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Molecular detection and characterization of bacteria from CSF samples of patients with suspected cerebrospinal meningitis in parts of northern Nigeria using metagenomic DNA extracts

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Abstract:

Background: The most commonly used approaches for detection and characterization of bacterial pathogens of meningitis in developing countries include culture, Gram stain, and latex agglutination. The positivity rate of culture is relatively low due to suboptimal storage and transportation conditions, culture practice, and/or antibiotic treatment administered before specimens are collected. Specimens that yield no growth in culture can still be analyzed using molecular methods, and metagenomic DNA (mDNA) extracted directly from clinical samples (CSF) can be used. We aimed to detect and characterize three major bacterial causes of cerebrospinal meningitis (CSM); *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* using mDNA extracted directly from CSF samples.
Methodology: Metagenomic DNA templates were prepared directly from CSF specimens collected from 210 patients with suspected CSM. A multiplex Real Time PCR (mRT-PCR) using the ABI StepOne Plus Machine and Taqman Probe chemistry was used in the molecular detection, while serogroup/serotype-specific singleplex RT-PCR was used to characterize all positives samples.
Results: Eighty-eight (41.9%) of the 210 samples were positive with the mRT-PCR assay for one or a combination of two of the three bacteria. Of these, 59 (67.1%) were *N. meningitidis,* 2 (2.3%) were *H. influenzae,* 3 (3.4%) were *S. pneumoniae*, 15 (17 %) had co-infections of *N. meningitidis* with *H. influenzae*, and 9 (10.2%) had co-infections of *H. influenzae* and *S. pneumoniae*. The serogroups of *N. meningitidis* encountered were A (13.5%), B (23%), C (8.1%), W135 (8.1%), X (5.4%), Y (32.4%), and non-groupable (9.5%). The serotypes of *H. influenzae* were Hia (3.8%), Hib (57.7%), Hic (3.85%), Hie (11.5%) and Hif (23.1%). The serotypes of *S. pneumoniae* were *Wxy1* (8.3%), *Wxy4* (33.3%), *Wxy5* (50.0%), and *Wxy9* (8.3%).
Conclusion: Multiplex RT-PCR is a fast and accurate method for detecting and characterizing serogroups/serotypes of major bacteria implicated in CSM. Isolating DNA directly from CSF improves turnaround time, which will speed up patient care and management.

Keywords: Cerebrospinal meningitis, metagenomic DNA, multiplex Real Time PCR, Northern Nigeria

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Détection moléculaire et caractérisation de bactéries à partir d'échantillons de LCR de patients suspectés de méningite cérébrospinale dans certaines parties du nord du Nigéria à l'aide d'extraits d'ADN métagénomique

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Abstrait:

Contexte: Les approches les plus couramment utilisées pour la détection et la caractérisation des agents pathogènes bactériens de la méningite dans les pays en développement comprennent la culture, la coloration de Gram et l'agglutination au latex. Le taux de positivité de la culture est relativement faible en raison des conditions de stockage et de transport sous-optimales, des pratiques de culture et/ou du traitement antibiotique administré avant le prélèvement des échantillons. Les échantillons qui ne donnent pas de croissance en culture peuvent toujours être analysés à l'aide de méthodes moléculaires, et l'ADN métagénomique (ADNm) extrait directement d'échantillons cliniques (LCR) peut être utilisé. Nous visions à détecter et à caractériser trois causes bactériennes majeures de la méningite cérébrospinale (CSM); *Neisseria meningitidis, Haemophilus influenzae* et *Streptococcus pneumoniae* à l'aide d'ADNm extrait directement d'échantillons de LCR.
Méthodologie: Des matrices d'ADN métagénomique ont été préparées directement à partir d'échantillons de LCR prélevés sur 210 patients suspects de CSM. Une PCR multiplex en temps réel (mRT-PCR) utilisant la chimie de la machine ABI StepOne Plus et de la sonde Taqman a été utilisée pour la détection moléculaire, tandis que la RT-PCR monoplex spécifique au sérogroupe/sérotype a été utilisée pour caractériser tous les échantillons positifs.
Résultats: Quatre-vingt-huit (41,9%) des 210 échantillons étaient positifs avec le test mRT-PCR pour une ou une combinaison de deux des trois bactéries. Parmi ceux-ci, 59 (67,1%) étaient *N. meningitidis*, 2 (2,3%) étaient *H. influenzae,* 3 (3,4%) étaient *S. pneumoniae*, 15 (17%) avaient des co-infections de *N. meningitidis* avec *H. influenzae* et 9 (10,2%) avaient des co-infections à *H. influenzae* et *S. pneumoniae*. Les sérogroupes de *N. meningitidis* rencontrés étaient A (13,5%), B (23%), C (8,1%), W135 (8,1%), X (5,4%), Y (32,4%) et non groupables (9,5%). Les sérotypes de *H. influenzae* étaient Hia (3,8%), Hib (57,7%), Hic (3,85%), Hie (11,5%) et Hif (23,1%). Les sérotypes de *S. pneumoniae* étaient *Wxy1* (8,3%), *Wxy4* (33,3%), *Wxy5* (50,0%) et *Wxy9* (8,3%).
Conclusion: La RT-PCR multiplex est une méthode rapide et précise de détection et de caractérisation des sérogroupes/sérotypes des principales bactéries impliquées dans le CSM. Isoler l'ADN directement du LCR améliore le temps de traitement, ce qui accélérera les soins et la gestion des patients.

