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Research article

Metagenome mining divulges virulent and multidrug resistant *Pseudomonas aeruginosa* ST242 and *Klebsiella michiganensis* ST*1b23 coinfecting an 8-month-old meningitis infant under ICU in Kampala, Uganda, East Africa

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ABSTRACT

Pediatric meningitis is a global health problem, with insufficiently known pathogens and antibiotic resistance (AMR) especially in low-resource settings. Here, we sought to uncover the virulence and AMR of pathogens associated with infant meningitis, treated with ceftriaxone, in Kampala, Uganda. In a bid to isolate *Klebsiella oxytoca*, we coincidentally recovered a co-culture and challenged it with antibiotic susceptibility testing (AST) on a panel of 14 antibiotics. We then combined metagenome binning with antiSMASH/PRISM genome mining to unveil the pathogens, their virulence and molecular targets in relation to meningitis. From AST, the co-culture exhibited resistance to multiple aminoglycosides, fluroquinolones, and β-lactams, including ceftriaxone, the inherently used drug. From metagenome annotation, the first bin was identified as *Pseudomonas aeruginosa* ST242 and the second as *Klebsiella michiganensis* ST*1b23. Among others, *P. aeruginosa* ST242 virulence factors include type 3 and type 6 secretion systems, biofilm, and nonribosomal peptides (NRPs) of the pyoverdine synthase operon, targeting claudin-5, a component of the tight junctions of the blood-brain barrier (BBB). The *P. aeruginosa* ST242 genome portrays intrinsic resistance to beta-lactamases (*blaOXA-50* and *blaPAO*), aminoglycosides [*aph(3′)-IIb)*], fluoroquinolones (*crpP*), tetracycline (*tmexD2*) and fosfomycin (*fosA*), among others. From *K. michiganensis* ST*1b23 genome mining we elucidated a yersiniabactin-related metabolite, targeting the ligand-binding domain of the human polymeric immunoglobulin receptor (pIgR) and other components of the BBB. The *K. michiganensis* ST*1b23 chromosome encodes the genes *blaOXY*-1 and *OqxA/B,* conferring resistance to β-lactams, fluoroquinolones, and trimethoprim

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respectively. Notably, we found one frameshift and nine substitution mutations conferring carbapenem and cephalosporin resistance mechanisms. Overall, our findings strongly suggest coinfection and a mechanistic crosstalk between *P. aeruginosa* ST242 and *K. michiganensis* ST*1b23 in the pathogenesis of meningitis in this case. Importantly, ceftriaxone could be an inappropriate treatment choice for these pathogens. Hence, serious surveillance and experimental studies will improve the management of pediatric meningitis.

1. Introduction

Bacterial meningitis represents one of the central nervous system (CNS) infections accounting for major causes of infant morbidity and mortality accounting for global rates of more than 10 % [1]. Common pathogens afflicting neonates, infants and children include *Streptococcus* spp, *Escherichia coli* (*E. coli*), *Neisseria meningitidis* (*N. meningitidis*) and *Klebsiella pneumoniae* (*K. pneumoniae*) [2–4]. Although, frequently associated with cystic fibrosis (CF), *Pseudomonas aeruginosa* (*P. aeruginosa*) is known to afflict immunocompromised and pediatric populations and is emerging to be associated with meningitis [5,6]. Owing to its extensive drug resistance, *P. aeruginosa* is listed as one of the priority pathogens of the World Health Organization (WHO), and its ubiquitous distribution from environmental to clinical niches poses critical public health concerns [7]. The most common virulence factors of *P. aeruginosa* include adhesion components such as pili and flagella, antiphagocytic structures including the capsule, iron acquisition (e.g. pyoverdine), secretion systems (T3SS, T6SS) and hydrolytic enzymes (phospholipases and lysins) [8]. While these virulence factors are predominant, the pathogen is equipped with genes conferring resistance to multiple conventional antibiotics, spanning from first to third generation beta lactams, fluoroquinolones, aminoglycosides and macrolides, among others [9,10]. The predominant mechanisms of resistance include drug metabolism, target modification, efflux and biofilm formation, to mention a few [5,9]. Whereas *P. aeruginosa* strains are genomically more studied from the developed world, counterparts from low-resource settings including sub-Saharan Africa are by far unexplored. This creates a paucity of locally established scientific knowledge about the clinical and public health relevance of *P. aeruginosa*, which hampers precision of medical diagnostics and therapeutics.

On the other hand, members of the *Klebsiella oxytoca* (*K. oxytoca*) complex have been commonly associated with the urinary tract infection, respiratory tract infection, blood stream, gastrointestinal tract infections and most commonly, antibiotic-associated hemorrhagic colitis [11,12]. The *K. oxytoca* complex consists of multiple species, including *K. oxytoca, K. grimontii, K. huaxiensis, K. pasteurii, K. spallanzanii* and *Raoultella ornithinolytica*, and *K. michiganensis,* which are considerably characterized [12]. Since *K. pneumoniae* is also a WHO's priority pathogen, most studies have been directed towards understanding of *K. pneumoniae*, leaving a scarcity of information on the health risks of *K. oxytoca*. However, a significant body of knowledge has emerged revealing shared genetic determinants of virulence and antimicrobial resistance between strains of *K. pneumoniae* and those of the *K. oxytoca* complex [13,14]. While reports have explained the involvement of *K. pneumoniae* in meningitis [15,16], the role of *K. oxytoca* complex including *K. michiganensis* in the central nervous system (CNS) infection such as meningitis remains elusive. In our recent work [17], we reported novel sequence types (STs) from various clinical specimens obtained from the National Hospital of Uganda, suggesting unnoticed circulating clones of *K. oxytoca* within the clinics. In this work, we sought to unveil the virulence and antimicrobial resistance repertoire of *K. michiganensis* and, serendipitously, *P. aeruginosa* to establish their implication in meningitis from an eight-month-infant, admitted to the intensive care unit (ICU) of the National Referral Hospital of Uganda. Here, we report metagenomic assembled genomes (MAGs) of *P. aeruginosa* and *K. michiganensis* from a blood sample of the patient, and we unveil complex virulence and multidrug resistance machineries underlying their role as pathogens implicated in systemic infection and meningitis in the infant.

2. Materials and methods

2.1. Patient's brief information

An 8-month-old male infant was admitted to the ICU for advanced respiratory support and other device supports including urinary catheter, nasogastric tube, central line, peripheral line and tracheal tubes. Presenting with neck rigidity, not feeding for three days, the patient was awake, had high grade fever, lethargy and was excessively crying. Diagnosed with sepsis and meningitis, the patient had no prior ICU history or any antibiotic regimen. The patient was thereby treated with an intravenous (IV) injection of ceftriaxone (1g).

2.2. Sample collection and partial isolation of bacterial pathogens

Blood sample was collected from the patient median cubital vein with a sterile syringe and processed as described in our recent work [18]. The sample was suspended in 5 ml Buffered Peptone Water (BPW), vortexed and incubated overnight at 37 °C in aerobic conditions. Thereafter, 10 μl of the sample was inoculated onto an in-house selective MacConkey agar with 305 μg/ml of ampicillin, followed by aerobic incubation at 35–37 °C for 24 h and identification from colony morphological characteristics of the colonies. Then biochemical tests; including urease citrate, hydrogen sulfide gas and indole motility tests were used in a semi-solid agar. Thereafter, mucoid indole positive rod-shaped colonies were selected for further experiments and downstream analysis.

