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Association of bacteria with hydrocephalus in Ugandan infants

Clinical article

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Object. Infantile hydrocephalus in East Africa is predominantly postinfectious. The microbial origins remain elusive, since most patients present with postinfectious hydrocephalus after antecedent neonatal sepsis (NS) has resolved.

Methods. To characterize this syndrome in Ugandan infants, the authors used polymerase chain reaction targeting bacterial 16S ribosomal DNA from CSF to determine if bacterial residua from recent infections were detectable. Bacteria were identified based on the relationship of genetic sequences obtained with reference bacteria in public databases. The authors evaluated samples from patients presenting during dry and rainy seasons and performed environmental sampling in the villages of patients.

Results. Bacterial DNA was recovered from 94% of patients. Gram-negative bacteria in the phylum Proteobacteria were the most commonly detected. Within this phylum, Gammaproteobacteria dominated in patients presenting after infections during the rainy season, and Betaproteobacteria was most common following infections during the dry season. *Acinetobacter* species were identified in the majority of patients admitted after rainy season infection.

Conclusions. Postinfectious hydrocephalus in Ugandan infants appears associated with predominantly enteric gram-negative bacteria. These findings highlight the need for linking these cases with antecedent NS to develop more effective treatment and prevention strategies. *(DOI: 10.3171/2010.9.PEDS10162)*

Key Words • hydrocephalus • central nervous system infection • tropical medicine • pediatrics • developing country

World Health Organization estimates suggest a worldwide yearly toll near 1.6 million neonatal deaths from infection.[28] Neonatal mortality disparity ranges from 5 cases per thousand live births in developed countries to 42 cases per thousand in some African countries. There are 2 classes of NS: early onset, within the 1st week of life from intrapartum organism transmission; and late onset, within weeks 2–4 of life from community and nosocomial sources.[28]

The occurrence of hydrocephalus in survivors of neonatal meningitis was well described by the 1960s, with incidences in developed countries as high as 31%.[17] By the 1970s, pneumoencephalography demonstrated that in such patients, there were intraventricular septa and loculations, which appeared related to a progressive ependymal inflammatory process.[24] Ventriculoscopy has recently provided direct observation of ependymal and choroid plexus scarring, postinflammatory aqueductal obstruction, and intraventricular deposits of pus and hemosiderin.[32] The latter finding appears to be the pneumoencephalographic detritus described in such cases by Handler and Wright,[14] who reported that PIH accounted for 30%–40% of cases in a South African population of infants with hydrocephalus,

Abbreviations used in this paper: NS = neonatal sepsis; PCR = polymerase chain reaction; PIH = postinfectious hydrocephalus.

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with a disproportionate percentage of these patients represented in the nonwhite peoples they treated.

It has recently been reported that most cases (> 60%) of infantile hydrocephalus in East Africa may be postinfectious.[32] Hydrocephalus is estimated to develop in between 1000 and 2000 Ugandan infants each year.[31] The microbial origins of these infections remain elusive, since the majority of patients present with PIH after antecedent NS has resolved and CSF cultures have been consistently negative.

The spectrum of bacteria causing NS in the developed countries is well known and relatively homogeneous in North America, Europe, Australia, and South Africa: group B *Streptococcus*, *Escherichia coli*, and *Listeria* monocytogenes.[21] When gram-negative enteric bacteria cause NS in industrialized countries, risk factors typically include neural tube defects and urinary tract anomalies.[27] In contrast, the spectrum of bacteria infecting neonates in the developing world is biased toward gram-negative organisms and appears to differ at each site from which high-quality (culture positive) data are available.[2,6,15] There is a striking absence of group B *Streptococcus* in many studies in the developing world[3,33] despite the fact that maternal carriage rates of this organism may be similar.[25] In Handler and Wright's cases,[14] the nonmeningococcal organisms were found to be gram-negative enteric bacteria (*Escherichia coli*, *Acinetobacter*, and *Enterobacter cloacae*).

The advent of modern molecular techniques permits us to identify the presence of microorganisms by the amplification of their genetic material through PCR. The amplified gene can then be used to establish a taxonomic relationship among microbes. For bacteria, the *16S rRNA* gene has become a standard for classifying bacteria,[12] often to the level of individual species. This new approach to bacterial identification has provided a significant clinical breakthrough; it provides a means of identifying bacteria that are not easily distinguishable based on biochemical properties and, most significantly, it allows for the detection of bacteria that cannot be cultured. In this study, faced with a large patient population with clinical infection residua but without successful bacterial culture results, we used amplification and sequencing of the bacterial *16S rRNA* gene to further study these patients.

We performed the following cohort study to delineate the nature of PIH in a Ugandan infant population presenting weeks to months after NS for neurosurgical treatment of their hydrocephalus.

Methods

All research was performed with institutional review board approval from the CURE Children's Hospital of Uganda and Penn State University.

Our criteria for PIH in infants presenting with hydrocephalus under 1 year of age were as follows: no history of hydrocephalus at birth and either a history of febrile illness and/or seizures preceding the onset of hydrocephalus, or endoscopic or imaging findings indicative of prior ventriculitis such as scarring, loculations, thickened ependyma, or purulent intraventricular debris (Fig. 1). In the overwhelming majority of infants presenting with PIH, their antecedent serious febrile illness had developed within the 1st month of life,[32] demonstrating that PIH very likely derives from preceding NS. One of the key motivations for our work is that CSF sampled at the time of endoscopic third ventriculostomy or shunt insertion has been consistently culture negative in more than 1000 such children treated at the CURE Children's Hospital of Uganda since the year 2000.

At the time of endoscopic third ventriculostomy or shunt insertion, we collected samples of CSF from 3 cohorts of infants meeting the above criteria for PIH: Cohort 1, specimens from 25 consecutive patients collected in January 2008 for DNA analysis; Cohort 2, specimens from 25 consecutive patients obtained in July 2008 for culture; and Cohort 3, specimens from 25 consecutive patients obtained in October 2008 for DNA analysis. Each specimen was obtained from an individual patient. There are 2 rainy seasons in Uganda: generally April–May and October–December. Cerebrospinal fluid samples from Cohort 1 were collected during the dry season in January 2008, and those from Cohort 3 were obtained at the beginning of the rainy season. Because the infants were ages 1–6 months and the mean/median time from illness to the onset of PIH was 0.8/0.5 months,[32] patients present-

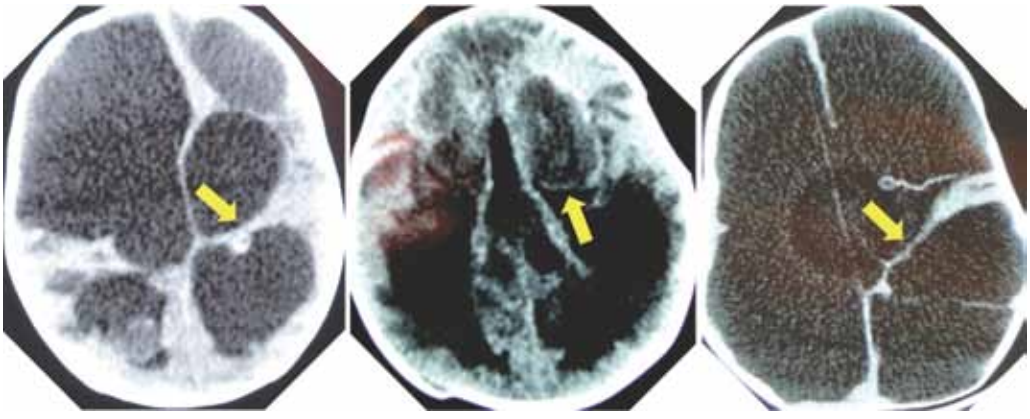

Fig. 1. Characteristic CT scans from 3 Ugandan infants younger than 6 months of age at the time of their treatment for PIH. In addition to severe hydrocephalus, frequent evidence of severe scarring and loculations within the ventricular system is visible *(arrows)*.

ing in January were presumptively infected during the rainy season, and the October cohort was presumptively infected during the dry season.

