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The Bacterial and Viral Complexity of Postinfectious Hydrocephalus in Uganda

A full list of authors and affiliations appears at the end of the article.

Abstract

‡To whom correspondence should be addressed: Steven J. Schiff, W311 Millennium Science Complex, The Pennsylvania State University, University Park, PA 16802 USA, 814-863-4210, steven.j.schiff@gmail.com.

*,§Contributed Equally

†Deceased

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Competing interests: None.

Data and materials availability: The assembled genome of Paenibacillus thiaminolyticus Mbale was deposited in GenBank with Accession CP041404. Sequencing data for bacterial 16S DNA, in silico host-depleted mRNA, and VirCapSeq data, along with sample metadata are available at the NCBI archive under project ID PRJNA605220. There was a Materials Transfer Agreement between the provider CURE Children's Hospital of Uganda and recipient Penn State University, where Penn State University retains rights to the derivatives, *Paenibacillus thiaminolyticus* Mbale and for the Mbarara University of Science and Technology, CURE Children's Hospital of Uganda, and Penn State University to own any new products discovered through the use of the materials, *Paenibacillus thiaminolyticus* Mbale. There was a Materials Transfer Agreement between the provider Penn State University and recipient Columbia University, for Penn State University to retain ownership of the materials, *Paenibacillus thiaminolyticus* Mbale, and joint ownership of modifications. These MTAs are archived at Science Translational Medicine. All custom code utilized in this study will be made available to investigators upon request. All data associated with this study are in the paper or supplementary materials.

List of the Supplementary Materials:

Postinfectious hydrocephalus (PIH), often following neonatal sepsis, is the most common cause of pediatric hydrocephalus world-wide, yet the microbial pathogens remain uncharacterized. Characterization of the microbial agents causing PIH would lead to an emphasis shift from surgical palliation of cerebrospinal fluid (CSF) accumulation to prevention. We examined blood and CSF from 100 consecutive cases of PIH and control cases of non-postinfectious hydrocephalus (NPIH) in infants in Uganda. Genomic testing was undertaken for bacterial, fungal, and parasitic DNA, DNA and RNA sequencing for viral identification, and extensive bacterial culture recovery. We uncovered a major contribution to PIH from *Paenibacillus*, upon a background of frequent cytomegalovirus (CMV) infection. CMV was only found in CSF in PIH cases. A facultatively anaerobic isolate was recovered. Assembly of the genome revealed a strain of *P. thiaminolyticus*. In mice, this isolate designated strain *Mbale*, was lethal in contrast with the benign reference strain. These findings point to the value of an unbiased pan-microbial approach to characterize PIH in settings where the organisms remain unknown, and enables a pathway towards more optimal treatment and prevention of the proximate neonatal infections.

One Sentence Summary:

We have discovered a novel strain of bacteria upon a frequent viral background underlying postinfectious hydrocephalus in Uganda.

Introduction

Hydrocephalus is the most common indication for neurosurgery in children. Of the estimated 400,000 new cases each year, about half are estimated to be postinfectious, with the largest number of cases in low- and middle-income countries, especially sub-Saharan Africa (1). Neonatal sepsis (2) often precedes postinfectious hydrocephalus (PIH) (3), although the manifestations of hydrocephalus typically emerge in the months following the neonatal period as sufficient cerebrospinal fluid (CSF) accumulates so that cranial expansion garners medical attention. Thus, although these infants will typically die in early childhood without advanced surgical management, they are omitted from neonatal mortality surveillance (4).

The spectrum of microbial agents that underlie PIH remains poorly characterized. It is known that seasonal *Neisseria* epidemics can produce such cases within the African meningitis belt (5), and there have been reports of a tendency towards gram negative coliform bacteria in infants in other Southern (6) and Eastern (7) African locations where *Neisseria* is uncommon. Nevertheless, there has never been a well-controlled examination of the agents underlying PIH, and no knowledge of the roles that viruses, parasites, or fungi might play in addition to bacteria. If the microbial agents causing PIH were better characterized, emphasis could shift from high technology palliation of CSF accumulation (8) to prevention.

In this study, we examined blood and CSF from 100 consecutive cases of PIH and control non-postinfectious hydrocephalus (NPIH) in infants under 3 months of age at the CURE Children's Hospital of Uganda (CCHU) in Mbale, Uganda. Since 2001, this pediatric neurosurgical hospital has treated thousands of PIH and NPIH cases, with nearly uniformly

negative recovery of putative pathogens through standard bacterial culture. In this study, we gathered high quality blood and CSF samples for molecular analysis, and comprehensive testing was undertaken for bacterial, fungal, and parasitic DNA, genomic and RNA transcript sequencing for viruses, in addition to extensive bacterial culture recovery efforts for taxonomic identification, genome assembly and virulence characterization.

Results

Demographics & clinical characteristics

Between March and November 2016, 115 consecutive patients were screened, and 15 were excluded for the following reasons: 8 did not give consent, 6 lived outside of Uganda (South Sudan and Kenya), and 1 weighed less than 2.5 kg. A total of 100 patients 3 months of age or younger with hydrocephalus were enrolled; 64 with PIH and 36 with NPIH. PIH patients were on average several weeks older than NPIH patients (whose disorders were generally recognized at birth), and had higher peripheral and CSF white blood cell counts, lower blood hemoglobin and hematocrit levels, but were evenly distributed by sex and HIV exposure status (Table 1).

Prior to admission for hydrocephalus, 15 infants received antibiotics, but we only have records detailing antibiotic type for 2 of these 15 patients (gentamicin with ampicillin in one case and gentamicin with ceftriaxone in the other case). Three patients received antibiotics for treatment of active infection at CCHU after admission (ceftriaxone with gentamicin in one case, and ceftriaxone alone in the others). Such antibiotic treatment is adjunctive to abscess management (drainage and irrigation of larger accessible abscesses), prior to more definitive surgical treatment of hydrocephalus with endoscopic third ventriculostomy or insertion of a ventriculoperitoneal shunt (8).

