 unique polymerase gene and *ctrA* alleles were identified: 6 for *csaB*, 28 for *csb*, 10 for *csc*, 1 for *cseC*, 9 for *csw*, 1 for *csxA*, 13 for *csy*, and 35 for *ctrA*. All serogroupable isolates formed one cluster, based on their *ctrA* genes; *ctrA* is one of the genes shared across serogroups. Conversely, capsule polymerase gene-based phylogeny demonstrated clear distinctions between the different serogroups; this was particularly true for *csa* and *csxA*, which were distant from the other polymerases. However, *csw* and *csy* formed a cluster derived from the same branch of the tree, indicating that they are closely related (28). We quantified the sequence diversity of the capsule genes within our collection through pairwise comparisons by BLAST analysis (see Table S2 in the supplemental material). As in Fig. 3, polymerase gene alleles showed only up to 30% sequence identity between serogroups, with the exception of *csy* and *csw*, which had ≥98% sequence similarity.

Serogroup determination using WGS, SASG, and RT-PCR. To evaluate the WGS-based method in *N. meningitidis* serogroup prediction, we compared the WGS results with those from SASG and PCR. Among the 453 global isolates from the convenience collection, WGS, SASG, and RT-PCR assigned serogroups for 422 (93.2%), 429 (94.7%), and 441 (97.4%) isolates, respectively (Fig. 4). The agreement of the serogroup assignments between WGS and SASG for serogroups A, B, C, W, and X ranged from 99.3 to 100%, with the lowest agreement being for serogroup Y isolates (30 of 33; 90.9%) (Table 2). In contrast, only 61.3% agreement (19 of 31 isolates) between these two methods was found among isolates assigned as nongroupable by WGS. There were 16 discrepant

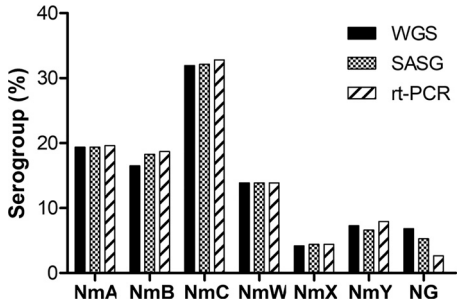

FIG 4 Serogroups of *N. meningitidis* (Nm) invasive isolates determined by WGS, SASG, and RT-PCR. A total of 453 isolates were tested by all three methods.

TABLE 2 Agreement between methods used for serogroup assignments

Serogroup by WGS	No. of isolates	Agreement with SASG			Agreement with RT-PCR		
		No. of isolates	% of isolates	95% CI	No. of isolates	% of isolates	95% CI
A	88	88	100.0	97.6−99.7[a]	88	100.0	98.7−100[a]
B	74	74	100.0		74	100.0	
C	145	144	99.3		144	99.3	
W	63	63	100.0		63	100.0	
X	19	19	100.0		19	100.0	
Y	33	30	90.9		33	100.0	
NG[b]	31	19	61.3	42.2−78.2	11	35.5	51.6−89.8
Total	453	436	96.5	94.3−98.0	432	95.4	93.0−97.1

[a]The 95% confidence interval (CI) for serogroups A, B, C, W, X, and Y.
[b]NG, nongroupable.

serogroup assignments between WGS and SASG (Table 3). It is worth noting that 4 isolates had an intact *cps* locus and were classified as *N. meningitidis* serogroup C ($n = 1$) and *N. meningitidis* serogroup Y ($n = 3$) by WGS. However, they were classified as nongroupable by SASG (Table 4). These four isolates were retested by SASG, and all were confirmed to be nongroupable. Two *N. meningitidis* serogroup Y isolates did not agglutinate with antisera. One *N. meningitidis* serogroup Y isolate agglutinated with serogroup W and specific antisera, and the *N. meningitidis* serogroup C isolate showed autoagglutination. The remaining 12 isolates with discrepant serogroup calls were groupable by SASG but nongroupable by WGS due to phase-variable off ($n = 6$), internal stop codons ($n = 3$), an IS*1301* insertion ($n = 1$) in the polymerase gene, and partial genes ($n = 2$) in capsule regions B and C (Table 3).