All CSF samples were collected under strict sterile conditions in the operating room. Samples were collected upon entrance to the brain, almost always with an endoscope but occasionally with a shunt catheter. Several milliliters of CSF were collected using a syringe and placed in a sterile bowl on a sterilely draped Mayo stand, and sterile and DNA-free pipette tips were used on a calibrated pipettor to place 50-ml aliquots of CSF onto DNA collection cards or into tubes. All handling of the DNA specimens was performed using an aseptic technique with gloves to reduce sample exposure to contaminants. Two collection techniques were utilized for DNA sampling: Whatman FTA cards and Biomatrica CrudE tubes. The recovery of DNA sequences was similar for both methods of stabilization, and the results were pooled in this report. Both methods permit the stabilization of nucleic acids for long-distance transport at room temperature.

Separate samples of each specimen were sent to the laboratory in Mbale for bacterial culture, where chocolate agar, and in some cases blood agar, failed to demonstrate any growth. In cohort 2, we used bacterial transport tubes (Becton Dickinson BBL CultureSwab Plus Collection and Transport System containing Amies agar gel media with oxygen-scavenging agents) for the transportation of both aerobic and anaerobic organisms to Penn State to attempt recovery of fastidious organisms. No bacteria were cultured from these specimens, either at Mbale or at Penn State, affirming the high quality of the sterile technique used and the absence of culturable bacteria in the samples.

Extraction of DNA from the FTA cards was conducted using the procedure described by Biek et al.[7] Briefly, the entire area containing sample was washed, minced, and eluted with 300–400 μl water at 95°C for 60 minutes by using sterile reagents and instruments. The paper and eluate were transferred to a spin column to remove solid material, the filtrate was adjusted with 1 M Tris to 10 mM Tris pH 8, and DNA recovery was achieved using a Qiagen DNeasy blood and tissue kit.

The PCR reaction was conducted using primers 16S-8F (5′-AGAGTTTGATCCTGGCTCAG) and 16S-534R (5′-ATTACCGCGGCTGCTGGC). Polymerase chain reaction products were sequenced directly or cloned into the pGEM-T vector (Promega), and cloned products were sequenced.

Sequences were aligned using the DNASTAR package, version 7, and MEGA, version 4.[26] For accurate reconstruction of the relationships of bacteria based on the *16S rRNA* gene, a model of how the sequences evolve must be developed. Over 50 models of sequence evolution were evaluated, and the best model for each dataset was selected using the Akaike Information Criterion[1] as implemented in the Modeltest, version 3.7.[22] The phylogenetic trees, which display the genetic relationships among the individual sequences of *Acinetobacter* species, were produced using a maximum likelihood tree method implemented in the program PhyML, version 2.4.4.[11] Using the same program, the strength of support for each node in the tree was estimated with 100 bootstrap replicates (a statistical resampling method) in the same program. To infer the relationships among individual bacterial sequences within each *Acinetobacter* species, parsimony networks were reconstructed using TCS, version 1.2.1.[5] Gaps were treated as missing data, and all the sequences were set to connect at the 95% confidence limit.

Results

To determine if bacteria were present in the CSF samples, we amplified the bacterial *16s rRNA* gene by PCR and sequenced the products with or without cloning. The composite phylogeny (Fig. 2 and *Appendix* Figs. 5–11, for phylogenetic representation of each bacterial phylum), which shows all unique sequences obtained from these patients, demonstrates that the bacterial diversity in the CSF samples was high and that many could not be classified. Surprisingly, in the 21 patients in Cohort 1 for whom the gene for bacterial 16S rRNA was amplified, Gammaproteobacteria (a class of gram-negative bacteria in the phylum Proteobacteria, which includes *Pseudomonas*, *Escherichia*, and *Acinetobacter*) were identified in 19; *Acinetobacter* species were represented in 95% of these samples (Fig. 3). We subsequently took samples from another 25 consecutive children presenting with PIH in July (Cohort 2) to attempt to improve our culture recovery of gram-negative bacteria such as *Acinetobacter,* but we were unsuccessful despite the incorporation of multiple media types (Table 1). For Cohort 3, we again focused on DNA extraction and *16S rRNA* gene amplification to determine bacterial presence. Proteobacteria (a phylum containing Beta- and Gammaproteobacteria) were also dominant in this cohort, although Betaproteobacteria (gram-negative bacteria such as *Burkholderia*) were more common (14 of 21 patients) than Gammaproteobacteria (8 of 21 patients). There were also more sequences that could not be classified. The groupings of all Gamma- and Betaproteobacteria *16S rRNA* gene sequences based on evolutionary relatedness obtained in Cohorts 1 and 3 are shown in *Appendix* Figs. 5 and 6.

Given the findings of enteric bacterial DNA in these children's CSF, we speculated that environmental exposure might play a role in PIH. Complicating the living conditions in proximity to domestic farm animals are certain cultural practices among some East African peoples (Masai in Kenya and Tanzania or related Nilotic peoples in northern Uganda) involving bovine dung use on umbilical stumps, which has been associated with neonatal tetanus. From such reports[18] emerge estimates of NS rates, which have approached 80 cases per 1000 births in regions of East Africa that are close to where we work.

We collected samples for culture from hut floors, nearby animal dung, and water supplies from the villages of 8 of 17 Cohort 1 patients with evidence of *Acinetobacter* species in their CSF. Our culture efforts targeted gram-negative bacteria, and a wide variety of species were recovered. Out of 39 surveillance cultures, *Acinetobacter* was recovered from 2 hut floors and 1 cow dung sample. The *16S rRNA* genes were amplified from these 3 positive cultures to identify the bacterial species. We note