Preoperative CT scans were scored, demonstrating that PIH patients were more likely to have evidence of CSF fluid loculations, debris within fluid spaces, ectopic calcification, and brain abscesses (Table 1 and Fig. S1). The homes of PIH patients were concentrated within central and eastern Uganda, in a swampy plateau north of Lake Victoria, and south and north of the banks of Lake Kyoga, while NPIH patients were more uniformly distributed geographically (Fig. 1 and Fig. S2, p=0.03 by linear discrimination).

Bacterial pathogen detection

For bacterial pathogen discovery, both Sanger sequencing of 16S rDNA V1-V4 region and next generation sequencing of V1-V2 and V4 regions were performed on fresh frozen and preserved specimens in two different laboratories, which enabled us to account for known variation in microbial community amplicon sequencing (9), and demonstrate reproducibility of our findings.

Using DNA from fresh frozen CSF, conventional PCR targeting the V1-V4 16S rDNA gene region (Table S1) revealed 16S amplification in 27/64 PIH and only 3/36 NPIH patients. V1-V4 Sanger sequencing of subcloned amplicons identified *Paenibacillus* as a predominant organism within the PIH cohort (23/64), but not within the NPIH cohort (0/36). The full results from this method are summarized in Table S2. For quantification of *Paenibacillus* in

CSF, *Paenibacillus* genus-specific qPCR was performed confirming and quantifying 22 of the 23 samples that were positive for *Paenibacillus* by Sanger sequencing and identifying 4 additional positive cases (Fig. 2A and 2B). Next generation sequencing on 16S rDNA V4 region was performed on all samples from which amplification libraries could be obtained with composite MiSeq primers (26/64 PIH and 3/36 NPIH) (Fig. 2C). Only a few nucleotides distinguished *P. thiaminolyticus* and *P. popilliae* within the V1-V4 region, hindering species-level discrimination between these taxa. From the V1-V4 sequencing data a phylogenetic tree was constructed, revealing that the majority of the *Paenibacillus* V1-V4 sequences were most closely related to *P. thiaminolyticus* and *P. popilliae*, and sequences from one subject that most closely matched *P. alvei* (Fig. S3).

Using DNA from samples in genomic preservative, next generation sequencing was performed on V1-V2. Overall, representative sequences from the 1,767 operational taxonomic units (OTUs) matched 159 genera. The majority of OTUs were sparsely represented except for a number of known skin flora, e.g., *Propionibacterium* spp. (Fig. 2D). Over half of the reads in 20% of the patients were attributed to the genus *Paenibacillus*. *Paenibacillus* spp. were present, defined as a minimum of 50 reads, in 38 PIH patients and 2 NPIH controls (Fig. 2D).

To associate taxa with infection we aggregated annotated OTUs at the genus level and performed differential abundance analysis. In performing linear regression analysis (Supplemental Methods: Differential Abundance Analysis), *Paenibacillus* was the only genus associated with PIH following multiple testing correction (Table S3). *Paenibacillus* spp. 16S rDNA abundance was used as a biomarker for classifying PIH patients and was consistent between V1-V2 and V4 (Fig. 2E). A receiver operating characteristic analysis yielded an area under the curve of 70.9% (95% DeLong CI = 60.6%−81.1%) for V1-V2 (Fig. 2F, Fig. S4A), with an optimal threshold just below 50 reads.

The spatial distribution of PIH and PIH *Paenibacillus* positive cases was significantly different from control NPIH cases (Fig. 1, Fig. S2).

Other putative pathogens detected by 16S in individual patients at high abundance included sequences consistent with *Bacillus subtilis* and *Streptococcus agalactiae* (Fig. 2C and 2D, Table S2). Diversity decreased as *Paenibacillus* abundance increased (Fig. S4B). The majority of CSF samples had similar microbial background leading to no clear visual separation of PIH and NPIH when diversity (beta) was visualized with principal coordinates analysis (PCoA) plots to reduce the dimension (Fig. S4C). Limiting to the set of OTUs annotated as *Paenibacillus* spp. revealed two or three clusters of patients with similar *Paenibacillus* abundance distributions in positive patients (Fig. S5). Further analysis on the microbial communities, including comparison of 16S regions and the characterization of isolates' taxonomy, is described in Supplementary Methods (microbial characterization: taxonomic assignment and overview and differential abundance analysis).

Viral pathogen detection

Utilizing the targeted viral detection capture technique VirCapSeq-VERT (10), we observed evidence of 11 viral strains distributed across 36% of samples — 32.8% (PIH) and 41.6%

(NPIH) (Table S4). Only Human Herpesvirus 5 (cytomegalovirus, CMV) was present at substantial abundance, confirmed by requiring positive findings on at least two replicates in two different qPCR methods (Supplementary Materials, Table S4) applied to all 100 preserved CSF and blood samples. CMV was confirmed in 27/100 patients (27/99 blood: 18/64 PIH, 9/35 NPIH), but CMV was found only in the CSF in blood CMV positive PIH cases (8/100 CSF: 8/64 PIH, 0/36 NPIH) (Fig. 3A). RNA sequence data confirmed 4 cases of CMV by sequence matches to multiple mRNA transcripts (Fig. 3A).

The spatial distribution of CMV positive cases was not significantly different from control NPIH cases (Fig. 1, Fig. S2).

Paenibacillus correlates with clinical signs

Several clinical measurements were positively associated with *Paenibacillus* presence including CSF cell count and CT scan scores.

In 12 months of follow-up, there were 5 deaths: 3 PIH and 2 NPIH patients. Each of the PIH deaths were in patients with *Paenibacillus*.