2.3. Antibiotic susceptibility testing, DNA extraction and metagenome sequencing

Antibiotic susceptibly testing (AST) was performed using a panel of 14 antibiotics (Table 1) using the Kirby-Bauer disk diffusion. The results were interpreted according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2022). Multi-drug resistance (MDR) was defined according to Magiorakos et al. [19], i.e. non-susceptibility to at least one in three or more antimicrobial classes.

We then used a Zymo Research miniprep kit to extract DNA, adhering to the manufacturer's guidelines. Then a metagenomic DNA library was constructed using a NextEra DNA XT prep kit by random fragmentation of the DNA sample, followed by 5′ and 3′ adapter ligation. These adapter-ligated fragments were then PCR-amplified using random primers and gel-purified, followed by quality check on a chip of the Agilent Technologies 2100 Bioanalyzer using a DNA 1000. The library was then subjected to shotgun whole metagenome sequencing using the Illumina Novaseq 6000 platform, which generates an average of 151 bp read sequence length.

2.4. Sequence read quality control, assembly and metagenome classification

Adapters and low-quality reads were filtered with FastQC (v0.11.9) and trimmed with Trimmomatic (v0.39) based on minimum sequence quality score of 10 and minimum read sequence length of 50 [20], to remain within the Illumina Novaseq 6000 sequencing depth. Initial individual genome assembly approach was performed with Unicycler (v0.4.8), with minimum contig length of 300 bp. High-quality sequence reads were first analyzed based on the 16S rRNA metabarcoding using the SILVA database (v138) within the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) platform https://www.bv-brc.org/, followed by microbiome profiling using the Kraken 2 standard database (v2. 0.8-beta), at the confidence interval of 0.1. To recover individual genomes from the metagenome sample, we reassembled the reads with the MEGAHIT software package (v1.2.9), followed by supervised binning algorithms to reconstruct the bacterial metagenome-assembled genomes (MAGs), then validation the CheckM package (v1.1.6) [21]. For downstream characterization of the capsular polysaccharides, plasmid and sequence type of each (MAG), we subjected bins to the Pathogenwatch platform [22] available at https://pathogen.watch/. This integrates PlasmidFinder (https://cge.food.dtu.dk/services/PlasmidFinder/) with multilocus sequence typing (MLST, https://pubmlst.org/organisms/plasmid-mlst) and Kleborate (https://kleborate.erc.monash.edu/) to characterize the species, capsular genotype, plasmid and sequence type of each bin (MAG). To further infer phylogenetic grouping of each MAG, we used the TYGs web (https://tygs.dsmz.de/) whole-proteome-based GBDP distances to generate corresponding phylogenies with FastME v2.1.6.1 [23].

2.5. Virulence factor, serotyping and genomic antimicrobial resistance analysis

Following recovery of each MAG, we performed comprehensive genome analysis using the BV-BRC to gain an insight into relative species, specialty genes including virulence and antimicrobial resistance genes from various reference databases including virulence factor database (VFDB), comprehensive antibiotic resistance database (CARD), Victors, NDARO and Transporters, among others [24] We then analyzed specific virulence factors using the virulence factor analyzer (VFanalyzer) package under the VFDB [25]. Finally, we applied ResFinder (v4.5, http://genepi.food.dtu.dk/resfinder) to detect acquired and intrinsic antimicrobial resistance mechanisms of each MAG.

2.6. Metagenome-assembled genome mining and prediction of molecular targets of putative toxins

We salvaged the antibiotics and secondary metabolite analysis shell' (antiSMASH, v7.0) [26] and PRediction Informatics for

Table 1
Antibiotic susceptibility results from the co-culture plate containing *P. aeruginosa* strain RSM9152B-1 and *K. michiganensis* strain RSM9152B-2: R = Resistant, S= Susceptible, I= Indeterminate AST results. The third column in the right column represents zone of inhibition (ZI).

Antibiotic	AST result	ZI
Amoxicillin/Clavulanate	R	14
Cefuroxime	R	6
Ceftriaxone	R	6
Ampicillin	R	6
Cefepime	R	12
Ceftazidime	R	8
Chloramphenicol	R	6
Cotrimoxazole	R	6
Ciprofloxacin	R	16
Imipenem	S	28
Amikacin	I	14
Gentamicin	R	6
Fosfomycin	R	22
Piperacillin/tazobactam	I	17

Secondary Metabolomes (PRISM, v4.0) algorithm [27], to characterize the biosynthetic gene clusters (BGCs) and generate scaffold structures of putative polyketide (PK) and/or nonribosomal peptide (NRP) toxins, as described in our recent work [28]. We then retrieved the most relevant potential targets from the Protein Databank (PDB) https://www.rcsb.org/, and processed them using BIOVIA Discovery Studio (https://www.3ds.com/products/biovia/discovery-studio) as described recently [18] before docking with Autodock Vina under CB-Dock 2 [29]. Docking parameters were set as follows: mode = 2, energy range = 5 and exhaustiveness = 10.

3. Results

3.1. Phenotypic results from antibiotic susceptibility testing

From AST (Table 1), the culture showed resistance to beta lactams (amoxicillin/clavulanic acid. cefuroxime, ceftriaxone, cefepime,