Mots clés: méningite cérébro-spinale, ADN métagénomique, PCR multiplex en temps réel, nord du Nigéria

Introduction:

Over the years, laboratory results confirming cases of cerebrospinal meningitis (CSM) in Nigeria have been under reported due to the methodology used in processing cerebrospinal fluid (CSF) samples for the diagnosis of meningitis. The most commonly used approaches for detection and characterization of bacterial meningitis pathogens in developing countries include culture, Gram stain, and latex agglutination. Although culture is considered as the gold standard for case confirmation in hospitals/clinics, the positivity rate is relatively low due to suboptimal storage and transportation conditions, cultural practice, and/or antibiotic treatment administered before specimens are collected (1).

Bacterial meningitis remains a serious global health problem and a life-threatening condition that requires prompt recognition and treatment. Beyond the newborn period, the most common causes of bacterial meningitis are *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* (1). Specimens that do not yield any culture can still be analyzed by molecular methods that can be applied on DNA extracted from clinical samples, typically, blood and CSF (1).

Molecular detection is by way of the PCR technology. In PCR, the method does not require live or intact cells, and is a valuable tool for detecting bacterial pathogens from clinical specimens where bacteria die or lyse easily due to inappropriate storage conditions or prior antibiotic treatment (2). Real Time PCR (RT-PCR) combines amplification and detection in one step through the use of fluorescent dyes. The PCR strategy typically employed to detect the causative agent(s) in a suspected case of bacterial meningitis is to first run each of the species-specific assays concurrently on the metagenomic DNA (mDNA) extracted from the clinical specimens. The appropriate serogroup/serotype specific assays should then be run on any positive specimen.

Multiplex RT-PCR (mRT-qPCR) refers to the simultaneous amplification of multiple target regions in a sample using different pairs of primers (2). It allows for development of multiplex assays for detection of several genes in the same reaction mix by using specific probes with different fluorescent dye labels, and mRT-qPCR assays are available for detection of *N. meningitidis, H. influenzae,* and *S. pneumoniae* in a single reaction (1).

Species-specific RT-PCR assays have been developed for use on DNA extracted from clinical specimens, typically, blood and CSF and from bacterial isolates. A RT-PCR assay which targets the Cu and Zn superoxide dismutase gene, *sodC,* to detect all meningococci, regard-

less of encapsulation status, has been developed and validated. The *sodC* assay detects encapsulated meningococci but it is also useful for detecting non-groupable meningococci that do not contain an insert *ctrA* (3). Serogroup-specific RT-PCR assay for *N. meningitidis* captures the major disease-causing serogroups which include A, B, C, Y, and W135 (4). The gene *syn* for capsule biosynthesis (5) is used for genotyping for serogroups B (*synD*), C (*synE*), Y (*synF*) and W135 (*synG*). The *sacB* gene is targeted for serogroup A and the *xcbA* gene, which most likely encodes the capsule polymerase, is targeted for serogroup X (6,7).

The *hpd* gene encodes protein D, a highly conserved, surface-exposed lipoprotein that is present in all encapsulated and non-encapsulated *H. influenzae* (8,9). Validated *hpd* RT-PCR assay is capable of detecting all six serotypes (a-f) and non-typeable (HiNT) *H. influenzae* with high sensitivity and specificity (10). For *H. influenzae* serotype-specific RT-PCR assay, the genes are named *acs, bcs*, etc, for "a capsule synthesis", "b capsule synthesis" etc. The genes targeted for RT-PCR assays specific to each serotype are as follows: *acsB* (Hia), *bcsB* (Hib), *ccsD* (Hic), *dcsE* (Hid), *ecsH* (Hie), and *bexD* (Hif).

The RT-PCR assay for detection of *S. pneumoniae* using a specific segment of the autolysin gene (*lytA*), is highly conserved with the species and has been shown that this assay best separates *S. pneumoniae* from the genotypically similar *S. mitis*, *S. oralis*, and *S. pseudopneumoniae* (11). A number of RT-PCR assays for serotyping *S. pneumoniae* are recommended for determining serotypes from clinical specimens when DNA may be present in low amounts and insufficient for conventional multiplex PCR serotyping (1).

The objective of this research was to utilize RT-PCR techniques to detect and characterize three major bacterial causes of cerebrospinal meningitis (CSM); *N. meningitidis, H. influenzae,* and *S. pneumoniae*, in parts of northern Nigeria using metagenomic DNA (mDNA) extracted directly from CSF.

Materials and method:

Study settings

The study sites were Federal Capital Territory (FCT): National Hospital, Abuja, and all District/General Hospitals in the FCT (Asokoro, Wuse, Maitama, Garki, Gwarinpa, Bwari, Kubwa, Kuje, and Nyanya); and some States in Northern Nigeria (Kebbi, Plateau, Sokoto and Zamfara States) during outbreak seasons of February – May 2017 and January – April, 2018.

Study design:

This was a cross-sectional study of hospitalized patients with suspected cerebrospinal meningitis. The study work flow is shown in Fig 1.

