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Invasive Genotypes of *Haemophilus Influenzae* Strains Implicated with Cerebrospinal Meningitis Outbreak in Parts of Northern Nigeria

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Abstract- The genetic profile of a given strain of bacteria generated by a specific genotyping method can be as unique as a fingerprint. Information on the circulating invasive genotypes of *Haemophilus influenzae* in Northern Nigeria is unavailable in the literature. We aimed at determining the invasive genotypes of *H. influenzae* strains implicated with cerebrospinal meningitis outbreak in parts of Northern Nigeria. The multilocus sequence typing 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 12 genotyped isolates, six (50 %) had detectable genotypes: genotype *adk* in two cases (16.7 %) found circulating amongst serotypes e (*ecsH*) and f (*bexD*); genotype *fucK*, two cases (16.7 %) amongst serotypes b (*bcsB*) and f (*bexD*); and genotype *mdh* in two cases (16.7 %) amongst serotypes b (*bcsB*) and e (*ecsH*). The three genotypes are now documented as invasive strains. Therefore, for a preventive vaccination programme against cerebrospinal meningitis caused by *H. influenzae* to be successful in Nigeria, these identified invasive genotype strains should be included in the composition of the vaccines for administration.

Keywords: *bacterial genotypes, genetic profile, serotypes, H. influenzae, CSM, outbreak, northern Nigeria.*

I. Introduction

Haemophilus influenzae is a Gram-negative, pleomorphic coccobacillus responsible for life-threatening invasive diseases such as septicemia and bacterial meningitis in young children [Peltola, 2000]. *H. influenzae* may be encapsulated (typable) with one of six polysaccharide capsules designated type Hi a *(acsB)*, b *(bcsB)*, c *(ccsD)*, d *(dcsE)*, e *(ecsH)*, and f *(bexD)* or unencapsulated (non-typable *H. influenzae*, NTHI). Beyond the newborn period, *H. influenzae* is one of the most common cause of bacterial meningitis [CDC, 2011, Chap. 2]. Of the infections caused by *H. influenzae,* meningitis is of particular importance because of its potential to cause lasting neurological damage, even with appropriate supportive and antibiotic treatment (Rodrigues et al., 1971). Bacterial meningitis is a life-threatening condition that has remained a serious global health problem [CDC, 2011, Chap. 1 & 2].

In a very recent study by Peletiri et al., (2021b), *H. influenzae* was reported as the second major aetiology of bacterial meningitis in parts of Northern Nigeria. In that same report, the authors reported the varying circulating *H. influenzae* serotypes. However, information on the circulating genotypes of *H. influenzae* in Northern Nigeria is unavailable in the literature. The genetic profile of a given strain generated by a specific genotyping method can be as unique as a fingerprint [Wenjun et al., 2009].

The multilocus sequence typing (MLST) protocol 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, and has been developed with a global epidemiology perspective [Chan et al., 2001]. MLST indexes the sequences 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 *H. influenzae* based on DNA sequencing of seven housekeeping enzyme genes (adk, atpG, frdB, fucK, mdh, pgi, and recA) for characterization of capsulated and uncapsulated *H. influenzae* isolates, metagenomic DNA (mDNA) or genomic DNA (gDNA) extracts [Meats et al., 2003]. We aimed at determining the invasive genotypes of *H. influenzae* strains implicated with cerebrospinal meningitis outbreak in parts of Northern Nigeria.

II. Materials and Methods

a) Ethics Approval

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 (FHREC/2017/01/27/03-04- 17); Kebbi State Ministry of Health, Nigeria (MOH/KSREC/VOL.1/56/No101.3/2015); Plateau 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 (ZSH REC/ 02/03/2017) [Peletiri et al., 2021a; 2021b].