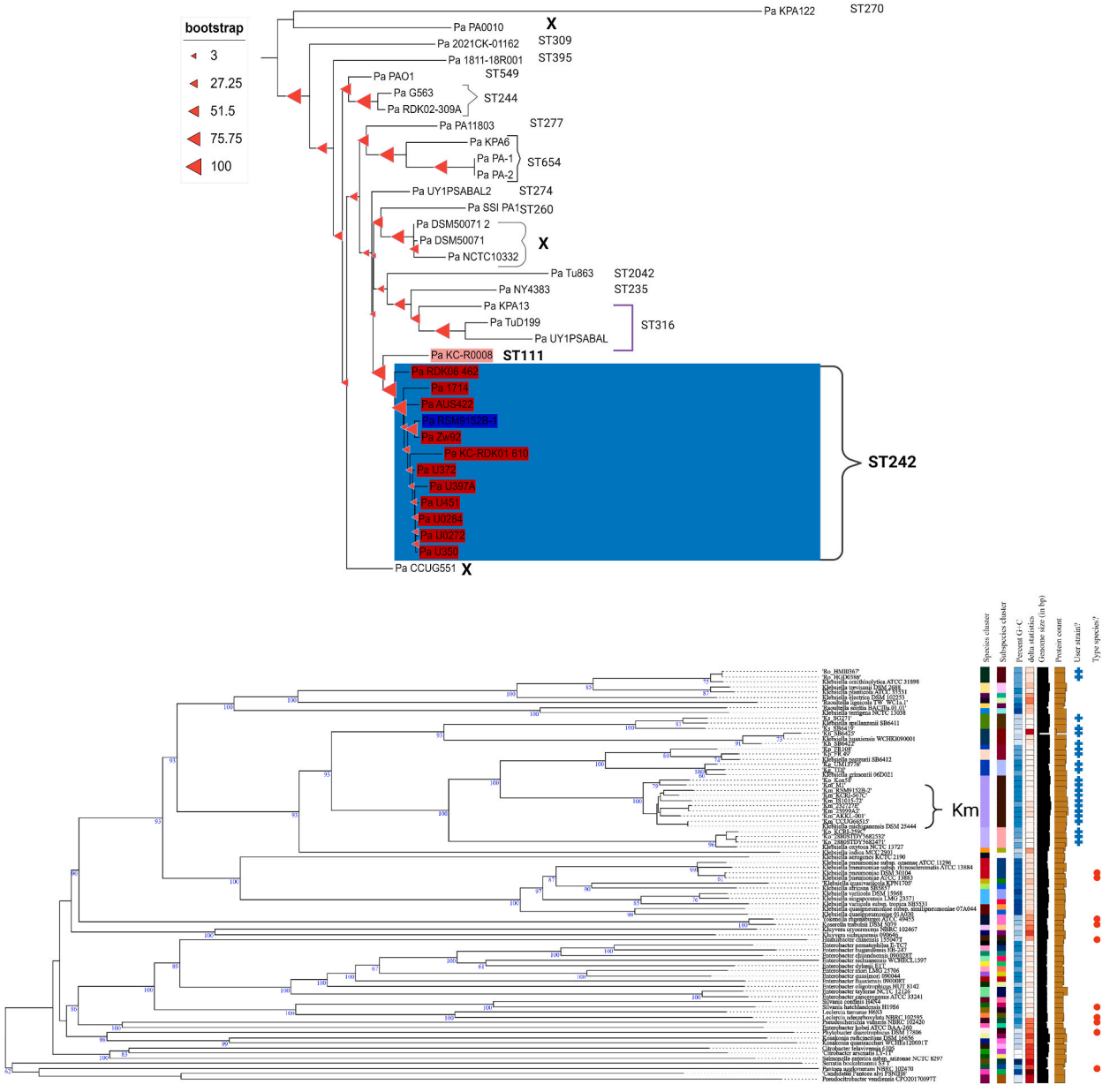

Fig. 1. Phylogrouping from TYGS tree inferred with FastME 2.1.6.1 [23] from whole-proteome-based GBDP distances. A) *Pseudomonas aeruginosa* RSM9152B-1 position in cluster with the ST242, is highlighted in blue. B) *Klebsiella michiganensis* RSM9152B-2. The branch lengths are scaled via GBDP distance formula d5. Branch values are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 52.2 %. The tree was midpoint-rooted.

ampicillin, and ceftazidime), aminoglycosides (gentamycin and fosfomycin), chloramphenicol and ciprofloxacin. While indeterminate results were observed with amikacin and piperacillin/tazobactam, the culture was susceptible to imipenem.

3.2. Metagenome assembly and phylogrouping

The Illumina Novaseq 6000 platform generated 20,169,956 paired-end raw reads, which after quality control, were decreased to 20,145,504. While our expectation was that the colonies contain a pure culture of *Klebsiella oxytoca/Klebsiella michiganensis*, our initial Unicycler assembly resulted in a total of 105 contigs, equivalent to 11,580,645 bp and GC content of 61.61 %, which is approximately twice as much as the genome of *Klebsiella michiganensis*. Surprised by this overwhelmingly large genome size, we suspected contamination, and therefore, considered a metagenomic workflow to identify every possible species. From 16S rRNA SILVA-metabarcoding, 72 % of the sequence reads were classified into the genus *Klebsiella*, followed by 17 % classified into the genus *Pseudomonas* (Supplementary Fig. S1A). Here we noted that most of the sequences represented the abundance of *Klebsiella.* However, when we performed microbiome profiling from the Kraken 2 database, we found that 81 % of the sequence reads were annotated as *Pseudomonas aeruginosa*, with only 11 % classified as *Klebsiella michiganensis* (Supplementary Fig. S1B). From MEGAHIT assembly, we recovered 282 contigs, comprising a metagenome of 12,617,060 bp (GC = 61.19 %), which upon binning resulted in two bins (MAGs) classified as *Pseudomonas aeruginosa* and *Klebsiella michiganensis* with complete genomes. (Supplementary Table S1).

While both bins were found to be of high quality (each consisting of 68 contigs), the first bin (*P. aeruginosa*) had a genome size of 6,384,627 bp whereas the second (*K. michiganensis*) comprised of 6,175,780 bp. Both MAGs were deposited into the National Center for Bioinformatics and Biotechnology Information (NCBI), Assembly database (accession nos. GCA_037202035.1, and GCA_037202025.1). Multilocus sequence typing of bin 1 included seven genes namely acetyl-coenzyme A synthetase (*acsA*), shikimate dehydrogenase (NADP (+)) (*aroE*), DNA mismatch repair protein MutL (*mutL*), NADH-quinone oxidoreductase subunit D (*nuoD*), phosphoenolpyruvate synthase (*ppsA*) and anthranilate synthase component 1 (*trpE*). From the database https://pubmlst.org/paeruginosa/, this MAG was classified as *P. aeruginosa* ST242, which best matches with *P. aeruginosa* strain 1714 (GenBank accession no. RRBZ01000078.1), sequenced from a patient with cystic fibrosis in the United Kingdoms, in addition to clinical sequence types isolated from China [9]. Shown in Fig. 1A, other isolates of the same ST242 include those reported as multidrug resistant strains from Tanzania (RDK06-462, RDK01610, KC-RDK01610), French (Zw92), and Australia (AU422, and U372, U397A), to mention a few. All the closest relative strains were isolated from patients with cystic fibrosis.

On the other hand, MLST of bin 2 involved seven housekeeping genes glyceraldehyde-3-phosphate dehydrogenase A (*gapA*), translation initiation factor IF-2 (*infB*), malate dehydrogenate (*mdh*), glucose 6-phosphate isomerase (*pgi*), outer membrane porin E (*PhoE*), RNA polymerase subunit B (*rpoB*) and protein ton B (*tonB*). From this analysis, the *K. michiganensis* MAG was found to possess a novel allele for the gene *pgi (*Table 2*),* hence, a novel sequence type (ST*1b23). The ST*1b23 is related to a clone from our recently reported clinical *K. oxytoca* isolates [17]. From Kaptive analysis we found that *K. michiganensis* RSM9152B-2 carries a serologically uncharacterized capsule locus type KL161 and an unknown O-polysaccharide antigen (O1/O2v1), While the capsular polysaccharide KL161 matches with those of hypervirulent *K. pneumoniae* strains from other parts [30,31] the type O1/O2v1 has been recently reported among clinical *K. pneumoniae* strains from health-care settings in Uganda [32]. Plasmid typing from the PlasmidFinder database https://cge.cbs.dtu.dk/services/PlasmidFinder/and the corresponding plasmid types revealed two plasmids (Supplementary Table S1). Detailed genotyping with Kleborate revealed missing genes KL161_02_*cpsACP*, KL161_12_*rmlD*, KL161_13_*wcuP*, and KL161_14, KL161_15 KL161_16_*wzx*. Further lipopolysaccharide O antigen characterization with Kleborate revealed that the unknown O locus (O1/O2v1) lacks the genes O1/O2v1_03_*wbbM*, O1/O2v1_05_*wbbN*, and O1/O2v1_07_*kfoC*. Phylogenetically (Fig. 1B), the closest relative strains of *K. michiganensis* RSM9152B-2 include the hospital *K. michiganensis* strain KCRI-567C (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_900407255.1/) isolated from wound swab in Tanzania, and IS1015-72 (https://www.ncbi.nlm.nih.

Table 2
Multilocus sequence typing results *K. michiganensis* RSM9152B-2. Inference is based on percentage identity from alignment against reference alleles of housekeeping genes against the pubMLST database. Alleles with less than 100 % identity is denoted by an asterisk (*).

Locus	Identity	Coverage	Alignment Length	Allele Length	Gaps	Allele
gapA	100	100	450	450	0	gapA_3
infB	100	100	318	318	0	infB_41
mdh	100	100	477	477	0	mdh_17
pgi	99.7685	100	432	432	0	pgi_9*
phoE	100	100	420	420	0	phoE_20
rpoB	100	100	501	501	0	rpoB_31
tonB	100	100	405	405	0	tonB_25

gov/assembly/GCF_003402095.1/) from environmental samples in Sweden, among others.