Paenibacillus spp. abundance was inversely correlated with patient age, consistent with residua from neonatal infection (Fig. 4A, Fig. S6, Kruskal-Wallis, $P<0.05$). Only PIH patients had high CSF cell counts (>5/μL); all were positive for *Paenibacillus* in the top abundance quartiles (Fig. 4B, Fig S6). Infants with hydrocephalus may have considerable extra weight in their heads from CSF relative to their body mass, and after calculating and subtracting excess fluid volume-for-age (11, 12), we found no difference between this corrected weight-for-age with hydrocephalus or *Paenibacillus* status (Fig. 4C). Seizures were more commonly reported in patients prior to admission (25 vs 13) and during hospital admission (9 vs 1) in patients who were *Paenibacillus* positive. The mean (SD) number of estimated days from initial febrile episode to when the head was noted to be growing was 21.4 (16.4) versus 29.3 (25.4) for *Paenibacillus* positive versus negative cases. Bloody CSF was noted in 15 CSF samples, but did not account for Paenibacillus positivity (1/7 positive in PIH), or the presence of CMV in CSF (0/15 positive in PIH or NPIH).

Brain image CT scan scoring representative of brain abscesses, calcifications, loculations, and debris was calculated using preoperative imaging. PIH patients without measurable *Paenibacillus* had higher scores than NPIH patients, and PIH patients positive for *Paenibacillus* had significantly higher scores compared to PIH patients without *Paenibacillus* detected (Fig. 3B, 3C, Table S5, Figures S1, S6 and S7). All of the CSF positive CMV patients were PIH patients (Table S6), and each had at least one of the four signs comprising the CT score: 6/7 fluid loculations, debris within fluid spaces, or ectopic calcification, and 5/7 abscess (Table S7). We fit an ordinal logistic regression model that included PIH vs NPIH status and *Paenibacillus* presence. Patients with PIH had increased proportional odds for high CT score, OR (95% CI) = 11.66 (4.29, 33.94) as well as *Paenibacillus* presence, OR (95% CI) = 7.6 (3.06, 19.88). Testing for CMV presence did not show increased proportional odds of high CT scan scores (Fig. 3D), OR (95% CI) = 3.30 (0.52, 29.66), when controlling for hydrocephalus etiology and *Paenibacillus* presence.

Growth and characterization of Paenibacillus strain

From 600 initial cultures from fresh frozen CSF (Table S8), 12 isolates were recovered from 7 patients (Table S9). Two isolates of *Paenibacillus* were recovered from cultures using small volumes (50 μl) of fresh frozen patient samples, and 1 from the blind culture of a lytic anaerobic bottle (BD BACTEC). These were identified as *Paenibacillus* spp. using MALDI-TOF (Table S9). Of the three inoculum recoveries, two grew from subculture or blind culture onto solid media, identifying them as facultative anaerobes, while the third was never successfully subcultured. Marker gene analysis of the three *Paenibacillus* isolates were identified as *P. thiaminolyticus*, *P. amyloliticus*, and *Paenibacillus* sp. (Fig. 5, Supplementary Methods: phylogenetic tree placement). The 16S rRNA genes from the whole genome sequences of all 3 isolates were compared to the 16S amplicon V1-V2 OTU cluster representative sequences. The *P. thiaminolyticus* isolate was most similar to that of OTU 99373, the most dominant OTU annotated as *Paenibacillus* within *Paenibacillus*-positive patients (Fig. S5). This identified the *P. thiaminolyticus* strain (hereafter, strain *Mbale*) as our isolate of interest for the subsequent tests for virulence in mice. Further, we compared this strain by 16S rRNA gene similarity, average nucleotide identity (ANI) (http://enve-omics.ce.gatech.edu/ani/) (13), and biochemical testing against the *P. thiaminolyticus* type strain NRRL B-4156^T (=JCM 8360^T,GenBank accession CP041405). The 16S rRNA genes of this isolate had 99.2%−99.4% identity, and the whole-genome average nucleotide identity (gANI) value was 97.06%, well above the 94–96% species threshold (14). Biochemical testing (https://apiweb.biomerieux.com, Table S10) had a 99.5–99.9% identity confirming that this isolate belongs to *P. thiaminolyticus* (15, 16). Antibiotic sensitivity was tested and the *Mbale* strain was broadly sensitive to common antibiotics (Table S11).

Thiamine testing

We tested for thiamine deficiency assaying for thiamine diphosphate (TDP) levels in whole blood. PIH cases (positive and negative for *Paenibacillus*) had lower TDP blood levels than NPIH cases (Fig. 3E, t-test, $p<0.05$).

Pathogenicity of Paenibacillus strain Mbale

Comparative virulence was assessed between the reference strain NRRL B-4156 and the strain *Mbale* in age-matched C57BL/6J mouse littermates of both sexes inoculated intraperitoneally at postnatal days 21–28. The reference strain demonstrated no adverse effects on the mice, while the strain *Mbale* produced illness in all mice (16/16), with mortality or moribund states in 15/16 (93%) of animals inoculated at a comparable concentration of colony forming units (Fig. 6, Table S12). The *Mbale* strain produced acute tubular necrosis in the kidneys, bone marrow myeloid hyperplasia, and moderate lymphocyte apoptosis in the splenic periarteriolar sheaths, but there were no significant brain lesions (Fig. 6).

Discussion

Postinfectious hydrocephalus may be the largest single cause of childhood hydrocephalus and the need for neurological surgery in children worldwide. These cases are concentrated in low- and middle-income countries (1), and the dominant predisposing event is often neonatal

sepsis. Although such hydrocephalus is in principle preventable, the microbial spectrum that accounts for this disease, and the routes of infection, have never been well characterized.