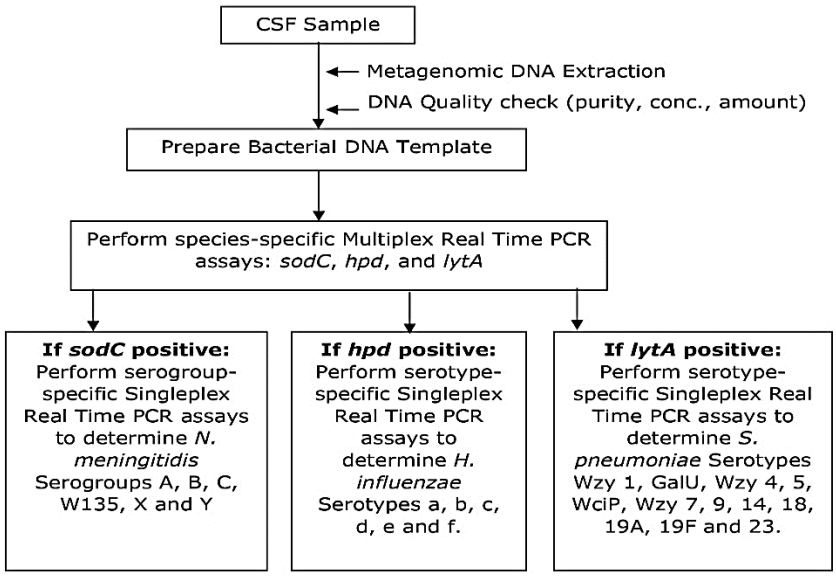

Fig 1: Study workflow

Subjects

All hospitalized patients (all ages and gender) with clinical symptoms of meningitis as reviewed by the attending physician who gave informed consent were included in the study. Patients who did not give informed consent and sites that did not grant approval were excluded from the study.

Sample size and sampling method

The sample size was determined using the Cochran formula (12) for calculating simple proportion; $n_o=z^2pq/e^2$, where 'n_o' is the minimum required sample size, 'z' is the selected critical value of desired confidence level at 95% (standard value of 1.96), 'p' is the estimated proportion of an attribute that is present in the population (estimated prevalence of meningitis in Zamfara State of 13.7% (13), 'q' is 1-p and 'e' is the desired level of precision (margin of error at 5%; standard value of 0.05). Therefore, the estimated sample size was 181.7 which was adjusted to 210 samples after calculating for 10% attrition. The subjects were recruited consecutively until the sample size was attained.

Ethical consideration

Ethical approvals were obtained from the Health Research Ethics Committees of National Hospital, Abuja (NHA/EC/034/2015**)**, Federal Capital Development Authority Health Services (FHREC/2017/01/27/03-04-17), Kebbi State Ministry of Health (MOH/KSREC/VOL.1/56 /No101.3/2015), Plateau State Ministry of Health (MOH/MIS/202/VOL.T/X,2017), Sokoto State Ministry of Health (SMH/1580/V.IV,2017), and Zamfara State Ministry of Health (ZSH REC/ 02/03/2017). A letter of introduction from the Nigeria Centre for Disease Control (NCDC), Federal Ministry of Health (Ref. MH/2768/ S.162/III) was obtained to cover for all outbreak sites in the country. Written informed consent for storage and future use of unused sample, and sample material and data transfer agreement were also obtained.

Laboratory analysis

Collection and transportation of CSF specimens, metagenomic DNA extraction, metagenomic DNA extract quality check as well as amount of mDNA present in CSF specimens were done as previously reported by Peletiri et al., (14).

i. Metagenomic DNA (sample requirement)

Bacterial mDNA was extracted directly from CSF samples using spin column method as previously described (14) and mDNA was stored at -20^{O}C until time for testing. DNA quantity and quality were checked fluorometrically using Qubit 3.0 Fluorometer. DNA concentration in ng/µL (as measured by Qubit Fluorometer 3.0) as DNA yield = DNA concentration x eluted volume (60µL) per 200µL of CSF and amount of DNA = DNA concentration x 5µL per qPCR reaction.

ii. Multiplex RT-PCR for *N. meningitidis, H. influenzae,* and *S. pneumoniae:*

A multiplex RT-PCR using Taqman probe chemistry that detects *sodC* gene for *N. meningitidis, hpd* gene for *H. influenzae,* and *lytA* gene for *S. pneumoniae* simultaneously in a single reaction tube using three different fluorescent dyes; FAM, HEX and Cy3 reporter dyes and Blackhole Quencher (BHQ), was developed using published primer and probe sequences (1,38). The American Type Culture Collection (ATCC) bacterial control strains (CultiControl™ Freeze-Dried Bacterial Strains) produced by Liofilchem® S.R.L., via scozia, Zona Industriale, 64026 Roseto Degli Abruzzi (TE), Italy, were procured through a supplier, Hospitest Laboratory Ltd, Lagos, Nigeria. The assay format used is as shown in Table 1.