3.3. Genome-wide virulence and AMR profile of P. aeruginosa RSM9152B-1

PathogenFinder revealed that RSM9152B-1 is a human pathogen (p = 0.755). The top five match protein sequences include the two-component sensor *PprA* (NCBI accession no. CAW29427), probable transcriptional regulator (CAW28296), usher CupA3 (CAW27924), probable siderophore receptor (AAG04754) and putative penicillin acylase (CAW28158), all with BLASTp hit identity of 100 %. Our VFanalyzer results revealed a complex virulence machinery of *P. aeruginosa* RSM9152B-1, marked by adherence (flagella, undefined LPS O-antigen, type IV pili and Type IV pili twitching motility related proteins). Other virulence factors include antimicrobial activity (phenazines biosynthesis), antiphagocytosis alginate biosynthesis and regulation), biosurfactant (rhamnolipid biosynthesis), enzymes (hemolytic and nonhemolytic phospholipase C isoforms). The iron uptake system of *P. aeruginosa* comprises of pyochelin and pyoverdine and their receptors), the biofilm and quorum sensing machinery comprising of the acylhomoserine lactone synthase (*hdtS*), N-(3-oxo-dodecanoyl)-L-homoserine lactone QS system (*lasI* and *lasR)* genes), N-(butanoyl)-L-homoserine lactone QS system (*rhlR*), which have been implicated in host mitochondrial damage and immune evasion mechanisms [33]. This biofilm formation facet also includes alginate and exopolysaccharide (EPS) biosynthetic operons. Here, we revealed that the alginate operon consists of nine (9) structural genes described in hypervirulent strains including those associated with meningitis [34]. Further, we found that *P. aeruginosa* ST242 possesses a T6SS machinery, comprising of the coupling protein (cp) secretion island-1 encoded type VI secretion system (H-T6SS). We also recovered nine open reading frames (ORFs) constituting the type 3 secretion system (T3SS), the injectosome machinery, which entails the TTSS translocated effectors (*exoS*, *exoT* and *exoY*). The latter have been recently shown be expressed by hypervirulent strains of *P. aeruginosa* [8], along with the well described *P. aeruginosa* TTSS components *exsA-E*, *pcr1-4*, *pcrGHRV*, *popBDN*, *pscC-U* [10]. Importantly, we also detected the *hcnABC* operon for production of hydrogen cyanide (HCN), which accounts for protection of the pathogen against oxidative stress [35], suggestively evading reactive oxygen species (ROS)-mediated pathogen killing by polymorphonuclear cells and/or macrophages.

From CARD, the AMR profile RSM9152B-1 was found to be abundantly characterized with efflux pumps (21 genes), accounting for a notable intrinsic MDR mechanism [36]. Presented in Table 3, other resistance mechanisms include target deletion (*gidB*), cell wall charge alteration (*GdpD*, *PgsA*), and target modification via the genes *(APH(3′)-II/APH(3′)-XV*, *CatB* family, *OXA-50* and *PDC* family proteins. ResFinder-annotated AMR genes from RSM9152B-1 (Fig. 2A) include those known for the beta lactams such as amoxicillin

Table 3
Specialty genes representing AMR mechanisms and corresponding genes/genotypes annotated from CARD, retrieved from BV-BRC comprehensive annotation of each MAG/strain.

Strain	AMR Mechanism	Genes
K. michiganensis RSMS9152B-2	Antibiotic activation enzyme	*KatG*
	Antibiotic inactivation enzyme	APH(3′)-I, OXY family
	Antibiotic resistance gene cluster, cassette,or operon	*MarA, MarB, MarR*
	Antibiotic target in susceptible species	*Alr, Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p*
	Antibiotic target protection protein	*BcrC*
	Efflux pump conferring antibiotic resistance	*AcrAB-TolC, AcrAD-TolC, AcrEF-TolC, AcrZ, EmrAB-TolC, EmrD, MacA, MacB, MdfA/Cmr, MdtABC-TolC, MdtL, MdtM, SugE, TolC/OpmH*
	Gene conferring resistance via absence	*gidB*
	Protein altering cell wall charge conferring antibiotic resistance	*GdpD, PgsA*
	Protein modulating permeability to antibiotic	*OprB*
	Regulator modulating expression of antibiotic resistance genes	*AcrAB-TolC, EmrAB-TolC, H-NS, OxyR*
P. aeruginosa RSM9152B-1	Antibiotic inactivation enzyme	*APH(3′)-II/APH(3′)-XV, CatB* family, *OXA-50* family, *PDC* family
	Antibiotic target in susceptible species	Alr, Ddl, dxr, EF-G, EF-Tu, folA, Dfr, folP, gyrA, gyrB, inhA, fabI, Iso-tRNA, kasA, MurA, rho, rpoB, rpoC, S10p, S12p
	Antibiotic target replacement protein	FabG, fabV, HtdX
	Efflux pump conferring antibiotic resistance	*EmrAB-OMF, EmrAB-TolC, MacA, MacB, MdtABC-OMF, MdtABC-TolC, MexAB-OprM, MexCD-OprJ, MexCD-OprJ system, MexEF-OprN, MexEF-OprN system, MexHI-OpmD, MexHI-OpmD system, MexJK-OprM/OpmH, MexPQ-OpmE, MexPQ-OpmE system, MexVW-OprM, MexXY-OMP, TolC/OpmH, TriABC-OpmH*
	Gene conferring resistance via absence	*gidB*
	Protein altering cell wall charge conferring antibiotic resistance	*GdpD, PgsA*
	Protein modulating permeability to antibiotic	*OccD1/OprD, OccD2/OpdC, OccD3/OpdP, OccD4/OpdT, OccD5/OpdI, OccD6/OprQ, OccD7/OpdB, OccD8/OpdJ, OccK1/OpdK, OccK10/OpdN, OccK11/OpdR, OccK2/OpdF, OccK3/OpdO, OccK4/OpdL, OccK5/OpdH, OccK6/OpdQ, OccK7/OpdD, OccK8/OprE, OccK9/OpdG, OprB, OprB family, OprF*
	Regulator modulating expression of antibiotic resistance genes	*OxyR*

ampicillin (*blaOXA-50*), cefepime and ceftazidime (*blaPAO*). We also identified genes conferring resistance to aminoglycosides (*aph (3′)-IIb)*, fluoroquinolone ciprofloxacin *crpP*, fosfomycin *fosA*, and tetracyclines (*tmexD2*)., i.e. tetracycline, doxycycline, minocycline, tigecycline, well characterized from *Raoultella ornithinolytica* [37]. MobileElementFinder results revealed two insertion sequences IS-PA-1 and IS-PA-6, corresponding to fosfomycin resistance.

