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Keywords: pneumococcal meningitis, outbreak, genetic status, genotypes, streptococcus pneumoniae, northern Nigeria.

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Pneumococcal Meningitis Outbreak Tracked to PCR Confirmed Genotypes of *Streptococcus Pneumoniae* in Parts of Northern Nigeria

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Abstract- Pneumococcal meningitis caused by Streptococcus pneumoniae strains has been reported as the third primary aetiology of bacterial meningitis in parts of Northern Nigeria. However, information on the genotypes of *S. pneumoniae* strains circulating in Northern Nigeria is unavailable in the literature. Genotyping is being practiced widely in medical microbiology and has been shown to be an invaluable tool in tracking strains responsible for disease outbreaks. We aimed at determining the genotypes of *S. pneumoniae* tracked to a pneumococcal meningitis outbreak in parts of Northern Nigeria. The multilocus sequence typing (MLST) scheme, a DNA sequencing based genotyping method of sequencing segments of seven internal housekeeping genes, was used. The PubMLST.org database and Bacterial Isolate Genome Sequence Database software was equally used. Of the eight genotyped isolates, three (37.5 %) had detectable genotypes: genotype *aroE* in two cases (25 %), found circulating within serotype 4 (*Wzy* 4); and genotype *gki*, one isolate (12.5 %) found circulating within serotype 5 (*Wzy* 5). The MLST results provided an overview of circulating *S. pneumoniae* genotypes and their genetic status, which is a piece of important information for public health strategies such as vaccination. To develop more effective vaccines, it is imperative that the candidate vaccines must be evaluated against a set of carefully selected genotypes, which are representative of the pathogen isolated.

Keywords: pneumococcal meningitis, outbreak, genetic status, genotypes, streptococcus pneumoniae, northern Nigeria.

I. Introduction

The aetiologic agent of pneumococcal meningitis, Streptococcus pneumoniae has been reported as one of the most common causes of bacterial meningitis beyond the newborn period [CDC, 2011, Chap. 2]. In a very recent study by Peletiri and colleagues, *S. pneumoniae* was reported as the third primary aetiology of bacterial meningitis in parts of northern Nigeria. The authors reported encountering four serotypes of *S. pneumoniae* including serotype 1 (Wzy 1), serotype 4 (*Wzy* 4), serotype 5 (*Wzy* 5), and serotype 9 (*Wzy* 9) as the offending serotypes [Peletiri et al., 2021b]. Meanwhile, some authors had earlier reported serotypes 1, 5, and 19F [Kwambana-Adams et al., 2018] and serotypes 6, 19, and 20 [Suleiman et al., 2018] in Northern Nigeria. Information on the circulating genotypes of *S. pneumoniae* in Northern Nigeria is unavailable in the literature. Genotyping is also known as DNA fingerprinting [Wenjun et al., 2009].

The multilocus sequence typing (MLST) methodology is a DNA sequencing based genotyping method that generates the original sequence of nucleotides and discriminates among bacterial strains directly from polymorphisms in their DNA [Chan et al., 2001]. The MLST scheme indexes the sequence of seven internal housekeeping gene fragments to identify bacterial genotypes and associate them with biological properties [Chan et al., 2001; Jolley et al., 2004; Maiden et al., 1998]. MLST scheme is available for *S. pneumoniae* based on DNA sequencing of fragments of seven housekeeping loci *(aroE, gdh, gki, recP, spi, xpt,* and *ddl)* [Enright & Spratt, 1998]. The assignment of alleles at each locus is carried out using the MLST website (http://www.pubmlst.streptococcuspneumoniae).

The American Society for Microbiology Journals (journals.asm.org/nomenclature) gave explicit instructions to authors on bacteria genetic nomenclature and genotype designations. The genetic properties of bacteria are described in terms of phenotypes and genotypes. The phenotype describes the observable properties of an organism. The genotype refers to the genetic constitution of an organism, with reference to some standard wild type. Genotype designations are indicated by three-letter locus symbols with lowercase italic (e.g., *ara, his, rps)*. If several loci govern related functions, these are distinguished by italicized capital letters following the locus symbol (e.g., *araA, araB, araC*). We aimed at determining the genotypes of *S. pneumoniae* tracked to a pneumococcal meningitis outbreak in parts of Northern Nigeria.