WGS and RT-PCR serogroup assignments agreed 100% for serogroups A, B, W, X, and Y, 99.3% for serogroup C, and 35.5% for nongroupable isolates (Table 2). Of 31 isolates classified as nongroupable by WGS, 11 were classified as nongroupable by RT-PCR (Table 2). However, WGS and RT-PCR identified the same genogroups for the other 20

TABLE 3 Discrepancies in serogroup assignments between methods

Isolate identifier	Serogroup assignment[a]			Summary of WGS analysis
	WGS	SASG	RT-PCR	
M21952	Y	**NG**	Y	Y genogroup, all capsule genes intact and present
M22800	Y	**NG**	Y	Y genogroup, all capsule genes intact and present
M27885	C	C	**NG**	C genogroup, all capsule genes intact and present
M28990	Y	**NG**	Y	Y genogroup, all capsule genes intact and present
M29039	C	**NG**	C	C genogroup, all capsule genes intact and present
M19897	NG	**B**	**B**	B genogroup, phase-variable off in *csb*
M19898	NG	**B**	**B**	B genogroup, phase-variable off in *csb*
M23083	NG	**C**	**C**	C genogroup, phase-variable off in *csc*
M27489	NG	**B**	**B**	B genogroup, phase-variable off in *csb*
M28972	NG	**B**	**B**	B genogroup, phase-variable off in *csb*
M29089	NG	**B**	**B**	B genogroup, phase-variable off in *csb*
M22425	NG	NG	**C**	C genogroup, internal stop in *csc* and *ctrE*
M24771	NG	NG	**A**	A genogroup, internal stop in *csaB*
M26017	NG	NG	**C**	C genogroup, internal stop in *csc* and *ctrE*
M27796	NG	NG	**C**	C genogroup, internal stop in *cssA*
M27819	NG	NG	**B**	B genogroup, internal stop in *cssC*
M28980	NG	**B**	**B**	B genogroup, internal stop in *csb*
M29017	NG	NG	**Y**	Y genogroup, internal stop in *csy*
M29036	NG	**B**	**B**	B genogroup, internal stop in *csb*
M29121	NG	**B**	**B**	B genogroup, internal stop in *csb*
M29038	NG	NG	**B**	B genogroup, internal stop in *csb* and *cssA*, *csb* disrupted by IS*1301* insertion
M22829	NG	**X**	**X**	X genogroup, partial *ctrE*
M27267	NG	**C**	**C**	C genogroup, partial *ctrA*
M29048	NG	NG	**Y**	Y genogroup, missing *cssA*
M29090	NG	**B**	**B**	B genogroup, *cssB* disrupted by IS*1301* insertion

[a]Boldface indicates discrepancies in serogroup calls by SASG or RT-PCR compared to those by WGS. There were 16 disagreements between WGS and SASG and 21 disagreements between WGS and RT-PCR. NG, nongroupable.

TABLE 4 Discrepancies in serogroup assignments between WGS and SASG for isolates with a complete *cps* locus

Serogroup assignment			
	SASG		
WGS	1st test	2nd test	SASG result interpretation
C	NG[a]	NG	Autoagglutination
Y	NG	NG	No agglutination
Y	NG	NG	No agglutination
Y	NG	NG	Polyagglutination

[a]NG, nongroupable.

isolates (Table 3). In total, there were 21 discrepant serogroup assignments determined by WGS and RT-PCR (Table 3). One isolate (M27885) had complete capsule genes and was classified as *N. meningitidis* serogroup C by WGS but was nongroupable by RT-PCR. This isolate did not produce any amplification after being tested twice by RT-PCR. Further alignment of the primers and probes for *csc* revealed a 2- bp mismatch on the forward primer, but no mismatches were found on the reverse primer or the probe. The remaining 20 isolates with discrepant serogroup assignments were groupable by RT-PCR but nongroupable by WGS due to phase-variable off ($n = 6$), an internal stop codon ($n = 9$), an IS*1301* insertion ($n = 1$) in polymerase and/or other genes, partial genes ($n = 2$) in capsule regions B and C, and a combination of IS*1301* and an internal stop ($n = 1$) in the polymerase gene (Table 3).