We have therefore taken a very broad view to this disease, proposing that an unbiased identification of pathogens may be necessary to identify potential causal factors in developing world settings, drawn from the *neonatal septisom*e (17) — the assemblage of the pathogens underlying neonatal sepsis in such settings.

Postinfectious hydrocephalus is part of a spectrum of conditions that through activation of the immune system in the brain lead to acquired hydrocephalus. The other major component of post-inflammatory hydrocephalus of infancy is intracerebral hemorrhage of prematurity. Both infection and hemorrhage within the brain lead to hydrocephalus through related inflammatory mechanisms (18). PIH is not a disease caused by a single organism, and the use of an unbiased pan-microbial analysis in other regions will likely reveal other organisms as important causes of PIH.One of the problems with utilizing genomic techniques for pathogen detection from nominally sterile body fluids, such as blood and CSF, is that such low-biomass samples are plagued by bacterial DNA contamination from reagents and other sources that can dominate the results (19), and substantial effort has addressed both statistical (20) and spike-in strategies (21) to reduce such effects. By employing case-controls in our study consisting of contemporaneously recruited NPIH patients referred to the same hospital, we were able to rigorously contrast our analysis of infected samples to that of clinically uninfected controls. By replicating our bacterial discovery efforts on differently preserved samples, in independent laboratories with separate regions of 16S, we reproduced convergent results — demonstrating a dominant *Paenibacillus* PIH pathogen in this cohort.

Whether prior analysis of postinfectious hydrocephalus in this region was biased by reagent contamination is not known at this time (7), but we implemented several technical strategies to reduce the effect of background contamination (see Supplementary Material). Despite such contamination reduction efforts, the additional use of case-controls was critical to achieve convincing levels of differential abundance significance, along with validation through organism recovery in culture from cases. The organism culture recovery rate was low, potentially due to the use of fresh frozen samples and antibiotic administration prior to patient sampling.

While various *Paenibacillus* spp. have been occasionally isolated from CSF (22–24), *P. thiaminolyticus* has not been known to be a virulent pathogen. It was first identified while screening for bacteria in the gut that might contribute to thiamine deficiency in beriberi in Asia (25). There is a single case report of indwelling catheter-associated bacteremia in an elderly patient on hemodialysis (26). This bacterium produces thiaminase I and II and is adapted to live in low-thiamine environments. Thiamine deficiency is associated with Wernicke's encephalopathy (27), two forms of beriberi (28), and polioencephalomalacia-associated brain necrosis in ruminant animals (29). In the developing world, thiamine deficiency is common in children (30), and is exacerbated by the stress of infection (31). Our findings demonstrate that thiamine levels were lower in postinfectious children regardless of

infectious etiology, but, consistent with reports in animals (32), we did not demonstrate lower thiamine levels specifically due to infection with *P. thiaminolyticus*.

The underpinnings of the organism's virulence remain uncharacterized at present. We found it difficult to culture this facultatively anaerobic organism from clinical samples. Once established in culture, the organism could be passaged onto aerobic media. We speculate that with a predilection to form calcified loculations and abscesses within the brain, it may be growing anaerobically when sampled, and require initial anaerobic conditions before switching to aerobic metabolism. Alternatively, the lytic properties of the anaerobic broth used successfully in recovery might have released viable organisms from intracellular phagocytosis within white blood cells. In either case, this organism has acquired substantial virulence in comparison with the existing reference strain, as demonstrated by nearly complete lethality of high concentrations of this organism when inoculated into mice in contrast with the reference strain. Supporting this differential virulence are multiple phage insertions into its genome, and other protein coding and copy number variations, that await further characterization.

The expression of differential virulence found in mice was constrained by the rapid lethality. The apparent toxin effect may have precluded replicating the establishment of the brain infections during sepsis which are the hallmark of PIH. Although our findings and animal model do not meet Koch's postulates for disease causation (33), many infectious disease do not meet these criteria (34), and in particular PIH can be caused by different organisms. Nevertheless, *P thiaminolyticus* Mbale was the dominant organism present in these PIH cases, correlation with disease severity on brain imaging was substantial, and correlation with central and peripheral WBC counts was strong.

Although this organism appears quite sensitive to common antibiotics, the presence of calcified abscesses will make the penetration of antibiotics to achieve adequate bactericidal concentrations challenging. Ideally, such concentrations are achieved at initial point-of-care treatment for neonatal sepsis prior to infection of the brain. It is possible that within the immune-privileged brain (35), inadequate treatment during neonatal sepsis is a substantial factor in the persistent development of brain *P. thiaminolyticus* infection. How co-infection with CMV might affect disease severity in the setting of *P. thiaminolyticus* Mbale infection is unknown at this time.

The typical clinical picture of these PIH cases is that of a ventriculitis without prior meningitis. Whether lumbar puncture in such infants, without meningeal infection, could be diagnostic during their neonatal sepsis evaluation is presently unknown.

Notwithstanding that we find CSF purulence, high levels of matching DNA, and recovered organisms whose DNA matches that in infected cases closely, a full description of causality in this disease may be more complex. Our analysis was limited to detection of active infections. For the regions in which we were working, *in utero* exposure to infections such as malaria is common (36). But predisposing *in utero* infections which are no longer active after birth (whether parasitic, viral, or bacterial), would remain undetected in our genomic sampling. Our finding of a substantial viral background infection with CMV cannot

distinguish congenital from postnatally acquired viral infection, but the true incidence of *in utero* infections must have been higher than what we could observe through testing several months after birth. In addition, our analysis does not address the heterogeneity in innate immunity (37) or nutritional status (38) known to be predisposing factors of infection.