Table 1: Target genes for preparing NHS primer probe mix

Target gene	Oligonucleotide name	5' to 3' nucleotide sequence	Amplicon size (bp)
sodC	F351	GCA CAC TTA GGT GAT TTA CCT GCA T	127
	R478	CCA CCC GTG TGG ATC ATA ATA GA	
	Pb387	(FAM)-CAT GAT GGC ACA GCA ACA AAT CCT GTT T–BHQ1	
hpd	F729	AGA TTG GAA AGA AAC ACA AGA AAA AGA	113
	R819	CAC CAT CGGCAT ATT TAA CCA CT	
	Pbr762ibb	(HEX)-AAA CAT CCA ATC GTA ATT ATA G-(BHQ1)	
lytA	F373	ACG CAA TCT AGC AGA TGA AGC A	51
	R424	TCG TGC GTT TTA ATT CCA GCT	
	Pb400	(Cy3)-TGC CGA AAA CGC TTG ATA CAG GGA G-(BHQ1)	

NHS = *Neisseria/Haemophilus/Streptococcus*

All primers and probes were synthesized by Eurofins, Germany, and supplied in lyophilized form. Primers and probes were first reconstituted to 100 µM following the manufacturer's instructions and working concentrations of 10µM prepared using 1xTE buffer as diluent. The 10µM primers and probes are used in preparing the mRT-qPCR primer probe mix.

The mRT-qPCR primer probe mix was aliquoted into 3 tubes and stored at –80^{O}C until use. When required, each aliquot was thawed in a heating block set at 37^{O}C and mixed well by gentle vortex before use. Other reagents and materials include probe master mix (Promega, UK); ABI OneStep Plus RT-PCR System (Thermo Fischer, UK); P10, P100 and P1000 pipettes and tips; ABI 96 well qPCR plate; Quality Control DNA samples [DNA from *N*. *meningitidis* (ATCC® No. 13090™), *H*. *influenzae* (ATCC® No. 10211™), *S*. *pneumoniae* (ATCC® No. 49619)]; nuclease free water; 4-point serially diluted pooled DNA standards (A, B, C, and D); Thermal seal for PCR plate; and refrigerated centrifuge with plate holder (Heraeus, UK) were used in setting up reactions.

Multiplex reaction set up and real time amplification

The worksheet was created according to the number of samples to be tested. The ABI 96 well plate placed into a plate holder on ice pack rack. Into each well, 12.5µL of probe Master Mix was dispensed and 7.5µL of the mRT-qPCR primer–probe mix was added. 5µL of DNA sample or ATCC bacterial strains or QC or NTC was then added into appropriate well. The No Template Control (NTC) was the DNA sample of *Escherichia coli*. Elution buffer was used as Negative Control. The plates were sealed with thermal seal and centrifuged at 1000 rpm for 1 minute in refrigerated centrifuge at 5^{O}C. The plates were then placed into the plate holder in the ABI RT-PCR machine. The manufacturer's instruction was followed for setting up template run for mRT-qPCR.

TaqMan chemistry and standard mode, with absolute quantification were selected. The channels for FAM, Hex, and Cy3 as reporters and BHQ as non-fluorescent quencher were equally selected. The standard values for A to D: 10^6 - 10^2 copies/mL entered appropriately. The thermal profile used was TaqMan chemistry at 50^{O}C for 2 min, 1 cycle; 95^{O}C for 10 min, 1 cycle; 95^{O}C for 15 sec, 45 cycles; 60^{O}C for 1 min. Run was started and saved accordingly.

Result analysis, recoding and interpretation

After the run, the slope of the calibration curve, the PCR efficiency, y-interval and plateau were checked. The slope of the reaction ranged from -3.30 to -3.60; the y-intercept should be < 40. Cycle threshold (Ct) value < 36 was positive for the species; Ct value undetected or > 40 was negative; Ct value between 36–40 was equivocal and was retested with higher concentration of DNA.

iii. Singleplex RT-PCR for *N*. *meningitidis, H. influenzae,* and *S. pneumoniae*

All positive samples at mRT-qPCR run were further analyzed appropriately using singleplex PCR for *N*. *meningitidis, H. influenzae, or S. pneumoniae* accordingly. All the samples positive for *N*. *meningitidis* at mRT-qPCR level were subjected to singleplex RT-PCR for typing of sero-markers of *N*. *meningitidis* based on FAM dye as a reporter and Blackhole Quenchers (BHQ). The assay detects six sero-markers as shown in Table 2 (primers, probes and targets). All the samples positive for *H. influenzae* at mRT-qPCR level were subjected to singleplex RT-PCR for typing of sero-markers of *H. influenzae* based on SYBR chemistry (intercalating dye). The assay detects six sero-markers as shown in Table 3 (primers and targets). All samples positive for *S. pneumoniae* at mRT-qPCR level were subjected to singleplex RT-PCR for typing of sero-markers of *S. pneumoniae* based on SYBR chemistry (intercalating dye). The assay detects twelve sero-markers as shown in Table 4 (primers and targets).

Singleplex reaction set up and real time amplification

The worksheet was created according to the number of samples to be tested. The ABI 96 well plate placed into a plate holder on ice pack rack. For *N. meningitidis,* into each well, 10µL of 2x Probe Master Mix was dispensed and 7µL of appropriate primer-probe mix was added. 3µL of DNA sample or ATCC bacterial strains or QC or NTC was then added into appropriate well. For *H. influenzae* and *S. pneumoniae,* 10µL of 2x Ampigene Green Master mix was dispensed and 7µL of appropriate primer was added. 3µL of DNA sample or ATCC bacterial strains or QC or NTC was then added into appropriate well. All other steps were as stated above under multiple reaction set up, based on specific standard operating procedures (SOPs).