3.4. Genome-wide virulence and AMR profile of K. michiganensis RSM9152B-2

From PathogenFinder analysis, *K. michiganensis* RSM9152B-2 was confirmed to be a human pathogen (p = 0.865), with top hit proteins including formate C-acetyltransferase 3 (GenBank accession no. ACI07711, % identity 96.17 %), glycerol dehydratase large subunit (accession no. AAV76821, % identity 99.82 %), anthranilate synthase, component II (ACI10550, % identity 97.36 %), 4-hydroxyphenylacetate 3-hydroxylase (BAH61578, % identity 97.69 %) and putative acyl-CoA synthase (BAH63408, % 96.91 %), among others. Virulence factors predicted from VFanalyzer include adherence fimbriae (type I, II, III, IV), antiphagocytosis (capsule), iron uptake systems (enterobactin and yersiniabactin), secretion systems (T6SS-I, II) and serum resistance (LPS rfb locus), which are typical of *Klebsiella* pathogens [38]. Our genomic AMR profile from both Kleborate and ResFinder (Fig. 2B) detected four acquired AMR genes: *aph(3′)-Ia*, *blaOXY-1, OqxA* and *OqxB*. The gene *aph(3′)-Ia* confers resistance to aminoglycosides such as kanamycin, neomycin, lividomycin, and paromomycin while the beta lactamase gene *blaOXY-1*, mediates resistance to amoxicillin, amoxicillin/clavulanic acid, ampicillin, as well as ampicillin/clavulanic acid. In addition, *blaOXY-1* confers resistance to cefotaxime, cefoxitin, ceftazidime, piperacillin, piperacillin/tazobactam, ticarcillin and ceftazidime/avibactam, accounting for ESBL [39]. The two fluoroquinolone resistance genes *OqxA* and *OqxB* confer resistance to ciprofloxacin, nalidixic acid, trimethoprim, and chloramphenicol, well described as multidrug efflux pumps carried by several members of the family Enterobacteriaceae, including *E. coli*, *Salmonella* spp and *K. pneumoniae* [40]. We also found that the genome harbors 10 mutations with single nucleotide polymorphisms (SNPs) and one frameshift mutation for various AMR genes conferring intrinsic resistance cephalosporins and carbapenems (Table 4). In addition, we found chromosomal mutations for the porins *OmpK35* and *OmpK36*, corresponding to genes encoded *K. pneumoniae* strains KT775 (GenBank accession no. AJ011501.1) and C3 (accession no. Z33506.1) respectively and have been to ESBL [41].

3.5. Genome mining-based molecular mechanisms underlying the pathogenesis of meningitis by P. aeruginosa RSMB9152B-1 and K. michiganensis RSM9152B-2

The inflammatory mechanisms underlying meningitis are primarily mediated by the CNS-resident macrophages, the microglia [4,42]. The inflammatory cascade is further complemented by mononuclear leukocytes, along with infiltrating monocytes and macrophages [4]. These reactions are triggered by invasive destruction of the blood brain barrier (BBB) by toxins (ligands) such as adhesins, choline binding protein A (*S. pneumoniae*), outer membrane protein 2 (OmpP2, *H. influenzae*), laminin receptor (LR, *E. coli*) and internalin (*L. monocytogenes)* [43,44]. Among the most common target host receptors for these pathogen ligands include polymeric immunoglobulin receptor (pIgR), cadherin, platelet endothelial cell adhesion molecule (PECAM-1) and mesenchymal-epithelial transition (MET) [44,45]. However, the toxin ligands exerting these complex mechanistic effects are far from understood. In our genome mining of the two strains, we went on to delineate the most predominant BGCs for NRPs to find out if they would portray any relevant toxic potential *in silico*. From RSM9152B-1 antiSMASH analysis we recovered four BGCs corresponding to pyoverdine (NRP), lancadicin (NRP-PK), azetidomonamide A/B (NRP) and azotobactin (NRP). Upon PRISM structural analysis, we recovered 12 BCGs and focused on the first NRP BGC. The latter consists of five open reading frames (ORFs) with sequence length of above 500 amino acids. Although all the five genes were annotated as key enzymes in the NRPS pathway, two ORFs are dedicated to the biosynthetic

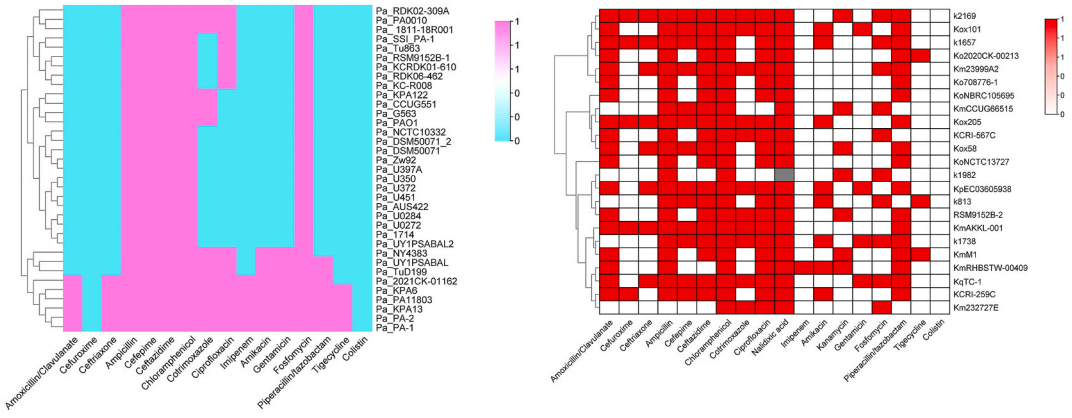

Fig. 2. Comparative multidrug resistance genotypes revealed from ResFinder. A) MDR profile of *P. aeruginosa* RSM9152B-1 against selected strains from Africa and the rest of the world. B) MDR profile of *K. michiganensis* RSM9152B-2 against selected strains from Africa and other parts of the world. The antibiotics selected include those that were used in the AST panel. The bar in the right of each heatmap indicates the presence (1) or absence (0) of a resistance genotype (gene or mutation).

Table 4
Mutations associated with cephalosporin and carbapenem resistance of *K. michiganensis* RSM9152B-2, detected from ResFinder analysis.

Mutation	Nucleotide change	Amino acid change	Phenotype	PMID
p.N49S	aac > tct	n - > s	cephalosporins	25245001
p.A183S	gcg > tcg	a - > s	cephalosporins	25245001
p.A190W - Frameshift	gct > -tgg	a - > w	cephalosporins	25245001
p.L191S	ctg > agc	l - > s	cephalosporins	25245001
p.F198Y	ttc > tac	f - > y	cephalosporins	25245001
p.F207W	ttc > tgg	f - > w	cephalosporins	25245001
p.A217S	gct > tct	a - > s	carbapenem	25245001
p.N218H	aac > cac	n - > h	carbapenem	25245001
p.L228V	ctg > gtt	l - > v	cephalosporins	25245001
p.E232R	gaa > cgg	e − > r	cephalosporins	25245001

assembly process, leading to structure scaffolds (1, 2, 3) as shown in Fig. 3 (A). While one compound (1) is cyclic, the rest two (2,3) are linear. From the VFanalyzer BLASTp search algorithm, we found that ORF1 and ORF2 match with pyoverdine biosynthesis genes (*pvdILDJ*) characterized from various *P. aeruginosa* strains [46], thus suggesting that the resolved structures could be derived from the pyoverdine biosynthetic pathway. When we applied BLASTp against the NCBI GenBank database, we found that the ORF1 corresponds (by 100 % identity) to the amino acid adenylation domain-containing protein (GenBank accession no. HEJ3098995.1) and NRPS (GenBank accession no. RQA33538.1). On the other hand, ORF2 is identical to the nonribosomal peptide synthase/polyketide synthase (NRPS/PKS), WP_225025786.1, by 99.97 %. These two identities confirm that the compounds (1,2,3) in Fig. 3 (A) are derived from the NRPS pathway. While ORF04350 (1266 aa) and ORF04411 (801 aa) were primarily identified as hypothetical proteins, they exhibit a percentage of identity of nearly 50 % to uncharacterized *P. aeruginosa* NRPS sequences (GenBank accession nos. RMS53773.1 and GAA17990.1). Exclusively, the ORF04304 (562 aa) is identical (97.58 %) to the linear gramicidin synthetase sequence (GenBank accession no. HBP5867846.1), suggesting an alternative modification towards versatile NRP structural alternatives.