The demographics of the *Paenibacillus* infections suggests localization to a circumscribed region in Eastern Uganda. This is a region associated with the north and south banks of Lake Kyoga, and the wetlands along the northern edge of Lake Victoria. It is characterized by large swamps and is a rice-growing region. Whether these infections are influenced by rainfall (39), which has been previously observed in PIH without organism identification (40), or share similarities with other environmental agents in similar topographies of the developing world (41), remains unknown.

Nevertheless, a pan-microbial approach has uncovered the presence of a difficult to grow pathogen not previously known to possess substantial virulence, which appears associated with calcified loculations and brain abscesses in infants, as well as with hydrocephalus following survival from neonatal sepsis. The presence of this organism upon a neurotropic viral background creates a scenario with frequent viral-bacterial coinfections where 6/8 CMV by CSF and 11/27 CMV by blood positive cases were co-infected with *Paenibacillus* spp. Prior studies in Ugandan adults have found that CMV viremia is frequently present in the setting of sepsis and is associated with increased risk of mortality (42–44). It has been hypothesized that immune modulation (45), rather than direct CMV effects, are responsible for the association of CMV with worse outcomes, especially for tuberculosis and cryptococcal meningitis. It is likely that, in many of these cases, latent CMV reactivates when overwhelming infection by another pathogen alters the immune system's ability to keep the virus sequestered (44, 45). However, due to the ages of our patients, all <90 days of age, a majority of the CMV viremias detected would be expected to be related to primary CMV infection (acquired either congenitally or during early postnatal life) and be more likely to cause, rather than be a cause of, alterations in immune function with related increases in bacterial pathogenesis and increased risk of severe bacterial infection (46).

Limitations to this study are important to note. PIH is a syndrome, and although we have identified a novel agent that appears to play a major role in the cases from Eastern Uganda, it remains unclear what other bacterial pathogens may play an important role in other regions of Uganda, or other countries within sub-Saharan Africa and beyond. Whether *Paenibacilus* spp. predisposes to invasion of the nervous system by CMV, or vice versa, remains unknown at present. The non-diagnostic PIH cases are mysteries, in that the ectopic calcifications seen commonly in the *Paenibacillus* positive cases would not have disappeared in the slightly older cohort of non-diagnostic patients – it is possible that they may have harbored different organisms. The prime limitation to our pan-microbial molecular approach seems to have been the age of the patients – only survivors of neonatal sepsis can develop PIH, and our data is consistent with a need to identify the causative organisms as early as possible, ideally during treatment of neonatal sepsis accompanied by serial brain imaging.

Addressing the estimated 160,000 annual cases of PIH generated largely throughout the developing world (1), and the larger pool of several million yearly neonatal sepsis cases (2),

is a critical global public health need. If a pan-microbial approach is required, then the current technology available to achieve this is at present neither readily scalable nor economically sustainable. On the other hand, the expansion of high-technology surgical facilities and pediatric neurosurgical care is also not readily scalable (47). Long-term, more sustainable and less expensive technologies to achieve pan-microbial surveillance in such settings, including maternal and environmental sources of infection, will be required to enable more optimal treatment and ultimately prevention of these devastating neonatal infections.

Methods

Study design and oversight.

The study was conducted at the CURE Children's Hospital of Uganda (CCHU), a freestanding pediatric neurosurgical hospital in eastern Uganda that serves as a countrywide referral center for patients with hydrocephalus. Infants were eligible for participation in the trial if they were 3 months of age or younger, met criteria for postinfectious or non-postinfectious hydrocephalus (PIH and NPIH respectively), and had a mother who was at least 18 years of age. The study was designed as a waste-fluid study at surgery with verbal consent. Ethics oversight was provided by the CCHU Institutional Review Board, the Mbarara University of Science and Technology Research Ethics Committee, and with oversight of the Ugandan National Council on Science and Technology. The study was approved by the Penn State University Institutional Review Board, and a Materials Transfer Agreement was in place between CCHU and Penn State University. A US Centers for Disease Control permit for the importation of infectious materials covered the transfer of specimens from CCHU to Penn State. An Institutional Biosafety Committee provided oversight of specimen handling at Penn State. A Materials Transfer Agreement between Penn State and Columbia University covered the transport of materials between these research sites.

Inclusion criteria for PIH: a) age 3 months or less, b) weight greater than 2.5 Kg, c) no history consistent with hydrocephalus at birth, and either i) a history of febrile illness and/or seizures preceding the onset of clinically apparent hydrocephalus, or ii) alternative findings such as imaging and endoscopic results indicative of prior ventriculitis including septations, loculations, or deposits of debris within the ventricular system (3), and d) mothers at least 18 years old to give informed consent.

Inclusion criteria for NPIH: a) age 3 months or less, b) weight greater than 2.5 Kg, c) findings of non-infectious origin of hydrocephalus on computed tomography (CT) scan or at endoscopy such as a lesion obstructing the Aqueduct of Sylvius such as tumor or cyst, aneurysm, or cavernous malformation, Dandy-Walker cyst, or other congenital malformation of the nervous system, or d) evidence of hemorrhage as cause of hydrocephalus such as i) bloody CSF and ii) absence of findings consistent with PIH or congenital origin of hydrocephalus, and e) mothers at least 18 years old to give informed consent.

background characterization, and *Paenibacillus* genus-specific qPCR for quantification was performed. At the other laboratory, a primer extension technique for 16S amplicon next generation sequencing of the V1-V2 region on DNA/RNA preserved samples was performed. Utilizing results from these two laboratories, 16S amplicon (regions V1-V2 and V4) reads sequenced from fresh frozen CSF and preservative samples were clustered at 97% similarity (49). For downstream analyses we accounted for sequencing variability using cumulative sum scaling normalized taxa abundances (50). A primer table is given in Table S1.

Targeted pathogen gene testing.