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b) Consent to Participate

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].

c) 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].

d) Sample Collection

The collection of cerebrospinal fluid (CSF) samples were as reported previously [Peletiri et al., 2021a].

e) Extraction and Quality Check of Metagenomic DNA

The metagenomic DNA extraction protocol and quality check was as previously reported by Peletiri and colleagues [Peletiri et al., 2021a].

f) Multiplex Real-Time PCR for H. influenzae Detection

The molecular detection of *H. influenzae* with multiplex Real-time PCR protocol was as previously reported [Peletiri et al., 2021b].

g) Singleplex Real-time PCR for H. influenzae Characterization

The molecular characterization of *H. influenzae* with singleplex Real-time PCR protocol was as previously reported [Peletiri et al., 2021b].

h) Sample Selection

Of the appropriately characterized 26 *H. influenzae* serotyped strains with singleplex Real- time PCR as reported by Peletiri and co-authors [Peletiri et al., 2021b], 12 (46.2 %) were properly selected for genotyping to ensure both geographical coverage (spread) and serotype representation or distribution.

i) Ultilocus Sequence Typing (MLST) Protocol for H. influenzae Genotyping

The Real-time PCR method of Multilocus Sequence Typing (MLST) using SYBR chemistry, is designed to genotype selected genotypic markers of *H. influenzae*. The assay detects seven genotypic markers: adk, atpG, frdB, fucK, mdh, pgi, and recA [Maiden, 2000; Maiden et al., 1998; Meats et al., 2003], and as in Table 1. The primers used for amplification by Real-time PCR were: adk-F (5'-GGTGCACC-GGGTGCAGGTAA-3'), and adk-R (5'-CCTAAGA-TTTTATCTAACTC-3'); atpG-F (5'-ATGGCAGGTGCAA-AAGAGAT-3'), and atpG-R (5'-TTGTACAACAGGC-TTTTGCG-3'); frdB-F (5'-CTTAT-CGTTGGTCTTGCCGT-3'), and frdB-R (5'-TTGG-CACTTTCCACTTTTCC-3'); fucK-F (5'-ACCACTTTCGG-CGTGGATGG-3'), and fucK-R (5'-AAGATTTCCC-AGGTGCCAGA-3'); mdh-F (5'-TCATTGTATGATATTG-CCCC-3'), and mdh-R (5'-ACTT-CTGTACCTGCATTTTG-3'); pgi-F (5'-GGTGAAAAAA-TCAATCGTAC-3'), and pgi-R (5'-ATTGAAAGACCAA-TAGCTGA-3'); recA-F (5'-ATGGCAACTCAAGAAGAAAA-3'), and recA-R (5'-TTACCAAACATCACGCCTAT- 3').

All primers were synthesized by Eurofins, Germany. Primers were supplied lyophilized; first reconstituted to 100 μM (working stock) following the manufacturer's instructions and working concentrations of 10 μM prepared using 1 x TE buffer as diluent.

j) MLST Protocol Set-up

i. *Sample Requirement*

Metagenomic DNA (mDNA) samples of serotyped *H. influenzae* with singleplex Real-time PCR, stored at – 20 ^{O}C (or at – 80 ^{O}C) until required for testing.

ii. *Reagents and Materials*

10 μM *H. influenzae* primer mixes labelled *adk, atpG, frdB, fucK, mdh, pgi*, and *recA* 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).

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

iv. *Result Analysis*

After the run, both 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

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checked to decide the result. If it melts at the same specific temperature as the positive cases, it should be considered positive.

v. *Sequenced Results from MLST*

The genome sequences obtained from genotyping (MLST protocol) was uploaded to the publicly available Haemophilus PubMLST.org database (http://pubmlst.org/haemophilus) [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.

vi. *Extracting Typing Information from a Local Genome File*

The MLST scheme selected from a drop-down box of the PubMLST.org database for Haemophilus, 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 twelve genotyped isolates with MLST, six (50 %) had detectable genotypes, while the remaining six (50 %) were undetectable genotype strains. Of the seven genotypes tested, we encountered three: *adk,* two cases (16.7 %); *fucK,* two cases (16.7 %); and *mdh,* two cases (16.7 %) (Table 2). The *H. influenzae* MLST scheme only allows any isolate to be compared with those in the MLST database, and (for encapsulated isolates) it assigns isolates to their phylogenetic lineage, via the internet. Results for *H. influenzae* genotypes sequence profile extracted from PubMLST.org powered by BIGSdb software as shown in Tables 3 to 5. Genotype *adk* sequence profile extracted for serotypes e (*ecsH*) and f (*bexD*) (Table 3); genotype *fucK* sequence profile extracted for serotypes b (*bcsB*) and f (*bexD*) (Table 4); and genotype *mdh* sequence profile extracted for serotypes b (*bcsB*) and e (*ecsH*) (Table 5).