Seven isolates were assigned as nongroupable by all three methods. WGS analysis showed that 4 of these isolates had a complete deletion of the *cps* locus (capsule null), while 2 isolates were nongroupable by either method due to missing polymerase. The remaining isolate belonged to the C genogroup but was missing sialic acid capsule biosynthesis genes *cssA*, *cssB*, and *cssC*, and only 14.8% of the polymerase gene sequence was identifiable due to an IS*1301* insertion.

DISCUSSION

We demonstrate the advantages of a WGS-based serogrouping method over conventional approaches for surveillance of invasive meningococcal isolates. Using a strain collection comprised of 453 global isolates, we provide evidence that our method enables automated, in-depth analysis of the genomic sequences and organization of the *cps* locus and comprehensive characterization for nongroupable assignments. This method is able to determine meningococcal serogroups with a high accordance with those determined by RT-PCR and SASG and analyze nongroupable isolates by identifying mutations that might impact capsule expression, such as internal stop codons, phase variation, partial gene deletion, missing genes, IS element insertion, and capsule null locus, consistent with other findings (29–32). While the WGS results showed low agreement with the RT-PCR and SASG results for nongroupable isolates, these isolates can be retested using other approaches to improve the accuracy of the serogroup assignment. In addition, a nonredundant data set of 250 isolates collected between 2012 and 2015 through domestic population-based surveillance (ABCs) was also analyzed using the developed method (data not shown). The results obtained from the ABCs collections were in concordance with those obtained from the global collection, including mechanisms of nongroupability; the rate of WGS agreement with SASG and RT-PCR was 98 to 100% for groupable isolates (for WGS and SASG, 225 of 227; for WGS and RT-PCR, 227 of 227) and 26 to 48% for nongroupable isolates (for WGS and SASG, 11 of 23; for WGS and RT-PCR, 6 of 23). This in-depth characterization by WGS is not feasible through conventional assays.

Further analyses of nongroupable isolates showed that disagreements in serogroup assignment between WGS and SASG or RT-PCR were mostly caused by the prediction of capsule gene truncation, often due to frameshift mutations occurring in phase-variable simple DNA repeats (identical repeated motifs with <10 nucleotides). Phase

variation is the frequent occurrence of reversible mutations, which may be an adaptation to constantly changing growth conditions within the host (33, 34). Within our strain collections, phase variation off was mainly found in serogroup B isolates. A noteworthy finding was that the capsule expression (determined by SASG) of 6 nongroupable isolates was not abolished by the off state of phase variation in the polymerase gene. Additional studies are warranted to investigate the changes in phase-variable on and off states during different phases of bacterial growth and the effect of these changes on *N. meningitidis* capsule expression. Another frequent mutation identified within nongroupable isolates was internal stops. Similarly, a total of 3 nongroupable isolates with internal stop codons in their polymerase or other capsule genes seemed to have sufficient capsule expression to be detectable by SASG. Generally, an internal stop codon would prevent gene translation into a functioning protein. An explanation might be that the stop codon may have been located at a position that still results in a functioning protein. Another consideration would be that reversible mutations, such as phase variation and internal stops, may result in a heterogeneous culture, where some colonies may have the intact form of the genes required for capsule expression, while the others would have the mutation within the capsule genes, preventing expression. This creates a situation where the colony that was sequenced might have had the mutation but the one tested by SASG did not, and vice versa.