Table 2: Target genes for preparing six sero-markers of *Neisseria meningitidis*

Target gene	Primer/probe name	Sequence (5' to 3')
Nm A	F2531	AAAATTCAATGGGTATATCACGAAGA
sacB	R2624	ATATGGTGCAAGCTGGTTTCAATAG
	Pb2591i	FAM-CTAAAAG''T"AGGAAGGGCACTTTGTGGCATAAT-BHQ
Nm B	F737	GCTACCCCATTTCAGATGATTTGT
synD	R882	ACCAGCCGAGGGTTTATTTCTAC
	Pb839i	FAM-AAGAGATGGGYAACAAC" T"ATGTAATGCTTTATTT-BHQ
Nm C	F478	CCCTGAGTATGCGAAAAAAATT
synE	R551	TGCTAATCCCGCCTGAATG
	Pb495i	FAM-TTTCAATGC"T"AATGAATACCACCGTTTTTTTGC-BHQ
Nm W135	F857	TATTTATGGAAGGCATGGTGTATG
synG	R964	TTGCCATTCCAGAAATATCACC
	Pb907i	FAM-AAATATGGAGCGAATGATTACAGTAACTATAATGAA-BHQ
Nm X	F173	TGTCCCCAACCGTTTATTGG
xcbB	R237	TGCTGCTATCATAGCCGCC
	Pb196	FAM-TGTTGCCCACATGAATGGCGG-BHQ
Nm Y	F787	TCCGAGCAGGAAATTTATGAGAATAC
synF	R929	TTGCTAAAATCATTCGCTCCATAT
	Pb10099i	FAM-TATGGTG" T"ACGATATCCCTATCCTTGCCTATAAT-BHQ

Table 3: Target genes for preparing six sero-markers of *Haemophilus influenzae*

Target gene	Primer name	Sequence (5' to 3')
Hi a	F261	GGT CTG CGG TGT CCT GTG T
acsB	R427	CCG GTC ATC TTT TAT GCT CCA A
Hi b	F192	TGA TGC ATT GAA AGA AGG TGT AAT TT
bcsB	R359	TGA TGC ATT GAA AGA AGG TGT AAT TT
Hi C	F7667	CAT TGG TGA TGG TTC AGT TAT TGG
ccsD	R7784	TAC AGC ATT CAG CAA TAA TGG G
Hi D	F2211	CCT AAA ATA CGG ACC TAG TGC AC
dcsE	R2255	CCG ATG AGA CCA AGT ATG GTT A
Hi e	F1523	ACT AAA ATA TGG CCC AAA CCC AC
ecsH	R1589	CCG ATG AGC CCA AGT ATG ATG A
Hi f	F7164	CCC TGA AAA GCG TTG ACT TTG
bexD	R7313	CCA ACT TCA GGA CCA AGT CAT TC

Results:

Of the 210 subjects, 129 (61.4%) were males comprising 104 (49.5%) children (<15 years of age) and 25 (11.9%) adults while the females were 81 (38.5%) comprising 66 (31.4%) children (<15 years of age) and 15 (7.1%) adults. Of the 210 CSF samples analyzed bacteriologically, 94 (44.8%) were Gram stain positive, while 17 (8.1%) were culture positive. Multiplex RT-PCR confirmed 45 (21.4%) of the 94 Gram stain positive samples as shown in Table 5.

Of the 17 culture positive samples, multiplex/singleplex RT-PCR confirmed 14 cases (82.4%). Of these 17 bacterial isolates, 5 (29.4%) showed same bacterial serogroup or serotype, 9 (52.9%) showed different bacterial serogroups including 3 (17.6%) that showed co-infection at PCR as against the single bacteria reported in culture, while 3 (17.6%) were PCR negative, as shown in Table 6.

Table 4: Target genes for preparing twelve sero-markers of *Streptococcus pneumoniae*