II. Materials and Methods

a) Ethics Approval and Consent to Participate

Ethical approval was obtained from the Health Research Ethics Committees of National Hospital, Abuja, Nigeria (NHA/EC/034/2015); Federal Capital Development Authority Health Services, Abuja, Nigeria

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(FHREC/2017/01/27/03-04- 17); Kebbi State Ministry of Health, Nigeria (MOH/KSREC/VOL.1/56/No101.3/2015); Plateaun State Ministry of Health, Nigeria (MOH/MIS/202/VOL.T/X, 2017); Sokoto State Ministry of Health, Nigeria (SMH/1580/V.IV, 2017), and Zamfara State Ministry of Health, Nigeria (ZSHREC/ 02/03/2017) [Peletiri et al., 2021a; 2021b]. Written informed consent for the storage and future use of the unused sample, and sample material and data transfer agreement were also obtained [Peletiri et al., 2021a; 2021b].

b) Sample Size Determination

The sample size was calculated using the Cochran formula [Cochran, 1977] for calculating simple proportion. At 0.05 alpha level of significance, 95% confidence level and patient population size of seventy-seven and a previous prevalence of 13.7 %, a sample size of 181.7, which was adjusted to 210 samples after calculating 10 % attrition [Peletiri et al., 2021a; 2021b]. The subjects were recruited consecutively until the sample size was attained [Peletiri et al., 2021a; 2021b].

c) Sample Collection

Cerebrospinal fluid samples collection was as previously reported [Peletiri et al. 2021a].

d) Extraction and Quality Check of Metagenomic DNA

Metagenomic DNA extraction methodology and quality check methods were as reported previously [Peletiri et al., 2021a].

e) Multiplex Real-Time PCR for S. pneumoniae Detection

Multiplex Real-time PCR protocol for molecular detection of *S. pneumoniae* was as previously reported [Peletiri et al., 2021b].

f) Singleplex Real-time PCR for S. pneumoniae Characterization

Singleplex Real-time PCR protocol for molecular characterization of *S. pneumoniae* was as previously reported [Peletiri et al., 2021b].

g) Sample Selection

Of the appropriately characterized 12 *S. pneumoniae* serotyped strains with singleplex Real- time PCR as reported by Peletiri et al., (2021b), eight (66.7 %) were properly selected for genotyping to ensure both geographical coverage (spread) and serotype representation or distribution.

h) Multilocus Sequence Typing (MLST) Protocol for S. pneumoniae Genotyping

The Real-time PCR method of Multilocus Sequence Typing (MLST) using SYBR chemistry, is designed to genotype selected genotypic markers of *S. pneumoniae*. The assay detects seven genotypic markers: aroE, gdh, gki, recP, spi, xpt, and ddl [Enright & Spratt, 1998; Maiden, 2000; Maiden et al., 1998], as in Table 1. The primers used for amplification by Real-time PCR were: aroE-F (5'- GCCTTTGAGGCGACAGC-3'), and aroE-R (5'TGCAGTTCAGAAAACATATTTCTAA-3'); gdh-F (5'-ATGGACAAACCAGCNAGCTTT-3'), and gdh-R (5'-GCTTGAGGTCCCATGACTNCC-3'); gki-F (5'-GGCA-TTGGAATGGGATCACC-3'), and gki-R (5'-TCTCCCGC-AGCTGACAC-3'); recP-F (5'-GCCAACTCAGGTCATCC-AGG-3'), and recP-R (5'-TGCAA-CCGTAGCATTGTAAC-3'); spi-F (5'-TTATTCCTCCTGA-TTCTGTC-3'), and spi-R (5'-GTGATTGGCCA-GAAGCGGAA-3'); xpt-F (5'-TTATTA-GAAGAGCG-CATCCT-3'), and xpt-R(5'-AGATCTGCC-TCCTT-AAATAC-3'); ddl-F (5'-TGCCTCAAGTTCCTTATG-TGG-3'), and ddl-R (5'-CACTGGGTGAAAACCATGG-CAT-3').