On the other hand, antiSMASH predicted three BGCs from *K. michiganensis* RSM9152B-2, i.e. yersiniabactin (NRP-PK), O-antigen (saccharide) and viobactin (NRP), from which PRISM unveiled five ORFs (50, 53. 54, 56 and 57) (Fig. 3B), which from VFanalyzer BLASTp, were confirmed to be part of the yersiniabactin biosynthetic pathway. This consists of the efflux pump ATP-binding cassette (ABC) *ybtQ*, yersiniabactin nonribosomal peptide synthetases *irp2* and *irp1*, the thioesterase *ybtT* and the adenylation enzyme *YbtE* well described in expression systems [47].

While the causal association of NRP/PKs in the pathogenesis of meningitis are largely still under scrutiny, a recent study using an avian pathogenic *E. coli* strain in a mouse model showed that that the NRP colibactin contributes to meningitis by disrupting the tight junction protein components claudin-5, occludin and ZO-1 [3]. Inspired by this study, we were curious to find out if the NRPs in our study portray the potential to target the tight junction component receptors. We used the targets claudin-5 (https://www.uniprot.org/uniprotkb/O00501/entry#structure), ligand-binding domain of the human polymeric immunoglobulin receptor (pIgR, https://www.rcsb.org/structure/1XED), platelet endothelial cell adhesion 1 (PECAM-1) D1D2 domain (https://www.rcsb.org/structure/5C14) and the human occludin fragment 413–522 https://www.rcsb.org/structure/1WPA). From molecular docking all the structures 1–4 express affinity for all the receptors tested, suggesting their potential for BBB disruption. Comparatively, compound 2 exhibited the highest affinity (−9.7 kcal/mol) for the target pIgR, followed by compound 3 (−8.2 kcal/mol). Further, we demonstrate that NRPs (1, 2, 3) can bind claudin-5 with affinities comparable to that of colibactin (5) (Fig. 4A–E). Details of binding pockets, binding affinities (kcal/mol), Cavity volume (Å3), and docking sizes (x, y, z) are presented in the Supplementary Table S2.

4. Discussion

Nosocomial infections account for the most prevalent cause of mortality among immunocompromised and pediatric populations in hospital settings globally. Common bacteria associated with meningitis include *N. meningitidis*, nontuberculous mycobacteria, *Staphylococcus* spp, and *S. meningitidis* [48–50]. Other pathogens include *M. tuberculosis*, *L. monocytogenes*, *E. coli*, and *H. influenzae*, with rare *K. pneumoniae* and *P. aeruginosa* [3,5,6,15]. Although our culture and biochemical tests were optimized to isolate *K. oxytoca* (*K. michiganensis*), we serendipitously ended up in a co-culture containing a metagenome of *P. aeruginosa* and *K. michiganensis*. However, we stress that our BSL2 is maintained under highly aseptic conditions throughout. Thus, the findings that each MAG nucleotides account for a complete genome indicate that our working clean bench was not contaminated, rather, both pathogens were originally recovered from the blood sample. These findings are strongly suggestive of co-infection and potential collaboration in AMR and the pathogenesis of pediatric meningitis. Further, we clearly demonstrate that both *P. aeruginosa* and *K. michiganensis* possess virulence factors attributable to the observed patient disease. The possession of secretion system effectors suggests a huge potential for invasion and dissemination of both pathogens from the blood stream into the CNS via BBB disruption. In addition, the extensive biofilm machinery observed in *P. aeruginosa* is indicative of effective colonization, immune survival and alginate or LPS-induced neuroinflammatory reactions [51]. While well described in many bacterial groups, type IV pili have been shown to mediate vascular damage in both humans and animal models of meningitis [52]. Notably from VFanalyzer, the lipopooligosaccharide (LOS) synthesis gene (*rfaF*) matched with that of *N. meningitidis,* characterized and associated with invasive and pathogenic potential of *N. meningitidis.* Thus, annotation of the RSM9152B-1 LOS gene suggests possible involvement of this virulence factor in the pathogenesis of meningitis in this case. Moreover, hemolytic and nonhemolytic phospholipases have been shown to play a crucial role in microbial invasion and host cell

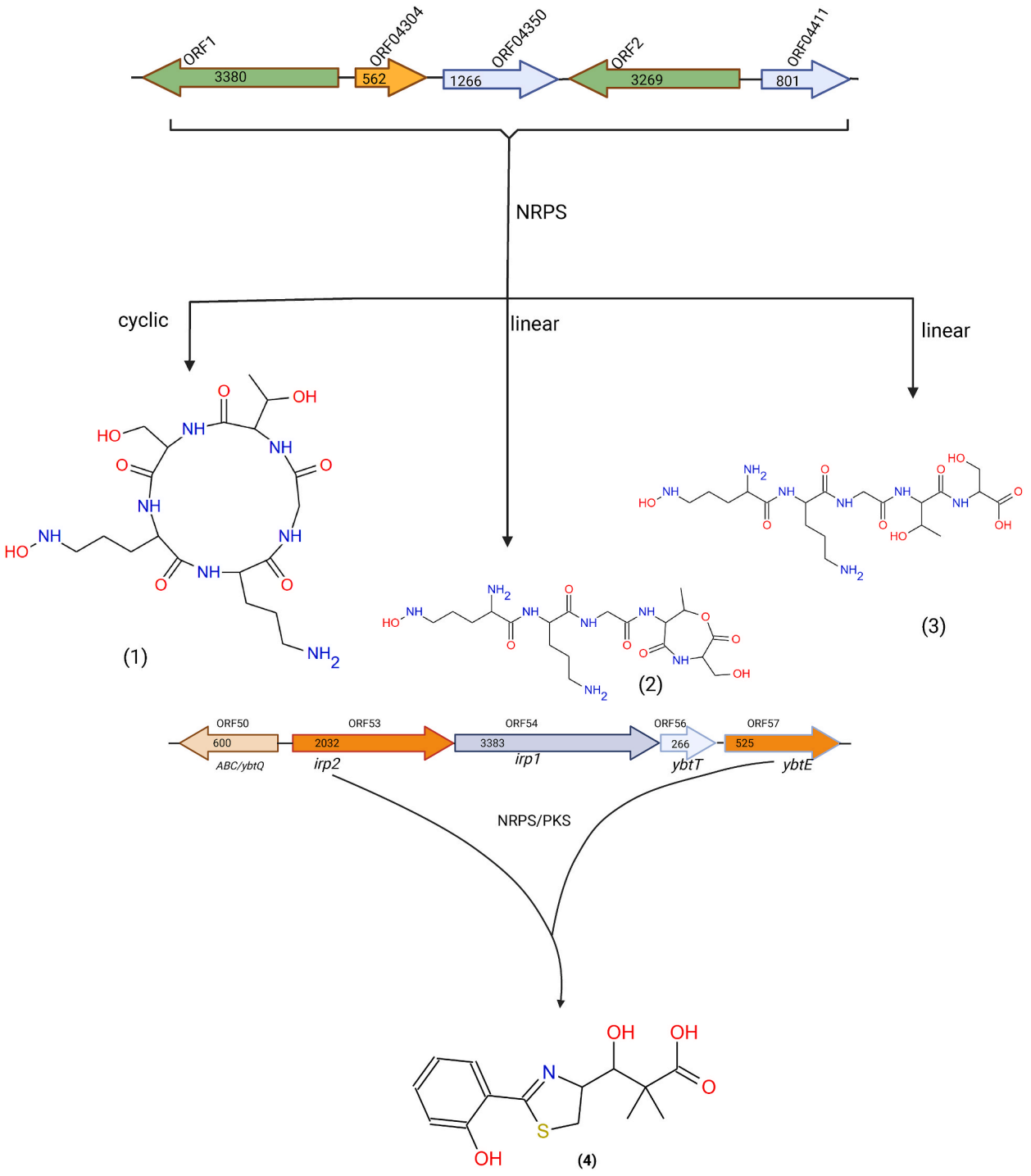

Fig. 3. Biosynthetic gene clusters (BGCs) divulged using PRISM-genome mining. A) NRPs (1, 2, 3, 3) encoded by the first MAG, *P. aeruginosa* RSM9152B-1 genome and B) the yersiniabactin BGC, representing core genes involved in the assembly of a NRP (4) encoded by the *K. michiganensis* RSM9152B-2 genome. In both cases only open reading frames (ORFs) playing role in the core assembly of the NRPs are represented.