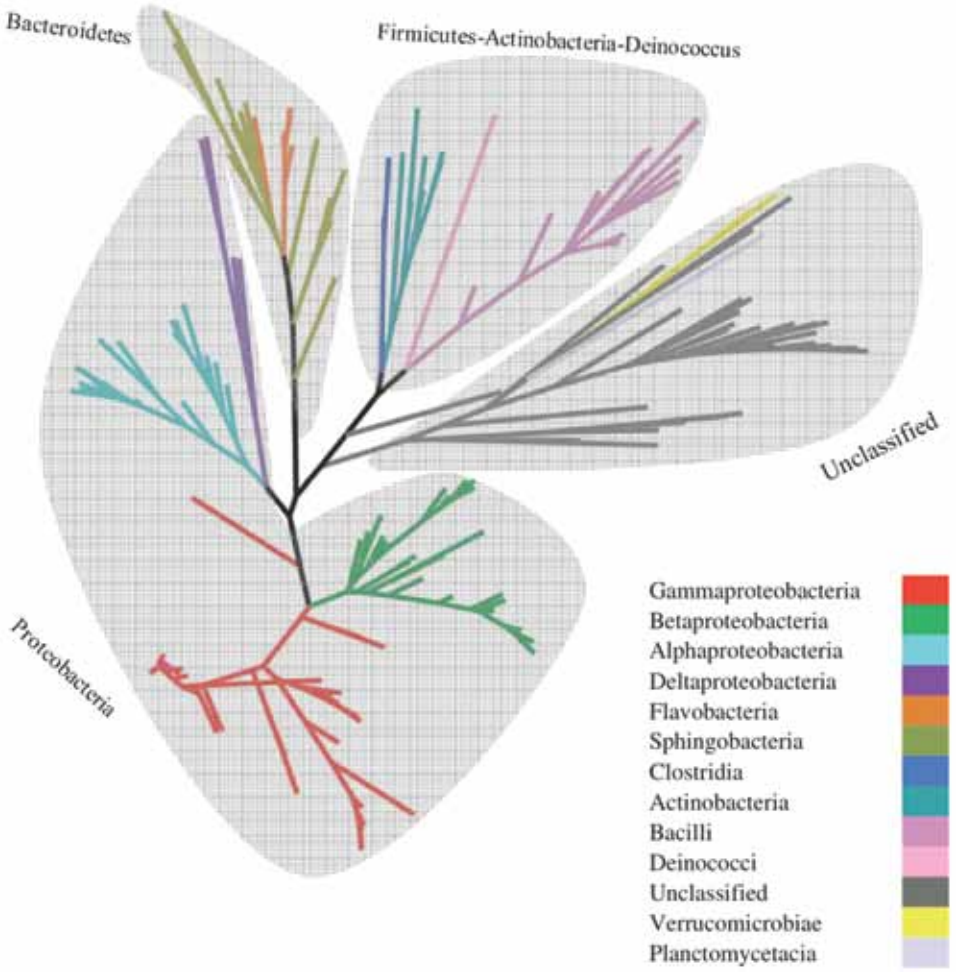

Fig. 2. Depiction of the phylogenetic relationship of all unique *16S rRNA* gene sequences obtained from patient CSF samples. Organisms are classified based on relatedness to known bacterial sequences. The data indicate the diversity of bacterial phyla detected.

that these environmental samples were collected during July 2008 and represent dry season specimens.

We further characterized the relationship between *Acinetobacter* species from patients and those from environmental sources and compared them with reference strains. These analyses demonstrate how closely related individual bacterial sequences are from each patient sample (Fig. 4 and *Appendix* Figs. 10 and 11, for more detailed analysis of individual samples). The majority of sequences from Cohort 1 formed a highly related cluster that is most similar to *A. junii*. Within the *A. junii* cluster, only 1 sample was derived from a patient in Cohort 3 (who was infected in the dry season). A second smaller cluster of Cohort 1 sequences matched more closely with *A. parvus*, which is a close relative of *A. junii*. *Acinetobacter* was only amplified from 2 other patients in Cohort 3; these sequences and those obtained from the environmental samples from the hut floor or dung collected during the dry season were most closely related to *A. gyllenbergii*. The other sequences from environmental samples were related to *A. schindlerii,* and no patient samples were found in this cluster. There are 2 important results from this analysis. First, the findings of multiple lineages of *Acinetobacter* from different patients suggest that the bacteria were not from a single source, which would be the case if the samples were contaminated postprocessing. Significantly, it appears that there is seasonality to the species of *Acinetobacter* identified in patients with PIH, and the prevalence of *A. junii* is particularly high in the rainy season.

Discussion

Our findings extend those of Handler and Wright[14] and now support the conclusion that the majority of infant hydrocephalus cases in an East African population are postinfectious. We found evidence of fragments of bacterial DNA in almost all patients, with a predominance of gram-negative enteric bacteria as well as evidence for shifting spectra of microbial species depending on the season of the year. The important clinical implication is that most of the hydrocephalus in this population may be preventable.

While previous studies have suggested that neonatal *Acinetobacter* infections are most commonly due to *A. baumannii* and are notorious for being nosocomial, little

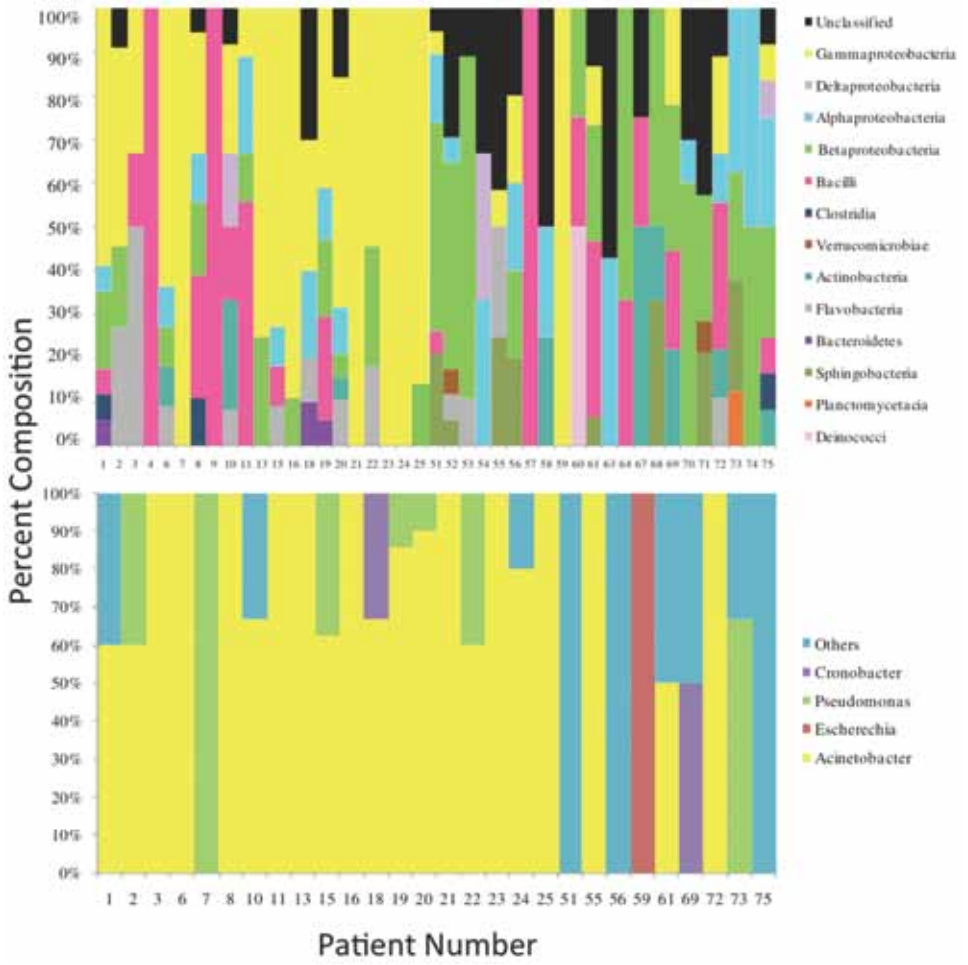

Fig. 3. Upper: Graph showing the percentage composition of the different bacterial classes in each patient. Taxonomic placement was determined using criteria of the Ribosomal Database Project (see Lebel et al., 1988). **Lower:** Percentage composition of different genera of Gammaproteobacteria in different patients. Samples from patients in Cases 26–50 were evaluated by culture only.