Target gene	Primer/probe name	Sequence (5' to 3')
Wzy 1	1-F	CTC TAT AGA ATG GAG TAT ATA AAC TAT GGT TA
	1-R	CCA AAG AAA ATA CTA ACA TTA TCA CAA TAT TGG C
GalU	3-F	ATG GTG TGA TTT CTC CTA GAT TGG AAA GTA G
	3-R	CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G
Wzy 4	4-F	CTT CTC CAA TTG CTT ACC AAG TGC AAT AAC G
	4-R	GCC CAC TCC TGT TAA AAT CCT ACC CGC ATT G
Wzy 5	5-F	ATA CCT ACA CAA CTT CTG ATT ATG CCT TTG TG
	5-R	GCT CGA TAA ACA TAA TCA ATA TTT GAA AAA GTA TG
WciP	6-F	AAT TTG TAT TTT ATT CAT GCC TAT ATC TGG
	6-R	TTA GCG GAG ATA ATT TAA AAT GAT GAC TA
Wzy 7	7-F	TCC AAA CTA TTA CAG TGG GAA TTA CGG
	7-R	ATA GGA ATT GAG ATT GCC AAA GCG AC
Wzy 9	9-F	GGG TTC AAA GTC AGA CAG TGA ATC TTA A
	9-R	CCA TGA ATG AAA TCA ACA TTG TCA GTA GC
Wzy 14	14-F	GAA ATG TTA CTT GGC GCA GGT GTC AGA ATT
	14-R	GCC AAT ACT TCT TAG TCT CTC AGA TGA AT
Wzy 18	18-F	CTT AAT AGC TCT CAT TAT TCT TTT TTT AAG CC
	18-R	TTA TCT GTA AAC CAT ATC AGC ATC TGA AAC
Wzy 19A	19A-F	GAG AGA TTC ATA ATC TTG CAC TTA GCC A
	19A-R	CAT AAT AGC TAC AAA TGA CTC ATC GCC
Wzy 19F	19F-F	GTT AAG ATT GCT GAT CGA TTA ATT GAT ATC C
	19F-R	GTA ATA TGT CTT TAG GGC GTT TAT GGC GAT AG
Wzy 23	23-F	GTA ACA GTT GTC GTA GAG GGA ATT GGC TTT TC
	23-R	CAC AAC ACC TAA CAC TCG ATG GCT ATA TGATTC

Table 5: Comparison of Gram stain and multiplex real-time PCR

Diagnostic test		**Multiplex RT-PCR**		Total (%)
		Positive (%)	Negative (%)	
Gram reaction	Positive (%)	45 (21.4)	49 (23.3)	94 (44.8)
	Negative (%)	43 (20.5)	73 (34.8)	116 (55.2)
		88 (41.9)	122 (58.1)	210 (100)

One hundred and eighty (85.7%) of the 210 CSF samples from which mDNA was extracted had concentrations of $\geq$ 0.005 ng/µL. The amounts of mDNA present in the 180 samples were; DNA concentration range of 0.03-50.5 ng/µL, DNA yield of 1.8 - 3030 µg and DNA amount of 0.15-252.5 ng/µL; while 30/210 (14.3%) had DNA concentrations less than 0.005 ng/µL (Table 7).

Multiplex RT-PCR was positive in 88 (41.9%) of the 210 CSF samples analyzed with mDNA concentration between 0.005-50.5ng/µL, for either one or co-infection of any two of the three bacteria; *N*. *meningitidis, H. influenzae,* and *S. pneumoniae*. Of these 88, 59 (67.1%) were *N*. *meningitidis,* 2 (2.3%) were *H*. *influenzae,* 3 (3.4%) were *S*. *pneumoniae,* 15 (17%) had co-infection of *N*. *meningitidis* with *H*. *influenzae* and 9 (10.2%) had co-infection of *H. influenzae* and *S*. *pneumoniae*, as shown in Table 8.

Table 6: Comparison of culture results and RT-PCR results from isolated bacterial DNA and CSF metagenomic DNA by multiplex and singleplex PCR assay

S/N	Culture results (bacteria isolated)	RT-PCR results			
		Bacterial DNA (from culture isolates)		Metagenomic DNA (from CSF samples)	
		Multiplex RT-PCR	Species-specific singleplex RT-PCR	Multiplex RT-PCR	Species-specific singleplex RT-PCR
1	Nm A	Nm	Nm B	Nm	Nm B
2	Nm Y	Nm	Nm Y	Nm	Nm Y
3	Nm C	Nm	Nm C	Nm	Nm C
4	Nm C	Nm	Nm C	Nm	Nm C
5	Nm C	Nm	Nm B	Nm	Nm B
6	Nm C	Nm	Nm B	Nm	Nm B
7	Nm C	Negative	Negative	Negative	Negative
8	Nm C	Negative	Negative	Negative	Negative
9	Nm C	Nm	Nm Y	Nm	Nm Y
10	Nm C	Nm	Nm C	Nm	Nm C
11	Nm C	Nm	Nm B	Nm	Nm B
12	Nm C	Nm	Nm Y	Nm	Nm Y
13	Hi B	Hi	Hi B	Hi	Hi B
14	Nm C	Nm	Nm Y	Nm, Hi	Nm Y, Hi f
15	Nm C	Nm	Nm Y	Nm, Hi	Nm Y, Hi b
16	Nm C	Hi	Hi f	Hi, Sp	Hi f, Sp
17	Nm C	Negative	Negative	Negative	Negative

Nm = *Neisseria meningitidis;* Hi = *Haemophilus influenzae*; Sp = *Streptococcus pneumoniae;* Negative = No bacteria detected. Of the 17 culture positives, mRT-PCR confirmed 14 cases; 5 were same bacteria while 9 had varied results. Culture confirmed by PCR is 14 (6.7%) of the 210 CSF samples analyzed

Table 7: Amount of mDNA present in extracted CSF samples for molecular analysis

No of samples	DNA concentration (ng/µL)	DNA yield (µg)	Amount of DNA (ng)
180	0.03 – 50.5	1.8 – 3030	0.15 – 252.5
30	< 0.005	--------	----------

Table 8: Detection rate of *Neisseria meningitidis, Haemophilus influenzae,* and *Streptococcus pneumoniae* by mRT-PCR