damage [53]. Thus, carriage of the enzymes *plcH* and *plcN*, could induce BBB and nerve injury, promoting neuroinflammatory events accounting for the symptoms observed in the current case. Whereas the primary role of enolase is in the glycolytic pathway, it was very recently shown to play role in the translocation of *Streptococcus suis* across the BBB [54], contributing to inflammatory symptoms of meningitis [43,53]. Here, we noted from VFanalyzer, that *P. aeruginosa* possesses the typical *Streptococcal* enolase enzyme, which could account for its potential role in the pathogenesis of meningitis in the current study. Combined with complex secretion systems (T3SS

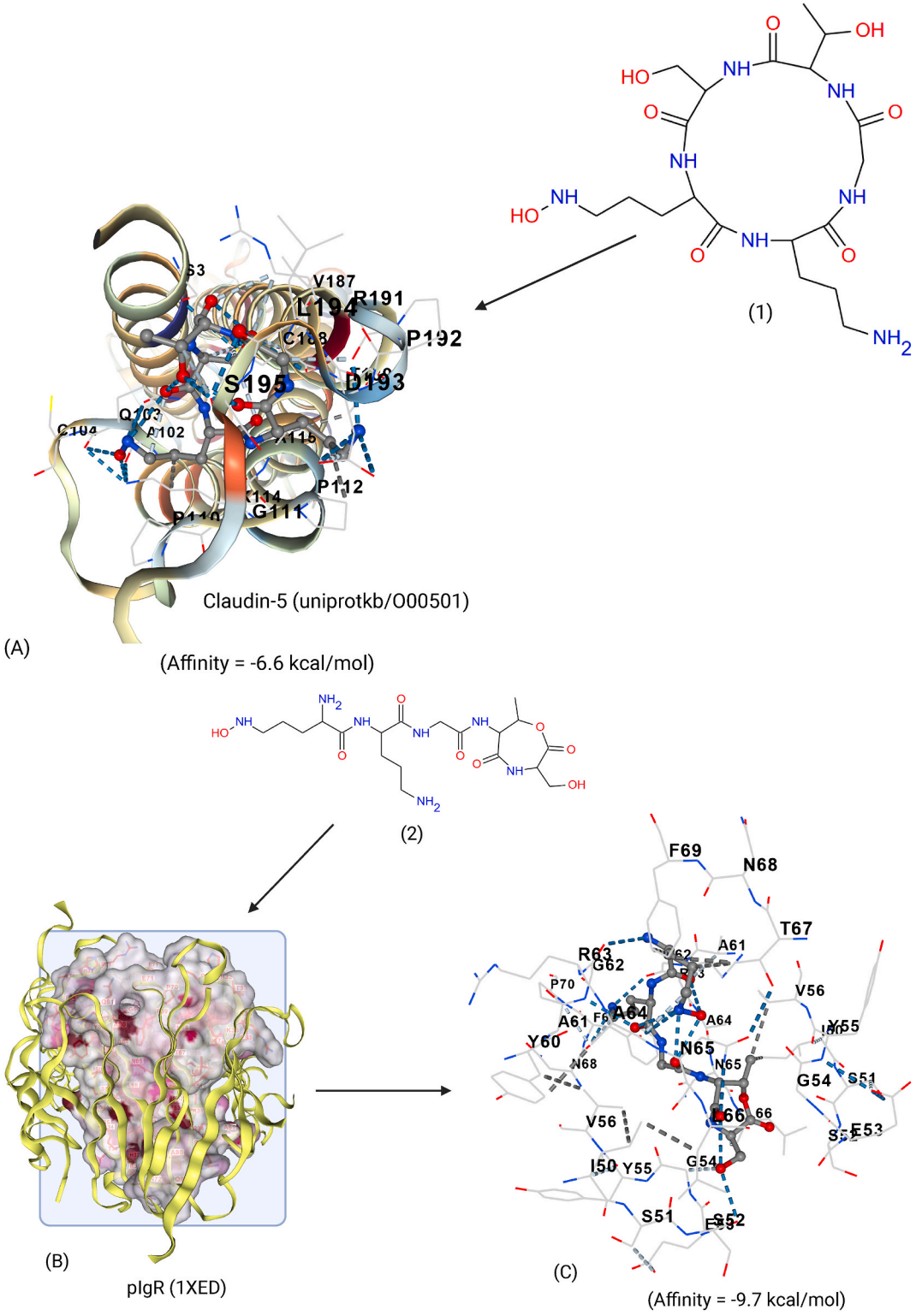

Fig. 4. Molecular docking results showing binding pockets and affinities for selected tight junction targets for NRPs; (A) compound 1 interaction with claudin-5, (B) binding pocket for compound 2 into pIgR, (C) compound 2 interaction with pIgR, (D) compound 3 interaction with PECAM-1, (E) compound 4 interaction with occludin.

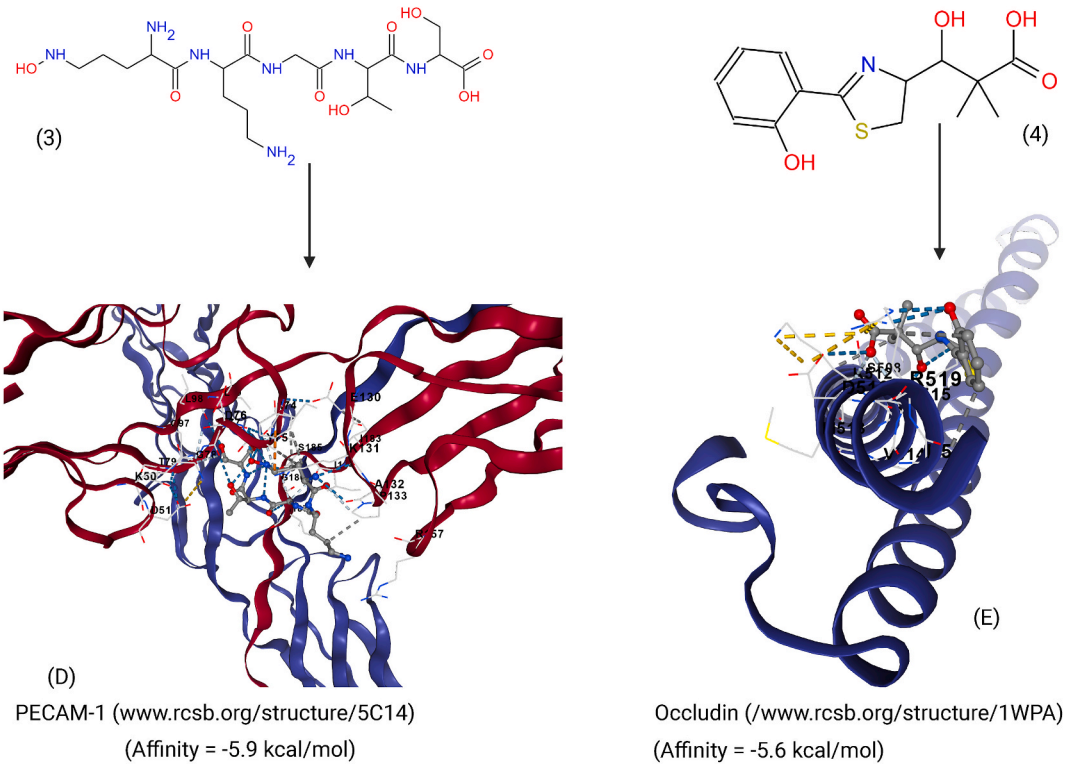

Fig. 4. (*continued*).