Targeted polymerase chain reaction (PCR) was performed in an attempt to detect the presence of Zika virus, chikungunya virus, human papilloma virus, parvovirus B19, toxoplasmosis, trypanosomiasis, malaria, and fungi (Table S1).

Virus detection.

A broad screen for viral presence was performed in two different ways: VirCapSeq oligomer concentration (10) and total RNA sequencing analysis. For the viruses that appeared abundant in either PIH or NPIH, PCR confirmation was performed.

Metabolites.

Thiamine diphosphate was quantified in fresh frozen blood using high-performance liquid chromatography with tandem mass spectrometry (LC/MS/MS) at Mayo Clinic Laboratories.

Microbiology.

In an attempt to culture the putative pathogen, 100 fresh frozen CSF samples were subjected to 6 different media outlined in Table S8. If colonies grew on solid media, gram stain and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) were performed to characterize the organism. For isolates identified as *Paenibacillus*, antibiotic sensitivity, biochemical testing, and whole genome sequencing were performed.

Genome assembly.

A hybrid method was utilized to reconstruct the genome of a *P. thiaminolyticus* isolate, combining short read sequencing, optical mapping (Bionano Genomics), and nanopore long contiguous sequencing (MinION, Oxford Nanopore Technologies). From the resulting whole genome sequences and optical mapping assembly a hybrid scaffold was generated (Bionano Hybrid Scaffold v1025201). For *P. amylolyticus* and *Paenibacillus* spp. isolates, and reference type strain *P. thiaminolyticus* NRRL B-4156 (Agricultural Research Service Culture Collection https://nrrl.ncaur.usda.gov/cgi-bin/usda/prokaryote/report.html?nrrlcodes=B-4156), only short read and nanopore sequencing were utilized for assembly.

Animal model virulence testing.

All animal experiments were performed with oversight by the Penn State Institutional Animal Care and Use Committee, and with Institutional Biosafety Committee approval at biosafety level 2 (BSL2). Virulence testing was performed on weanling P21-P28 C57BL/6J

mice using up to 10^9 colony forming units suspended in 100 μL saline, or saline only, injected into the peritoneum. Bacteria for injection were thawed and subcultured prior to each inoculation and quantified using standard colony forming unit methods (Supplementary Material). Animals were humanely euthanized with CO_2 if they developed altered or depressed mentation, or lost more than 20% of their body weight. A full complement of tissues were collected from each mouse following the guidelines set forth by international veterinary toxicology interest groups (51–53). Tissues were preserved in 10% neutral buffered formalin, embedded in paraffin blocks, cut into 3 μm sections, and stained with hematoxylin and eosin for analysis. All organs were evaluated by a veterinary pathologist (HA).

Supplementary Material

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Authors

Joseph N. Paulson[1,*], Brent L. Williams[2,23,*], Christine Hehnly[3,*], Nischay Mishra[2,23,*], Shamim A. Sinnar[4,5,*], Lijun Zhang[3,*], Paddy Ssentongo[4,6,7,*], Edith Mbabazi-Kabachelor[8,*], Dona S. S. Wijetunge[9,*], Benjamin von Bredow[9,*], Ronnie Mulondo[8,*], Julius Kiwanuka[10,*,†], Francis Bajunirwe[11], Joel Bazira[12], Lisa M. Bebell[13], Kathy Burgoine[14,15,16], Mara Couto-Rodriguez[2,17], Jessica E. Ericson[18], Tim Erickson[8], Matthew Ferrari[19,20], Melissa Gladstone[21], Cheng Guo[2], Murali Haran[22], Mady Hornig[23], Albert M. Isaacs[24], Brian Nsubuga Kaaya[8], Sheila M. Kangere[8], Abhaya V. Kulkarni[25], Elias Kumbakumba[10], Xiaoxiao Li[22], David D. Limbrick Jr.[26], Joshua Magombe[8], Sarah U. Morton[27], John Mugamba[8], James Ng[2], Peter Olupot-Olupot[15,28], Justin Onen[8], Mallory R. Peterson[4,6], Farrah Roy[29], Kathryn Sheldon[3], Reid Townsend[30], Andrew D. Weeks[31], Andrew J. Whalen[32], John Quackenbush[29,§], Peter Ssenyonga[8,§], Michael Y. Galperin[33,§], Mathieu Almeida[34,§], Hannah Atkins[35,§], Benjamin C. Warf[36,§], W. Ian Lipkin[2,23,§], James R. Broach[3,§], Steven J. Schiff[4,6,19,37,38,§,‡]

Affiliations

[1]Department of Biostatistics, Product Development, Genentech Inc. [2]Center for Infection and Immunity, Mailman School of Public Health, Columbia University [3]Institute for Personalized Medicine, Dept. of Biochemistry and Molecular Biology, The Pennsylvania State University College of Medicine [4]Center for Neural Engineering, The Pennsylvania State University [5]Department of Medicine, The Pennsylvania State University College of Medicine [6]Department of Engineering Science and Mechanics, The Pennsylvania State University [7]Department of Public Health Sciences, The Pennsylvania State University College of Medicine [8]CURE Children's Hospital of Uganda [9]Department of Pathology, The Pennsylvania State University College of Medicine [10]Department of Pediatrics, Mbarara University of Science and Technology [11]Department of Epidemiology, Mbarara University of Science and Technology [12]Department of Microbiology, Mbarara University of Science and Technology [13]MGH Medical Practice Evaluation Center, Division of