Bacteria detected	Number	Percentage
Neisseria meningitidis (only)	59	67.1
Haemophilus influenzae (only)	2	2.3
Streptococcus pneumoniae (only)	3	3.4
N. meningitidis + *H. influenzae* (co-infection)	15	17.0
H. influenzae + *S. pneumoniae* (co-infection)	9	10.2
Total	88	100

Table 9: Singleplex real time PCR result for *Neisseria meningitidis* serogroups

Serogroup detected	Gene	Number	Percentage
A	*sacB*	10	13.5
B	*synD*	17	23.0
C	*synE*	6	8.1
W135	*synG*	6	8.1
X	*xcbB*	4	5.4
Y	*synF*	24	32.4
*Negative (non-groupable)		7	9.5
Total		74	100

*All seven samples were *N. meningitidis* serogroup negative (non-groupable) after repeated PCR run with concentrated DNA extract

Table 10: Singleplex real time PCR result for *Haemophilus influenzae* serotypes

Serotype detected	Gene	Number	Percentage
a	*acsB*	1	3.8
b	*bcsB*	15	57.7
c	*ccsD*	1	3.8
e	*ecsH*	3	11.5
f	*bexD*	6	23.1
Total		26	100

Table 11: Singleplex real time PCR result for *streptococcus pneumoniae* serotypes

Serotype detected	Gene	Number	Percentage
1	*Wzy1*	1	8.3
4	*Wzy4*	4	33.3
5	*Wzy5*	6	50.0
9	*Wzy9*	1	8.3
Total		12	100

Of the 74 *N. meningitidis* detected by mRT-PCR (59 single infections and 15 from mixed infection with *H. influenzae),* the sero-groups are as shown in Table 9. Of the 26 *H. influenzae* detected by PCR (2 single infection, 15 co-infection with *N. meningitidis* and 9 co-infection with *S. pneumoniae),* the serotypes are as shown in Table 10. Of the 12 *S. pneumoniae* detected by PCR (3 single infections and 9 co-infection with *H. influenzae),* the serotypes are as shown in Table 11.

Discussion:

Our CSF culture results as confirmed by mRT-PCR showed 6.7% recovery rate. Previous reports from Nigeria using culture of CSF samples had shown bacterial isolation rates ranging from 1.7% to 16.7% (15-20). Results from culture with latex agglutination had rate of 2.9-15.3% (21,22) while results from latex agglutination only was between 9.1-20.4% (23). Therefore, our findings are in tune with

earlier reports. The detection rate of mRT-PCR using metagenomic DNA extract from CSF samples was 88 (41.9%) including 15 (17.0%) that had mixed infections (co-infection) of *N. meningitidis* with *H. influenzae*, and 9 (10.2%) with co-infection of *H. influenzae* and *S. pneumoniae*. No co-infection of *N. meningitidis* and *S. pneumoniae* was encountered. Though, reports on mixed bacterial meningitis in Nigeria are unavailable, literature search revealed such reports from elsewhere such as Kansas City Missouri USA, and Taiwan, with varying prevalence rates ranging from 1% to 11.7% (24-28). The mixed infection rate of 17% for *N. meningitidis* and *H. influenzae*, and 10.2% for *H. influenzae* and *S. pneumoniae* in our study agrees with reports elsewhere (27,28), though, our findings are a little higher for the *N. meningitidis* and *H. influenzae* association.

Mixed infections are reported to be rare but can occur with certain predisposing conditions (26,29) such as trauma, tumours or infections like acute paranasal sinusitis that may extend directly to the meninges. Mixed infections can also arise by direct entry of organisms via fistulae or as a result of a ruptured brain abscess (30). However, we do not know if these factors were present in our study population. This being that, apart from the fact that we least expected mixed infections in the course of this research, we did not follow up by visiting the various sites to have access to patients' hospital folders and identify other underlying issues including ascertaining whether they had these predisposing factors.

The use of PCR assay has been reported by several authors to be more sensitive than phenotypic methods. Favaro and colleagues (31) had previously reported a higher detection rate with PCR. In their study that analyzed 296 samples, 59 (19.9%) were positive by CSF culture and/or molecular assays, 46 (15.5%) by both CSF culture and PCR assay, while 13 (4.4%) by RT-PCR, but negative for the traditional assays, implying that only 46 (15.5%) of the samples were culture positive while 59 (19.9%) were PCR positive (31). In another study (32) of 451 CSF specimens analyzed, 80 (17.7%) had culture isolation of one of the three pathogens (40 *S. pneumoniae,* 36 *N. meningitidis,* and 4 *H. influenzae),* and 113 (25.1%) were positive by RT-PCR assay (51 *S. pneumoniae,* 57 *N. meningitidis,* and 5 *H. influenzae*). Our findings corroborated these previous reports that showed higher detection rate with RT-PCR method.