and T6SS) effectors, along with the biofilm facets, it is imperative to implicate this pathogen in the pathogenesis of meningitis in this patient.

Mechanistic studies on bacterial meningitis are increasingly associated with the destruction of the tight junctions constituting the BBB [42–44]. In this work, we were curious to gain an insight into the possible involvement of secondary metabolites such as NRPs in the pathogenesis of meningitis. The negative affinity values (e.g. −9.7, −8.6, −6.9 kcal/mol) observed in this study suggest that the ligands can readily interact with their cognate receptors such as claudin, pIgR, PECAM-1 and occludin to dismantle the BBB and enhance the pathogenesis of meningitis [42]. Interestingly, the compounds (1–4) are generated from BGCs of the established virulence machineries from both *Pseudomonas* and *Klebsiella* pathogens [46]. From our findings, we emphasize that toxic effects of NRPs could extend from iron acquisition to host cell protein damage.

While the virulence factors of the *K. oxytoca* complex are considerably explored [13,17], cases of *K. michiganensis* in meningitis are yet to be tapped. However, significant evidence shows that *K. pneumoniae* is implicated in certain meningitis cases [2]. Shared virulence factors such as the capsular KL161 and O- antigen (e.g. O1/O2v) between *K. pneumoniae* and *K. michiganensis* could account for the possible pathogenicity of the RSM9152B-2 strain in the current study. Additionally, virulence factors such as the T6SS and yersiniabactin are crucial for host cell invasion [31], underlying the role of *K. michiganensis* in meningitis observed in this case. These findings strongly highlight the need for comprehensive characterization of the *K. oxytoca* complex from meningitis patients, which could improve our understanding of the bacterial etiology of meningitis, especially in pediatric populations. Most importantly, our encounter of the novel ST*1b23 is an alarm for the existence of unnoticed and uncharacterized pathogens associated with clinical cases with idiopathic etiology. Moreover, combining these findings with our recently reported *K. oxytoca* strains including the same ST*1b23 [17] further underscores the importance of extensive genomic studies to characterize clinical strains from local settings for better risk prediction and disease management of the more unexplored pathogens.

From AMR analysis, our findings have clearly shown that both pathogens possess a high rate of multidrug resistance, with ESBLs accounting for the outstanding AMR phenotypes. One of the notorious features of *P. aeruginosa* is its intrinsic mechanisms of MDR, which is predominantly determined efflux pumps and chromosomal SNPs [10,55]. Here, the predominance of efflux pumps such as *MexAB-OprM*, *mexBCYVW*, *MexCD-Opr* and *Omp35/36* could account for the MDR repertoire observed in this work. Given a coinfection status, it is critical to speculate that the observed results resistance to aminoglycosides and cephalosporins, could have been contributed by crosstalk AMR mechanisms between the two pathogens. This strongly highlights the importance of rigorous application of combined diagnostic methods to clearly delineate the most relevant pathogens afflicting the patient in question. On the other hand, whereas *P. aeruginosa* was found genetically susceptible to trimethoprim, the observed AST cotrimoxazole (trimethoprim/sulfamethoxazole) resistance in Fig. 1 could be contributed by the *OqxA/B* genes carried by the genome of *K. michiganensis* RSM9152B-1. This suggests supplemental resistance of the pathogens under collaboration. In addition, combining AST and genomic analysis, our findings

suggest that neither of these pathogens could be treated with most beta lactams such as ampicillin, amoxicillin, amoxicillin/clavulanate, cefotaxime, cefoxitin, ceftazidime, piperacillin, piperacillin/tazobactam, ticarcillin and ceftazidime/avibactam. Although we could not find a specific resistance gene for ceftriaxone, our phenotype AST results showed a resistance, implying that the administered IV could not effectively eliminate the pathogens. This could be attributed to the effect of cephalosporin resistance genotypes such as the outer membrane protein Omp*35/36*, beta lactamases *blaOXY*-1-7 and *OXA-50*, as well as the multidrug resistance proteins *mex*BCYVW from both pathogens. Evidence shows that these genes confer resistance to a wide spectrum of cephalosporins including cephalexin, cefuroxime, cefixime, ceftazidime and ceftriaxone, among others underlying ESBLs from *Klebsiella* and *Pseudomonas* spp [10,31]. Moreover, it is crucial to note that both pathogens exhibited resistance to ciprofloxacin and multiple aminoglycosides such as kanamycin and neomycin. These findings indicate that appropriate options for treatment are missing unless established datasets are generated from locally established surveillance reports.

5. Conclusion

From metagenomic assembly, our study suggests coinfection, a pathogenic crosstalk and synergistic multidrug resistance between *P. aeruginosa* ST242 and *K. michiganensis* ST*1b23, which could account for pathogenesis of meningitis in the infant. In the meantime, we establish that ciprofloxacin, kanamycin, chloramphenicol and several cephalosporins including ceftriaxone (although used in this case), are not the best options for these pathogens. For the first time from East Africa, our study contributes to new insights into the risks of pediatric meningitis and recommends rigorous surveillance projects for better management of infections among pediatric populations with meningitis and comorbidities.

CRediT authorship contribution statement

Reuben S. Maghembe: Writing – original draft, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Maximilian A.K. Magulye:** Writing – review & editing, Validation, Software, Methodology, Data curation, Conceptualization. **Abdalah Makaranga:** Writing – review & editing, Validation, Software, Data curation. **Gideon Nsubuga:** Writing – review & editing, Methodology, Investigation. **Simon Sekyanzi:** Writing – review & editing, Methodology, Investigation, Funding acquisition. **Edward A. Moto:** Writing – review & editing, Visualization, Validation, Resources. **Savannah Mwesigwa:** Writing – review & editing, Visualization, Supervision, Conceptualization. **Eric Katagirya:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Conceptualization.

Ethical approval

Our project was approved by the Higher Degree and Graduate Research Ethics Committee (HDREC) of Makerere University College of Health Sciences (MakCHS): Approval number SBS-2021-47. The permission of study was obtained from Mulago National Referral Hospital Management, Mulago ICU leadership and Department of Medical Microbiology. Laboratory numbers but not names were used for identification of samples and consent from the parents by proxy were obtained before sample collection. Collected samples were coded and secured with restricted access.

Data availability statement

Both metagenome-assembled genomes were deposited into the National Center for Bioinformatics and Biotechnology Information (NCBI) Assembly database (accession nos. GCA_037202035.1, and GCA_037202025.1) and can be accessed under the BioProject link https://www.ncbi.nlm.nih.gov/bioproject/1070028/.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39455.

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