is known about the routes of neonatal infection in a developing world setting. In reexamining WHO data from Ethiopia, The Gambia, Papua New Guinea, and the Phillipines,[33] we note that the spectra of bacteria from infants younger than 1 year of age is similar to the Gammaproteobacteria observed in our infants with PIH. Further WHO data revealed a predominance of Gammaproteobacteria in community-acquired meningitis among infants younger than 90 days of age, with the most common agent being *Acinetobacter*, in infants from slums clustered about Manilla.[10]

In an intriguing study from the Sudan, Aziz[4] demonstrated strong seasonality of PIH coinciding with yearly meningitis epidemics. Although presumably meningococcal in origin, there was no associated bacteriological identification in these data, and neither is it possible to determine the precise timing of the causative infection from their records (they correlated the time of surgical treatment with meningitis peaks). Our cases in Uganda originated south of the seasonal African meningitis belt[23] and appear to be linked to prior NS, and we found no bacterial DNA consistent with the prior presence of *Neis-*

TABLE 1: Matrix of culture techniques applied by Penn State personnel to attempt recovery of viable organisms*

Medium	Aerobic†	Anaerobic‡
blood agar	RT (approx 22°C); 30°; 37°C	37°C + 5% CO_2 + 37°C + 5% CO_2 + 5% H_2 + 90% N_2
chocolate agar	RT (approx 22°C); 30°; 37°C	37°C + 5% CO_2 + 37°C + 5% CO_2 + 5% H_2 + 90% N_2
MacConkey agar	RT (approx 22°C); 30°; 37°C	37°C + 5% CO_2 + 37°C + 5% CO_2 + 5% H_2 + 90% N_2

* approx = approximately; RT = room temperature.
† Three temperatures at which tests were run.
‡ Conditions under which tests were run.

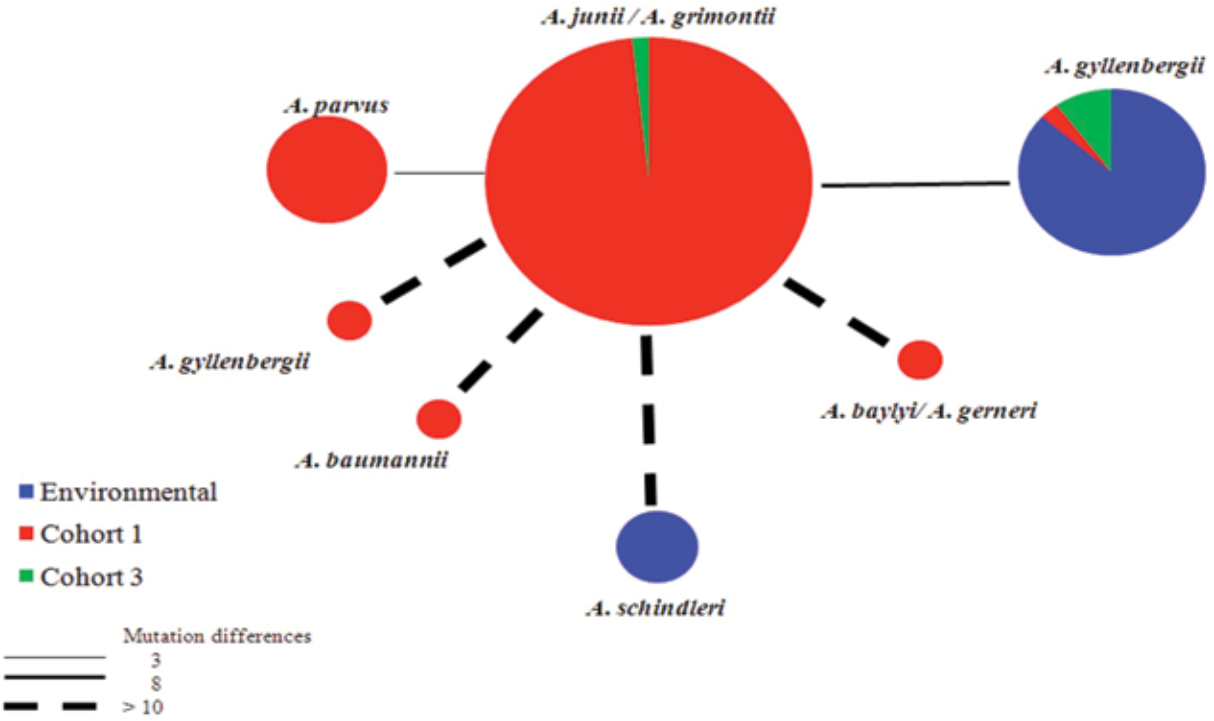

Fig. 4. Genetic clustering among the *Acinetobacter* species detected from Cohorts 1 and 3 and environmental samples. The size of each *circle* is proportional to the number of sequences. The *lines* connecting the circles represent the number of mutations (see key in the figure) that separate groups of related sequences. The species level *Acinetobacter* identification was based on the phylogenetic tree shown in Fig. 10. Also see Fig. 11 for the individual relationships among all patient and environmental samples.

seria meningitidis in any of the samples. The implication is that there are probably multiple bacterial routes to PIH specific to geography and time in sub-Saharan Africa that require untangling.

Although we isolated staphylococcal DNA from 6 patients (*Appendix* Fig. 8), we rarely encountered streptococcal strains (3 patients), as opposed to *Acinetobacter* isolates (22 patients). Consistent with these data, we have found no evidence of the nontyphoid *Salmonella* meningitis seen in older infants with postmalarial anemia.[19] Similarly, we have not observed the predominance of *Streptococcus pneumoniae* meningitis seen in association with HIV/AIDS,[19] perhaps due to the modest percentage of HIV/AIDS cases (3%–4%) among these Ugandan children. Among Betaproteobacteria, the *Burkholderia* species were most common (*Appendix* Fig. 6), and yet we are aware of only 1 case report of community-acquired *Burkholderia* species meningitis in the literature.[13]

Contamination is always a concern when interpreting DNA samples such as ours in the absence of viable organisms. Our efforts to ensure sample integrity included the following. First, samples collected at the time of surgery were stored using 2 methods: filter paper and CrudE tubes. These samples were prepared for sequencing at different times and in different labs at Penn State. The initial set of samples was prepared in a lab conducting diagnostic microbiology, and the subsequent set was prepared in a microbiology basic research lab. Both labs used sterile procedures. None of the organisms identified in patient samples were those under investigation in either lab. Second, we sequenced both directly from PCR-amplified fragments and from clones of PCR products. The latter is more sensitive to detecting bacterial diversity and is an excellent means of assessing contamination with ubiquitous laboratory organisms. None were detected. Third, our phylogenetic analyses indicated that species identified from different patients were not identical. If sample contamination had occurred during processing, the contaminating bacteria should be overrepresented and identical. While our analysis does show that identical sequences related to *A. junii* were recovered from several patients, there is sufficient diversity among the *Acinetobacter* sequences recovered to suggest that they do not arise from a common origin. Thus, even removing the identical *Acinetobacter* sequences, our interpretation of the relations among the *Acinetobacter* sequences and the proportion of different bacterial classes would not change. Fourth, in our efforts to culture viable organisms from Cohort 2, we found no growth in any sample retrieved under sterile conditions at surgery, which is consistent with both the quality of our retrieval technique and the local microbiological findings at the Mbale hospital over the past 10 years.