IV. Discussion

Bacterial genotypes obtained from the housekeeping genes are meant to establish if a disease outbreak is short term (local epidemiology) or long term (global epidemiology) [Maiden et al., 1998]. Global epidemiology of pathogenic bacteria requires the direct comparison of isolates obtained in laboratories in different areas of the world in order to track the development of potential outbreaks [Chan et al., 2001]. Of the three genotypes encountered, genotype *adk* was found to be circulating amongst *H. influenzae* serotypes e (*ecsH*) and f (*bexD*) (Table 2). *H. influenzae* genome sequence query on the PubMLST.org for typing by MLST allelic profile through a search by locus combinations showed "No records found". However, under 'genome collection page', which is a subset of records within the isolate database that may contain genomes assemblies; one can access these from the isolate database by filtering on the sequence size in a query [Jolley & Maiden, 2010].

Our query of genotype *adk* by filtering on the sequence size for serotype e (*ecsH*) with identified biotype revealed sequence type (ST) of MLST allele 18, ST 18 from three isolates submitted from Czech Republic, France, and Spain (Table 3). However, serotype f (*bexD*) showed varying sequence types (ST) of MLST (*adk*) allele 22, ST 124, 980, and 1126 (Table 3). All serotype e (*ecsH*) invasive strains belonged to ST 18 [Puig et al., 2014]. However, serotype f (*bexD*) strains were genetically diverse formed by ST 124, ST 980, and ST 1126 as against the report by Puig et al., (2014) which implicated only ST 124 in their study.

Genotype *fucK* was found to be circulating amongst serotypes b (*bcsB*) and f (*bexD*) (Table 4). Our query of genotype *fucK* by filtering on the sequence size for serotype b (*bcsB*) with identified biotype revealed varying sequence types (ST) of MLST (*fucK*) allele 5, ST 6, 54, and 913 (without biotype) from three isolates from Czech Republic, USA, and Nigeria (Table 4). Serotype f (*bexD*) showed varying sequence types (ST) of MLST (*fucK*) allele 11, ST 124, 980, and 1126 from three countries (Czech Republic, Norway, and Spain) (Table 4). Genotype *mdh* was found to be circulating amongst serotypes b (*bcsB*) and e (*ecsH*) (Table 5). Our query of genotype *mdh* by filtering on the sequence size for serotype b (*bcsB*) with identified biotype revealed two sequence types (ST) of MLST (*mdh*) allele 4, ST 6 and 54 from two countries (Czech Republic and USA) (Table 5). Serotype e (*ecsH*) with MLST (*mdh*) allele 10, showed a single Sequence Type, ST 18 from three countries (Czech Republic, France, and Spain) (Table 5).

Therefore, using the principle of inference, the strain designations of our isolates from the global perspective are: (1) *H. influenzae* serotype e (*ecsH*), biotype I, allele 18, ST 18, genotype *adk*; (2) *H. influenzae* serotype f (*bexD*), biotype I, allele 22, ST 124, 980, and 1126, genotype *adk*; (3) *H. influenzae* serotype b (*bcsB*), biotype I, IV, allele 5, 41, ST 6, 54, and 913, genotype *fucK*; (4) *H. influenzae* serotype f (*bexD*), biotype I, allele 11, ST 124, 980, and 1126, genotype *fucK*; (5) *H. influenzae* serotype b (*bcsB*), biotype I, IV, allele 4, ST 6, and 54, genotype *mdh*; (6) *H. influenzae*

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serotype e (*ecsH*), biotype I, allele 10, ST 18, genotype *mdh*.

The identification of *H. influenzae* 'genotype adk' amongst serotypes e (*ecs*H) and f (*bexD*), 'genotype *fuc*K' amongst serotypes b (bcsB) and f (*bexD*), and 'genotype *mdh*' amongst serotypes b (*bcsB*) and e (*ecsH*) indicates an established genetic similarity (or relatedness) between the respective serotypes. Such genetic similarities amongst *Neisseria meningitidis* serogroups has been reported (Lamelas et al., 2017; Peletiri et al., 2022).