All primers were synthesized by Eurofins, Germany. Primers were supplied lyophilized. Primers were first reconstituted to 100 µM (working stock) following the manufacturer's instructions and working concentrations of 10 µM prepared using DNA elution buffer (or TE buffer) as diluent.

i) MLST Protocol Set-up

i. *Sample Requirement*

Metagenomic DNA (mDNA) samples of serotyped *S. pneumoniae* with singleplex Real-time PCR, stored at – 20 OC (or at – 80 OC) until required for testing.

j) Reagents and Materials

10 µM *S. pneumoniae* primer mixes labelled *aroE, gdh, gki, recP, spi, xpt,* and *ddl* mix, respectively. qPCR Master Mix (SYBR); PCR water (Nuclease free water); ABI One Step Plus Real-time PCR System (Thermofisher, UK); ABI 96 well qPCR plate; P10, P100, and P1000 pipettes and tips; Thermal seal for PCR microtitre plate; Cold rack; Refrigerated centrifuge with plate holder (Heraeus, UK).

k) Setting up Reaction

A worksheet was created according to the number of samples to be tested. The ABI 96 well plate was placed into a plate holder on a cold rack. Note: Each genotypic marker was tested at a time. So, for each sample, there were seven separate reactions. Into each well, 15 µL of qPCR Master mix / Primer was dispensed. Five (5) µL of sample (mDNA), Positive control and Negative control (PCR water) was added into the appropriate well. The plate was sealed with a thermal seal and centrifuged at 1000 rpm for 1 minute in a refrigerated centrifuge (2 – 8^{O}C). The microtitre plate was placed into the holder in the ABI One Step Plus Real-time PCR machine. The manufacturer's instruction was followed in setting up the template –*S. pneumoniae* genotyping. To commence testing, SYBR Chemistry and Standard mode, with Absolute Quantification, were selected. The run was started and saved correctly. The thermal profile comprised of initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 5s, 60°C for

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30s, 72°C for 10s, and a final melt cycle of 72°C to 95°C at ramp rate of 0.3°C/s.

l) Result Analysis

After the run, the amplification curve and cycle threshold (Ct) values were inspected. Ct values of < 35 were positive; Ct values of 35 – 40 were equivocal; Ct values > 40 were negative. For the equivocal ones, the amplification curve and melting curve should be checked to decide the result. If it melts at the same specific temperature as the positive cases, it should be considered positive.

m) Sequenced Results from MLST

The genome sequences obtained from genotyping (MLST protocol) was uploaded to the publicly available Streptococcus PubMLST.org database(http://pubmlst.org/streptococcuspneumoniae) [Jolley & Maiden, 2014], powered by the Bacterial Isolate Genome Sequence Database (BIGSdb) software [Jolley & Maiden, 2010] for the determination of sequence type (ST), biotype, and global epidemiology status.

n) Extracting Typing Information from a Local Genome File

The MLST scheme from a drop-down box of the PubMLST.org database for Streptococcus was selected, and the analysis run started by clicking the submit button. Individual allelic matches identified along with the sequence type (ST) if the combination of alleles has been previously defined [Jolley et al., 2018]. Typing information can be readily extracted from whole genome sequence assemblies using the sequence query pages. Genome assembly contids pasted into the sequence query form of the database, and the required scheme or locus was selected. Any locus exact matches are displayed, and, if this corresponds to a defined combination of alleles, the profile definition (Sequence type (ST) / clonal complex (cc) for MLST) was displayed [Jolley et al., 2018].

III. Results

Of the eight genotyped isolates with MLST, three (37.5 %) had detectable genotypes, while five (62.5 %) were undetectable genotype strains. Of the seven genotypes tested, we encountered only two genotypes: genotype *aroE,* two cases (25 %) found circulating within serotype 4 (*Wzy* 4), and genotype *gki* in one case (12.5 %) found circulating within serotype 5 (*Wzy* 5) (Table 2). For genotype *aroE,* one sequence type (ST) 12750 was identified, while for genotype *gki,* two sequence types (ST) 11337 and 13103 were identified (Table 3). Results for *S. pneumoniae* genotypes sequence profile extracted from PubMLST.org powered by BIGSdb software as shown in Tables 3 to 5.

IV. Discussion

Knowledge of the genetic diversity of pathogens is being exploited more directly in the study of epidemiology. In molecular epidemiology parlance, genetic diversity is commonly referred to as 'typing' or 'genotyping'. Genotyping is being practiced widely in medical microbiology and has shown to be an invaluable tool in tracking strains responsible for disease outbreaks; particularly useful in studying and controlling nosocomial outbreaks and to ascertain whether the relapse of an infectious disease after therapeutic intervention, was due to treatment failure or recolonization of the host by a new strain [Virdi & Sachdeva, 2005]. *S. pneumoniae* genome sequence on the PubMLST.org for typing by MLST allelic profile through a search by specific schemes such as Penicillin-binding proteins (PBPs) and Pneumococcal surface protein A (PspA), results were not available. However, under the PubMLST pneumococcal genome library page, we could extract the global epidemiology status.