While comparing multiplex PCR and other traditional methods for diagnosis of acute bacterial meningitis, Yahia and Balach (33) reported that of 110 CSF samples analyzed, Gram stain for any bacteria was positive in 32 cases (29.1%) including five pathogens (*S. pneumoniae, H. influenzae* type b*, N. meningitidis,* Group B streptococcus and *Listeria monocytogenes)* in 11 cases (10%). Bacteria culture was positive in 38 cases (34.5%) including the five pathogens in 8 cases (7.2%). Multiplex PCR was positive in 60 cases (54.5%); 50 cases of acute bacterial meningitis were diagnosed by multiplex PCR while both Gram stain and bacterial culture missed the diagnosis. In another study, Kwambana-Adams and colleagues (19) reported PCR detection of pathogens in 95 (46%) of 208 CSF samples analyzed as against 9% culture recovery rate. Our finding of 41.9% detection rate by mRT-PCR method and 6.7% culture recovery are in agreement with these previous reports.

We were also able to identify the circulating serogroups and serotypes alike of the three bacteria under study using the metagenomic approach. Of the 74 *N. meningitidis* positive on PCR*,* 10 (13.5%) were serogroup A (*sacB*), 17 (23%) serogroup B (*synD*), 6 (8.1%) serogroup C (*synE*), 6 (8.1%) serogroup W135 (*synG*), 4 (5.4%) serogroup X (*xcbB*), 24 (32.4%) serogroup Y (*synF*), and 7 (9.5%) were non-groupable (serogroup negative). Over the years (2008 to 2018), several authors (a total of twelve) have implicated *N. meningitidis* serogroups A, B, C, and W135 as responsible for CSM in Northern Nigeria. Seven of these authors (19, 39-44) reported *N. meningitidis* serogroup C as the only offending pathogen. Only one author, Mado and colleagues (13), identified *N. meningitidis* serogroup A. Two authors (45,46) implicated both *N. meningitidis* serogroups A and C; Bassey and colleagues (23) reported *N. meningitidis* serogroups A, C, and W135 while Ujah et al., (47) reported four serogroups (*N. meningitidis* serogroups A, B, C, and W135) as the circulating serogroups in Jigawa State, Northern Nigeria.

None of the twelve authors reported ever encountering *N. meningitidis* serogroups X and Y in their research studies. Therefore, our results which identified *N. meningitidis* serogroup X (5.4%) and *N. meningitidis* serogroup Y (32.4%) as invasive serogroups are baseline data for reference. We reason that the recovery of these two serogroups (Nm X and Nm Y) which hitherto had not been reported in Northern Nigeria could be attributable to the use of metagenomic protocol in this research. It is noteworthy to mention here that previous reports implicating non-groupable (serogroup negative) strains of *N. meningitidis* in CSM cases in our study area are not available in the

literature. Therefore, our finding in the course of this research of 9.5% non-groupable or serogroup negative strains is another baseline data for reference. Hence, we ascribe that non-groupable (serogroup negative) strains of *N. meningitidis* should also be considered in outbreaks in Nigeria.

Of the 26 *H. influenzae* serotypes positive for PCR (2 single infections, 15 co-infection with *N. meningitidis* and 9 co-infection with *S. pneumoniae)*, serotype a *(acsB*) was 1 (3.8%), serotype b (*bcsB*) was 15 (57.7%), serotype c (*ccsD*) was 1 (3.8%), serotype e (*ecsH*) was 3 (11.5%) and serotype f (*bexD*) was 6 (23.1%). A previous report (48) implicated Hib as the only serotype encountered. Therefore, our finding in this current study is well revealing because of the six serotypes tested, only serotype d (*dcsE*) was not encountered. Of the 12 *S. pneumoniae* serotypes identified by PCR (3 single infections and 9 co-infection with *H. influenzae),* serotype 1 (*Wxy1*) was 1 (8.3%), serotype 4 (*Wxy4*) was 4 (33.3%), serotype 5 (*Wxy5*) was 6 (50%), and serotype 9 (*Wxy9*) was 1 (8.3%). Previous reports had shown varying results, while Kwambana-Adams and colleagues (19) implicated *S. pneumoniae* serotypes 1, 5 and 19F, Suleiman et al., (49) implicated *S. pneumoniae* serotypes 6, 19, and 20. Of the twelve serotypes tested in our study, only four positive serotypes were encountered.

Serotyping is of great importance for the development of vaccination strategies (34). The identification of the serogroup or serotype responsible for an outbreak is crucial for its containment (35). The serogroup or serotype data can impact policy decisions regarding selection of appropriate vaccination programs and can improve epidemiological data (36). Some types of meningitis such as those associated with *N. meningitidis, H. influenzae,* and *S. pneumoniae* can be prevented by immunization (37).

Conclusion:

Our results showed that multiplex RT-PCR using mDNA extracted directly from CSF samples offered a higher detection rate than culture for major bacterial pathogens involved in meningitis in northern parts of Nigeria. Multiplex RT-PCR is a highly sensitive, specific, fast and reliable method of detecting bacterial pathogens implicated in CSM. Utilizing both mRT-PCR and singleplex RT-PCR enabled serogrouping/serotyping of positive cases with a shorter turn-around time. These outcomes will speed patient care and management when applied in national responses to meningitis outbreaks.

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Authors' contributions:

PIC and IEI conceived, and led the design and writing of the manuscript. PIC and NE were responsible in the performance of molecular diagnostic (PCR) activities. PIC, IEI, AGM, and NE were responsible for the final editing of the manuscript.

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