V. Conclusion

The MLST results provided an overview of circulating bacterial genotypes of *H. influenzae* and its' genetic diversity status. This information is of great importance for public health strategies such as vaccination. From the global perspective analysis, the strains responsible for infection in Nigeria had global linkage. This could be as a result of travels and movement of persons from one geographical area to another as earlier reported (McGahey, 1905). Finally, as clarified by Puig et al., (2014), all the isolates of *H. influenzae* from CSF with clinical symptoms in the patient, are invasive strains; we document the three genotypes encountered in this research (*H. influenzae* genotypes *adk, fucK,* and *mdh*) as invasive strains reported for the first time in parts of Northern Nigeria. Therefore, for a preventive vaccination programme against cerebrospinal meningitis caused by *H. influenzae* to be successful in Nigeria, these identified genotype strains should be included in the composition of the vaccines for administration.

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Table 1: H. influenzae 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
Adenylate kinase	*adk*	477
ATP synthase F1 subunit gamma	*atpG*	447
Fumarate reductase iron-sulfur protein	*frdB*	489
Fuculokinase	*fucK*	345
Malate dehydrogenase	*mdh*	405
Glucose-6-phosphate isomerase	*pgi*	468
RecA protein	*recA*	426

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

Gene locus	Allele	Number Encountered	%	Circulating amongst serotypes	No. of serotypes
adk	477	2	16.7	e (*ecsH*) 1; f (*bexD*) 1	2
fucK	345	2	16.7	b (*bcsB*) 1; f (*bexD*) 1	2
mdh	405	2	16.7	b (*bcsB*) 1; e (*ecsH*) 1	2
Undetectable genotype strains		6	50.0		
Total		12	100.0		

Gene locus - Housekeeping genes
adk - Adenylate kinase
fucK - Fuculokinase
mdh - Malate dehydrogenase

Table 3: *H. influenza*e genotype *adk* sequence profile extracted for serotypese (*ecsH*) and f (*bexD*) from PubMLST.org powered by BIGSdb for Sequence Type (ST), biotype, and global epidemiology status

Id	Isolate	Country	Year	Serotype	Biotype	MLST (*adk*) Allele	ST
Serotype e(*ecsH*)							
1217	031/07	Czech Rep.	2007	e	I	18	18
1980	52576	Spain	2008	e	I	18	18
3311	LNP28998	France	2017	e	I	18	18
Serotype f(*bexD*)							
1218	032/07	Czech Rep.	2007	f	I	22	124
1775	702/95	Norway	2011	f	I	22	980
1973	51118	Spain	2005	f	I	22	
9873	30/18	Czech Rep.	2018	f	I	22	124

Table 4: *H. influenza*e genotype *fucK* sequence profile extracted for serotypesb (*bcsB*) and f (*bexD*) from PubMLST.org powered by BIGSdb for Sequence Type (ST), biotype, and global epidemiology status

Id	Isolate	Country	Year	Serotype	Biotype	MLST (*adk*) Allele	ST
Serotype b(*bcsB*)							
105	7854	USA	1990	b	IV	5	54
149	Hi.28/01	Czech Rep.	2001	b	I	5	6
1673	P10095	Nigeria	2010	b	Nil	41	913
Serotype f(*bexD*)							
1218	032/07	Czech Rep.	2007	f	I	11	124
1775	702/95	Norway	2011	f	I	11	980
1973	51118	Spain	2005	f	I	11	1126

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Table 5: *H. influenzae* genotype *mdh* sequence profile extracted for serotypesb (*bcsB*) and e (*ecsH*) from PubMLST.org powered by BIGSdb for Sequence Type (ST), biotype, and global epidemiology status

Id	Isolate	Country	Year	Serotype	Biotype	MLST *(mdh)* Allele	ST
Serotype b(*bcsB*)							
106	7863	USA	1990	b	IV	4	54
149	Hi.28/01	Czech Rep.	2001	b	I	4	6
Serotype e(*ecsH*)							
1217	031/07	Czech Rep.	2007	e	I	10	18
1980	52576	Spain	2008	e	I	10	18
3311	LNP28998	France	2017	e	I	10	18

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