Insertion and excision of IS elements may influence the expression of virulence factors, which play a role in meningococcal epidemiology and pathogenesis (35). We detected three different types of IS elements—IS*1301*, IS*1016*, and IS*Nme1*—within the *cps* locus of serogroups B, C, X, and Y that did not disrupt the adjacent genes or capsule expression. The insertion element IS*1301* seems to be prevalent only among the sialic acid-producing serogroups, B, C, Y, and W (3), which is in accordance with our results. Our collection included isolates with the known IS*1301* insertion within the *css-ctr* IGR in serogroups B and C and the IS*1016* insertion within the *csx-ctr* IGR in serogroup X (3, 36). Insertion of IS*1301* into the *css-ctr* IGR of an *N. meningitidis* serogroup C strain was shown to enhance resistance against complement-mediated killing (37). Notably, we found a serogroup Y isolate with a complete *cps* locus harboring IS*1301* located upstream of *csy*; this specific location of IS*1301* insertion has not been reported previously. Like IS*1301*, IS*Nme1* belongs to the IS*5* family of transposable elements (38). It is speculated to play a role in gene translocation, such as that of *lbpAB*, encoding a surface receptor for human lactoferrin, in the FAM18 (*N. meningitidis* serogroup C) strain (39). To the best of our knowledge, detection of the IS*Nme1* element downstream of the serogroup B polymerase gene has not been reported elsewhere.

While *N. meningitidis* serogrouping using SASG can be effective, variation in capsule expression and the requirement for antiserum specificity can result in strains being classified as nongroupable even if their genomes encode all genes for capsule synthesis. Six isolates from both collections fell into this category, in which WGS showed a complete *cps* locus, but SASG failed to determine serogroups, suggesting a low capsule expression. Additionally, nonagglutinating, autoagglutinating, and polyagglutinating strains can be classified as nongroupable by SASG, as previously reported (40). Two isolates showing nonagglutination and one isolate each with autoagglutination and polyagglutination were detected. The isolate with polyagglutination agglutinated in both anti-Y and anti-W antisera but was *N. meningitidis* serogroup Y by RT-PCR, similar to the phenomenon observed with other isolates reported previously (41, 42). Moreover, sequence analysis of the polyagglutinating isolate indicated the presence of a serine at position 310 in the polymerase (*csy*), which has been demonstrated to account for this dual antigenic specificity (42).

Even when the capsule is not expressed, RT-PCR assays may yield positive results if the primers and probe still bind to the sequence of the target gene. For instance, an isolate (M29048) nongroupable by WGS due to missing the sialic acid capsule biosynthesis gene *cssA* was assigned to serogroup Y by RT-PCR. This phenomenon causes relatively low rates of agreement between WGS and RT-PCR for nongroupable isolates.

However, WGS and RT-PCR agreed on the genogroup assignments, but unlike RT-PCR, WGS predicted a problem with a gene. The PCR-based assay is expected to report an isolate as nongroupable only if the isolate is capsule null and may yield positive results if the target gene of the primers and the probe or any other gene that impacts capsule expression is not mutated or deleted.

Despite many advantages, there are still limitations to using WGS for *N. meningitidis* serogrouping purposes. For instance, if the sequenced genome has low coverage or poor quality or is missing information for proper gene assembly, WGS-based methods do not accurately assign the correct serogroup, as seen with the genome sequences of five isolates from our collections that had failed the assembly quality metrics. Only after resequencing these isolates were we able to reassign the correct serogroups. Even some genomes with high-quality assemblies may present partial genes or mutations that are known to be reversible, challenging the confident prediction that a nongroupable call is correct without further analysis. Furthermore, variations in noncoding regions could affect gene expression. This could be the case with isolates whose genomes contain a complete *cps* locus but are nongroupable by SASG. In addition, unlike RT-PCR, the WGS-based approach for serogrouping may not be suitable on clinical specimens from which an isolate is not available. Lastly, our global collection contained a number of isolates that were collected from the same geographical locations, which may influence the level of agreement between methods.

Taken together, the automated WGS analysis method allows prediction of meningococcal serogroups and comprehensive analysis of bacterial genetic composition, which enable us to identify various genetic variations in entire gene sequences that may not otherwise be identified through conventional methods. Finally, to provide high-confidence serogroup assignment and in-depth characterization of the *cps* locus, the WGS testing scheme should be customized for groupable and nongroupable isolates for integration into routine meningococcal surveillance testing.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01609-18.

SUPPLEMENTAL FILE 1, XLSX file, 0.04 MB.

SUPPLEMENTAL FILE 2, XLSX file, 1.7 MB.

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