Because the treatment for NS involving meningitis/ventriculitis should be significantly more prolonged than for NS that does not involve the nervous system,[21] the delineation of NS routes to PIH becomes clinically and economically important. It is believed that much of the harm to the brain from well-treated meningitis is related to inflammation.[19] Vascular inflammation producing thrombosis of arachnoidal and subependymal veins is described in the pathogenesis of the type of PIH that we

have observed,[21] as are the protrusions of fibroglial tufts through denuded ependyma,[24] and is consistent with our findings of arachnoidal and ependymal scarring and severe leukomalacia in typical cases of PIH in Uganda (Fig. 1). The possibility of preventing the progressive formation of ventricular septa and loculations with corticosteroids in PIH was discussed by Schultz and Leeds.[24] Whether immune modulation[8,9,16] during NS might reduce this degree of damage to the brain in such patients remains an open question.

Significant issues suggested by our findings must now be addressed. We do not know if the isolates we characterized were causative of PIH or secondary environmental infections. We performed environmental sampling only for culturable organisms, focusing on gram-negative isolates. Patients in a certain number of cases presented with bloody CSF despite meeting the criteria for PIH, and whether such hemorrhages were postinfectious is unclear at present. We have not excluded that viruses might play a role in these hydrocephalus cases.[20] Although we screened all infants with PIH for the presence of malaria parasites on blood smears, we cannot exclude prior plasmodium infection from playing a role in this syndrome. At the time of this writing, we are pursuing clinical trials characterizing clinically PIH versus non-PIH in infants, as well as a trial characterizing the microbiology of neonatal sepsis in Ugandan infants, to further address these issues.

Conclusions

As medical technology expands in developing countries and demands for the treatment of hydrocephalic children increase, the present standard of medical care continues to focus on the technical neurosurgical treatment of PIH with a combination of ventriculoperitoneal shunts and, increasingly, endoscopic third ventriculostomy with the adjunctive use of choroid plexus cauterization.[29–32] Nevertheless, most of the children who survive these infections have severely damaged brains, and their future quality of life will remain seriously impacted. Our findings suggest that delineation of a microbial cause and prevention, rather than surgical fluid diversion, will be needed to substantially improve the present approach to these children.

Disclosure

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Author contributions to the study and manuscript preparation include the following. Conception and design: Schiff, Warf, Kapur, Poss. Acquisition of data: Schiff, Mugamba, Opio. Analysis and interpretation of data: Schiff, Li, Padhi, Ranjeva, Donaldson, Jayarao, Kapur, Poss. Drafting the article: Schiff, Poss. Critically revising the article: Schiff, Warf, Kapur, Poss. Reviewed final version of the manuscript and approved it for submission: all authors. Statistical analysis: Padhi, Poss. Administrative/technical/material support: Li, Johnson, Opio. Study supervision: Schiff, Mugamba, Jayarao, Kapur, Poss.

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Appendix

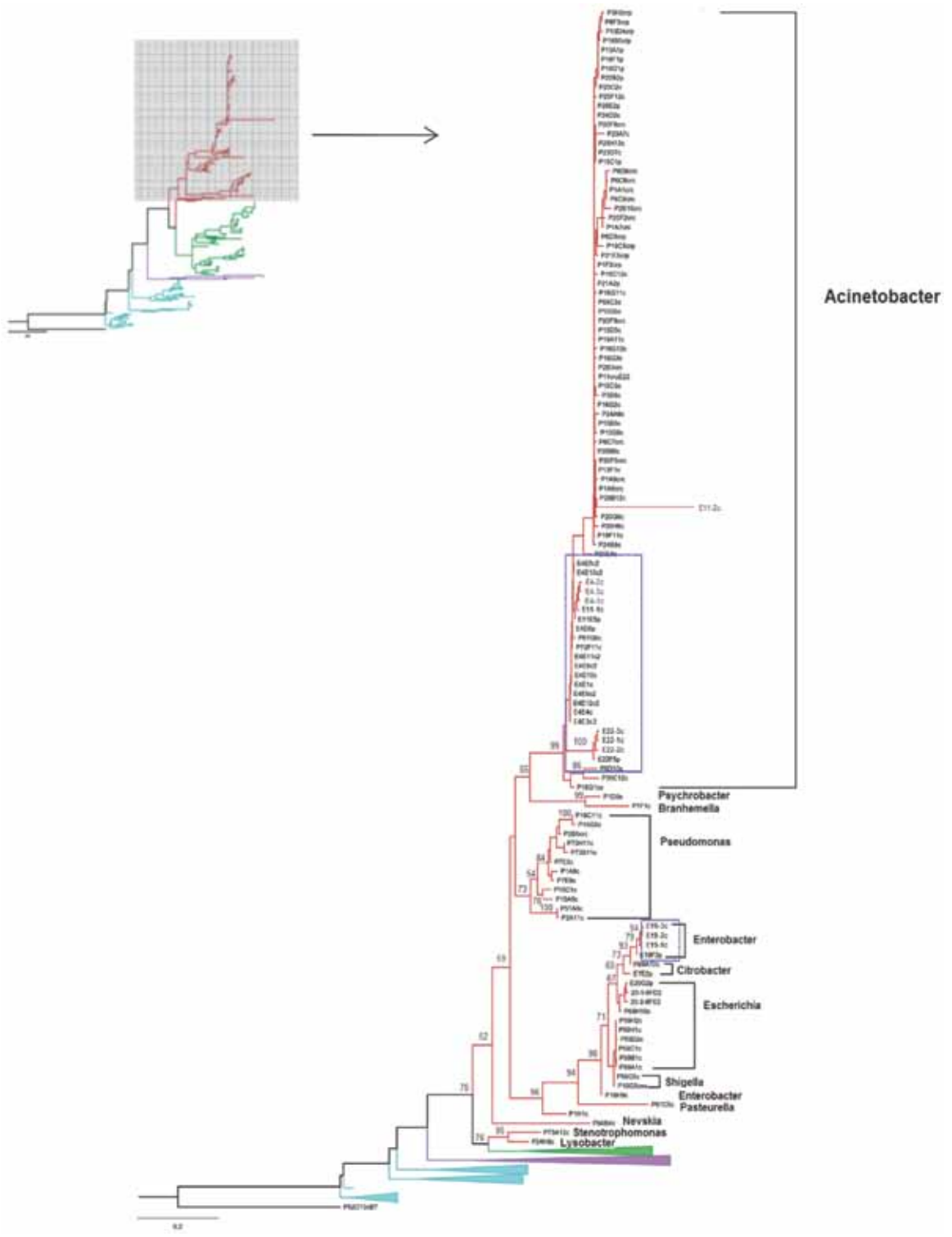

Fig. 5. Phylogenetic relationships among the different genera of Gammaproteobacteria sampled from patients (indicated by P) and the environment. Bootstrap supports indicate the confidence in the placement of a sequence and are noted at the base of each node. Environmental samples (indicated by E) are highlighted by *blue boxes*. The phylogenetic color scheme in Figs. 5–9 corresponds to that of Fig. 2.