Infectious Diseases, and Center for Global Health [14]Neonatal Unit, Department of Paediatrics and Child Health, Mbale Regional Referral Hospital, Mbale, Uganda [15]Mbale Clinical Research Institute, Mbale Regional Referral Hospital, Mbale, Uganda [16]University of Liverpool, Liverpool, UK [17]Biotia, 100 6th avenue, New York, NY, USA [18]Division of Pediatric Infectious Disease, The Pennsylvania State University College of Medicine [19]The Center for Infectious Disease Dynamics, The Pennsylvania State University [20]Departments of Biology and Statistics, The Pennsylvania State University [21]Institute for Translational Medicine, University of Liverpool [22]Department of Statistics, The Pennsylvania State University [23]Department of Epidemiology, Columbia University Mailman School of Public Health [24]Department of Neuroscience, Washington University School of Medicine [25]Department of Neurosurgery, University of Toronto [26]Department of Neurological Surgery, Washington University School of Medicine [27]Division of Newborn Medicine, Boston Children's Hospital and Department of Pediatrics, Harvard Medical School [28]Busitema University, Faculty of Health Sciences, Mbale Campus 2 [29]Department of Biostatistics, Harvard T.H. Chan School of Public Health [30]Department of Medicine, Washington University School of Medicine [31]Department of Women's and Children's Health, University of Liverpool and Liverpool Women's Hospital for Liverpool Health Partners, Liverpool, UK [32]Department of Mechanical Engineering, The Pennsylvania State University [33]National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health [34]MGP MetaGénoPolis, INRA, Université Paris-Saclay [35]Department of Comparative Medicine, The Pennsylvania State University College of Medicine [36]Dept. of Neurosurgery, Boston Children's Hospital, Harvard Medical School [37]Department of Neurosurgery, The Pennsylvania State University College of Medicine [38]Department of Physics, The Pennsylvania State University

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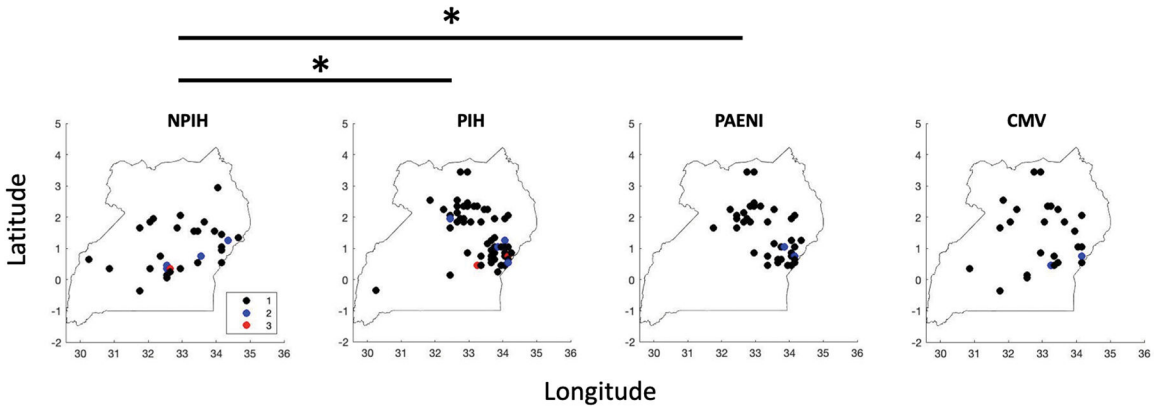

Figure 1. Comparative location of cases within map of Uganda.

The village centroid GPS locations are mapped to the 0.1 × 0.1 degree grid frequently used in satellite rainfall estimation (54), and where village name was uncertain, the centroid of the administrative parish or sub-county was used. The groups shown are mapped by clinical status (NPIH and PIH), and by organism type (*Paenibacillus*, PAENI; cytomegalovirus, CMV). Both the PAENI and CMV mappings included all NPIH and PIH cases with such diagnoses. Using Fisher's linear discrimination analysis, group comparisons by latitude and longitude mapping could significantly discriminate NPIH from PIH, or NPIH from PAENI, at the $p < 0.01$ level parametrically (Wilk's lambda), and at the $p = 0.03$ and $p = 0.01$ level respectively using a bootstrap method (see Figure S2). The number of cases mapped to a 0.1 × 0.1 degree grid (11 km per edge at the equator) are indicated with colored circles as 1, 2, or 3.