Our query of genotype *aroE* by filtering on serotype 4 (*Wzy* 4) revealed varying sequence types (ST) and alleles. While isolates from The Netherlands had two STs (ST 247, allele 16 and ST 205, allele 10), those from South Africa had the same ST and allele (ST 1221, allele 7) [Gladstone et al., 2020]. Though they were silent in the genomic status of these isolates; our search from the PubMLST.org database confirmed that serotype 4 (*Wzy* 4) was also found in these countries (Table 4). Our query of genotype *gki* by filtering on serotype 5 (*Wzy* 5) displayed a single ST 5659 and allele number 16 from South Africa alone submitted between 2006 and 2012 (Table 5) [Gladstone et al., 2020].

V. Conclusion

The MLST results provided an overview of circulating *S. pneumoniae* genotypes and their genetic diversity status, which is a piece of information for public health strategies such as vaccination. To develop more effective vaccines, it is imperative that the candidate vaccines must be evaluated against a set of carefully selected genotypes, which are representative of the pathogen population [Dykhuizen et al., 1993]. The tracking of identified genotypes of *S. pneumoniae* (*aroE* and *gki*) to a pneumococcal meningitis outbreak in parts of Northern Nigeria is being reported as baseline data for reference.

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Table 1: *S. pneumoniae* MLST scheme, including gene locus, amplicon length, and trimmed length of sequence used for allelic determination on http://www.mlst.net platform (CDC, 2011, Chap. 12).

Housekeeping genes	Gene locus	Trimmed length
Shikimate dehydrogenase	*aroE*	405
Glucose-6-phosphate dehydrogenase	*gdh*	460
Glucose kinase	*gki*	483
Transketolase	*recP*	450
Signal peptidase I	*spi*	474
Xanthine phosphoribosyltransferase	*xpt*	486
D-alanine-D-alanine ligase	*ddl*	441

Table 2: Prevalence of circulating *S. pneumoniae* genotypes amongst the various identified serotypes in parts of Northern Nigeria

Gene locus	Allele	Number encountered	%	Circulating amongst serotype	Number of serotype
aroE	405	2	25.0	*Wzy 4*	1
gki	483	1	12.5	*Wzy 5*	1
Undetectable genotype strains		5	62.5		
Total		8	100.0		

Gene locus - Housekeeping genes
aroE- Shikimate dehydrogenase
gki- Glucose kinase

Table 3: Genotypes of *S. pneumoniae* allelic profile for Sequence type (ST) and clonal complex (cc) extracted from PubMLST.org powered by BISGdb software

Gene Locus	Allele	Sequence Type (ST)	Clonal complex (cc)	BURST analysis Singletons	
				ST	Frequency
aroE	405	12750	Nil	Nil	
gki	483	11337	Nil	11337	1
		13103	Nil	13103	1

Table 4: *S. pneumoniae* genotype *aroE* sequence profile extracted for Serotype 4 (*Wzy 4*) from PubMLST.org powered by BIGSdb for global epidemiology status

Id	Isolate	Country	Year	Serotype	MLST (*aroE*) Allele	ST
49181	AMCSP09	The Netherlands	2008	4	16	247
49190	AMCSP18	The Netherlands	2008	4	10	205
116970	GPS_ZA_3068	South Africa	2014	4	7	1221
117961	GPS_ZA_1432	South Africa	2008	4	7	1221

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Table 5: *S. pneumoniae* genotype *gki* sequence profile extracted for Serotype 5 (*Wzy 5*) from PubMLST.org powered by BIGSdb for global epidemiology status

Id	Isolate	Country	Year	Serotype	MLST (*gki*) Allele	ST
117626	SA_GPS_SP259	South Africa	2012	5	16	5659
117672	SA_GPS_SP191	South Africa	2012	5	16	5659
117879	GPS_ZA_695	South Africa	2006	5	16	5659
117908	GPS_ZA_1145	South Africa	2010	5	16	5659
118148	GPS_ZA_1806	South Africa	2010	5	16	5659
118188	GPS_ZA_2259	South Africa	2011	5	16	5659

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