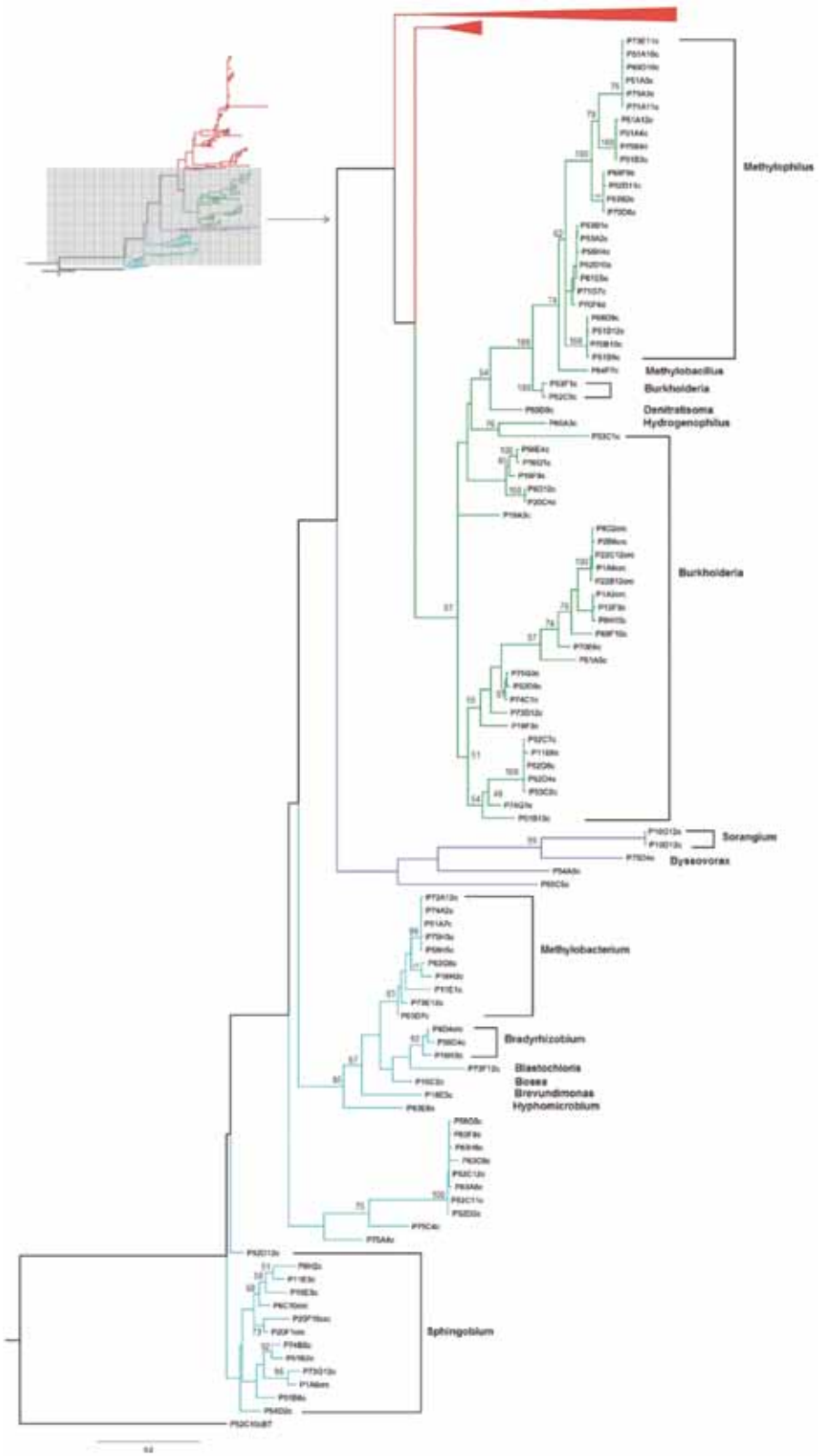

Fig. 6. Phylogenetic relationships among Beta-, Alpha-, and Deltaproteobacteria.

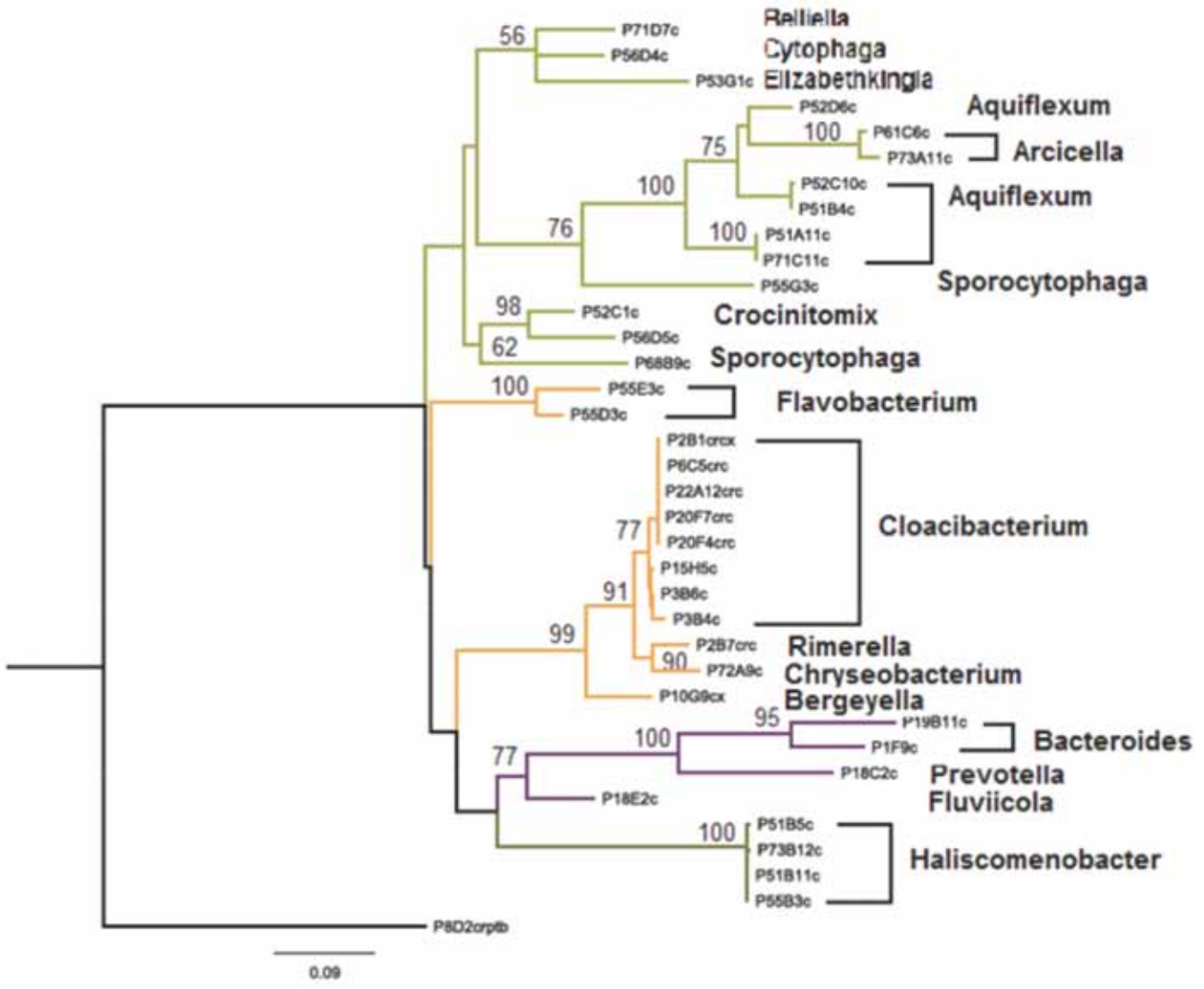

Fig. 7. Phylogenetic relationships among different isolates belonging to the phylum Bacteroidetes.