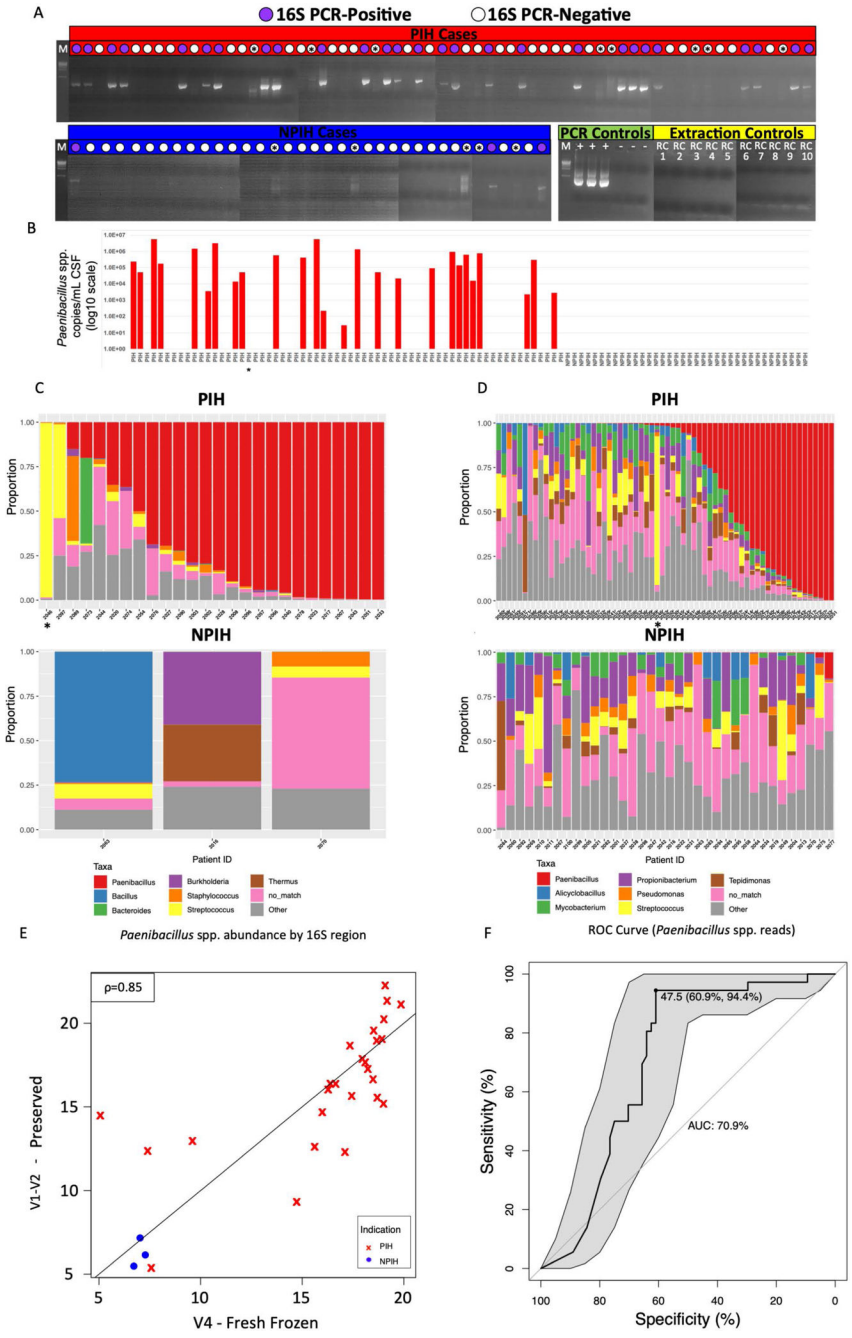

Figure 2. Detection and typing of bacteria using 16S rDNA.

A) Agarose gels showing 16S rDNA amplification products for PIH (red) and NPIH (blue), along with PCR positive (+) and negative (−) controls (green) and 10 separate extraction reagent controls (yellow). Brightness and contrast were adjusted for gels to maximize visibility of faint bands and smears. Asterisk (*) denotes lanes where faint non-specific amplification (smears) or bands of unexpected size were observed. All amplification products including non-specific amplifications were subjected to subcloning and sequencing. B) *Paenibacillus* qPCR quantification. C-D) Stacked bar relative abundance plots of (C) 16S V4 and (D) V1-V2 regions in microbial communities for the most dominant bacteria

observed within (C) 16S positive samples from (B) and in (D) all 100 samples. Star underneath PIH sample (C, D) highlights individual PIH sample with group B streptococcal infection. E) Scatterplot of cumulative sum scaling normalized *Paenibacillus* abundance for (abscissa) V4 16S sequencing of fresh frozen CSF samples and (ordinate) V1-V2 16S sequencing of biological replicates from preserved CSF samples. F) Receiver-operating-characteristic (ROC) curve using the number of *Paenibacillus* reads as the predictor for PIH or NPIH status. Area under the curve was 70.88% (95% DeLong CI = 60.61%−81.15%). Sensitivity and specificity are maximized at 47.5 reads in a given sample, consistent with the threshold employed of 50 reads.

Table 1:

Demographics and clinical characteristics of NPIH and PIH cohort.

Characteristics	All patients n=100	PIH n=64	NPIH n=36
Age in days, mean (SD)	57 (24)	66 (17)	43 (27)
Sex			
Male (%)	51 (51)	35 (55)	16 (44)
Female (%)	49 (49)	29 (45)	20 (56)
Peripheral blood WBC [1.0 × 10^3]/μL, mean (SD)	10.3 (3.6)	11.0 (3.8)	8.8 (2.6)
CSF WBC/μL, mean (SD) ≠	30 (62)	45 (74)	5 (0.5)
Hemoglobin g/dl, mean (SD)	11.5 (2.2)	10.7 (1.3)	13.0 (2.8)
Hematocrit %, mean (SD)	36.8 (7.4)	34.1 (4.1)	41.6 (9.3)
CT scan scoring * **[no. and % in each category]**			
0	32 (33)	8 (25)	24 (69)
1	15 (15)	7 (11)	8 (23)
2	13 (13)	10 (16)	3 (8)
3	11 (11)	11 (17)	0 (0)
4	27 (28)	27 (43)	0 (0)
HIV Exposure status [no. and %]			
Yes	5 (5)	3 (5)	2 (6)
No	95 (95)	61 (95)	34 (94)

* n=98

≠ n=99

Patient demographics and Clinical Characteristics. Comparison of demographic and clinical attributes between two groups: Postinfectious hydrocephalus (PIH, n=64) and non-postinfectious hydrocephalus (NPIH, n=36). PIH patients were older (66 days vs 43 days, p <0.0001), had higher peripheral and cerebrospinal fluid (CSF) white blood cell (WBC) counts, and were more likely to be anemic (hemoglobin 10.7g/dL vs 13.0 g/dL, p<0.0001) compared with NPIH patients. Preoperative computed tomography (CT) scans were available for 98 subjects. Of these, the PIH group was more likely to have a higher CT scan score reflective of brain abscess, calcifications, loculations and septations (p<0.0001). There were no significant differences in gender and human immunodeficiency virus (HIV) exposure frequencies between the groups. Continuous demographic variables were evaluated using the non-parametric Wilcoxon rank-sum (2-group comparisons) and Kruskal-Wallis (>2 groups) tests following Shapiro-Wilk's test for normality unless otherwise stated. Fisher's exact test was performed for categorical variables.