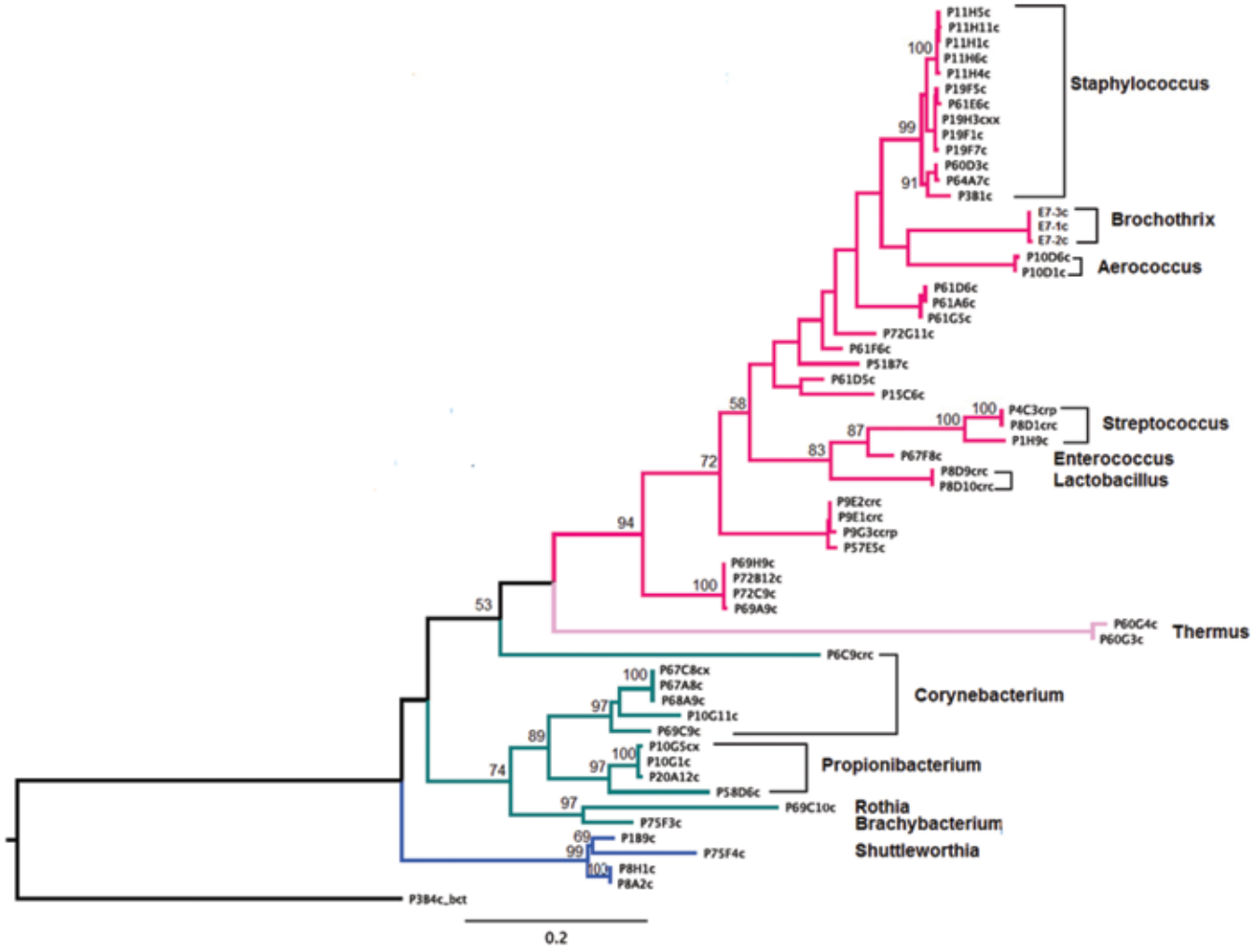

Fig. 8. Phylogenetic relationships among the gram-positive isolates belonging to the phyla Firmicutes and Actinobacteria and the genus *Deinococcus*.

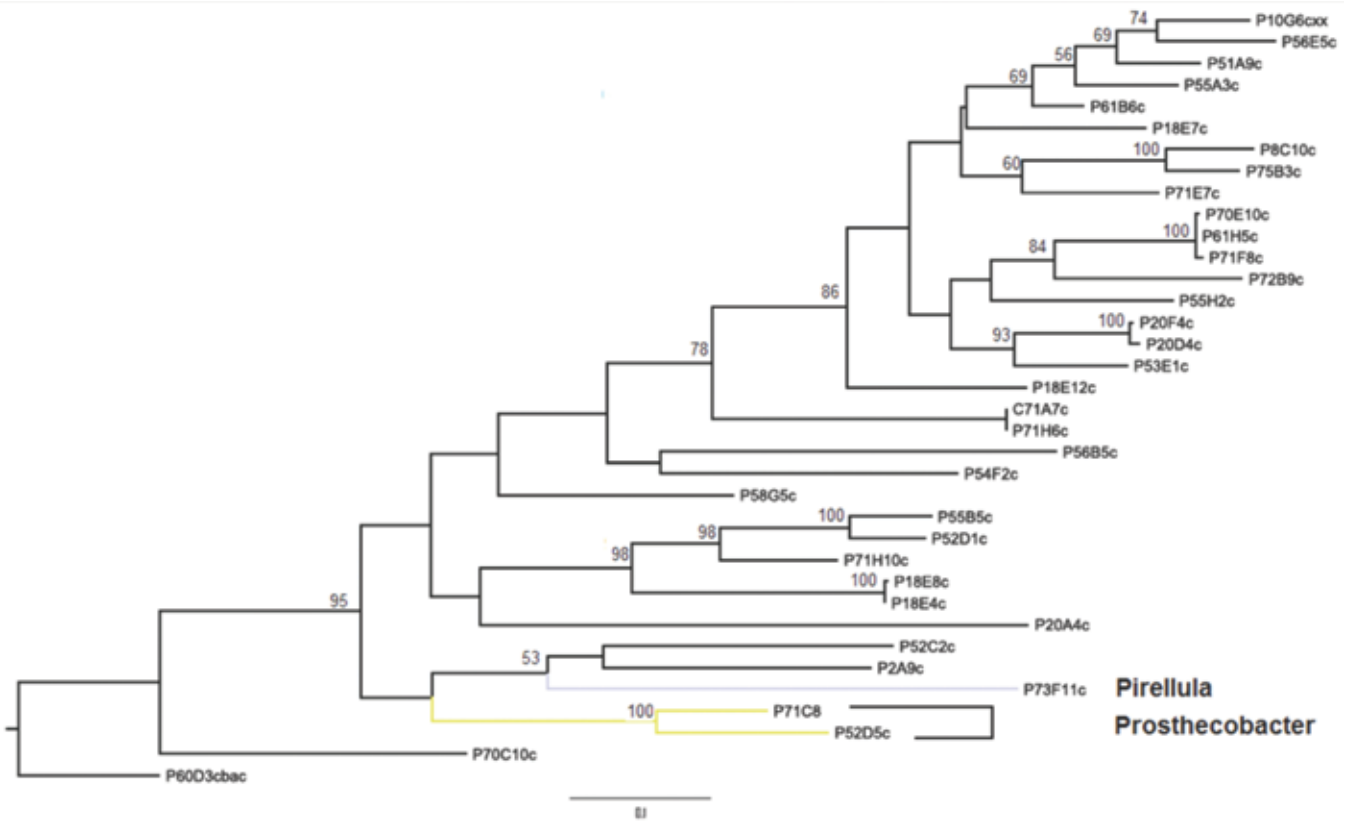

Fig. 9. Phylogenetic relationships among the unclassified isolates.

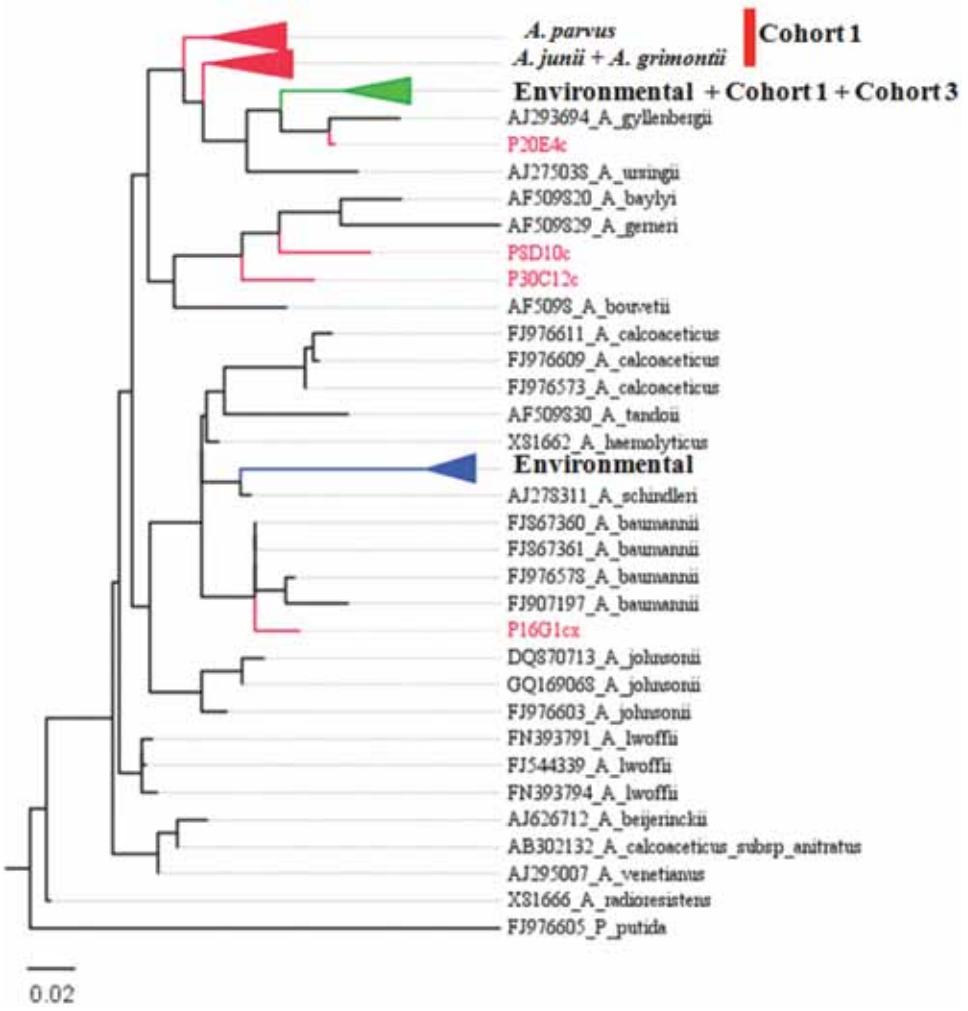

Fig. 10. Species affiliation of the *Acinetobacter* isolates collected from Cohorts 1 and 3 and the environment. Reference sequences are displayed with the GenBank accession numbers. *Red* and *blue* represent Cohort 1 and the environmental samples, respectively. *Green* represents clustering of a few samples from Cohorts 1 and 3 with the environmental samples. The names in *red* (starting with P) are individual patient samples from Cohort 1 that did not cluster in the other groups.

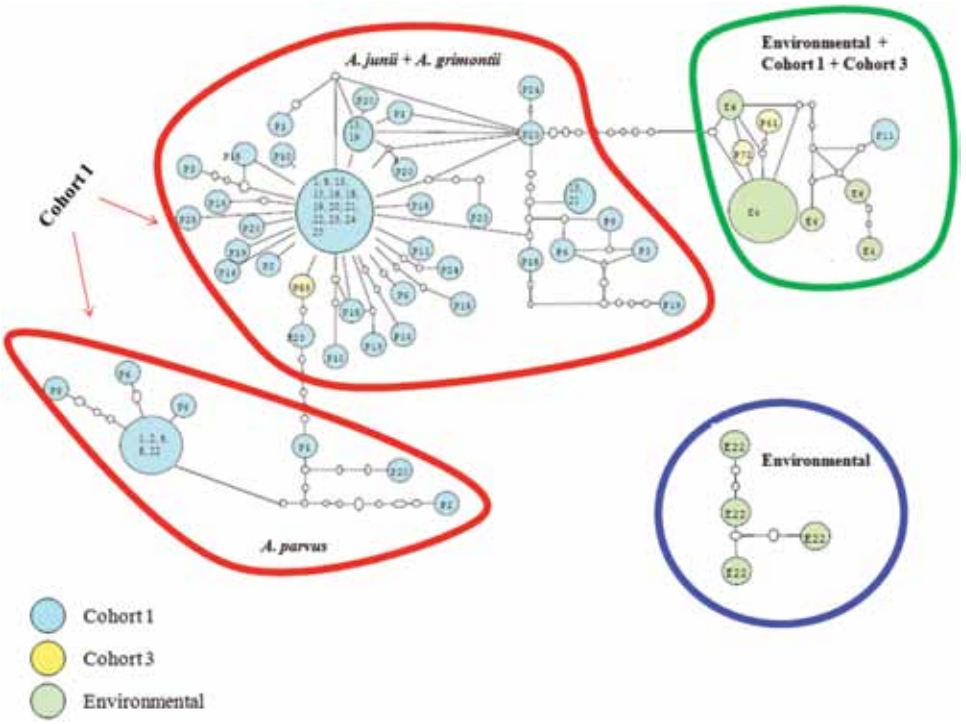

Fig. 11. Genetic relationships among the individuals within each cluster. There are 4 unique clusters. While 3 clusters are connected to each other, 1 cluster, mostly represented by the environmental samples, could not be connected to the other clusters. Each *colored circle* within each cluster represents a unique sequence, and the *small white circles* represent a hypothetical sequence not observed in the sample that is needed to connect samples with 95% confidence. The *solid line* between 2 consecutive circles represents 1 mutational difference. The size of the circle is proportional to the number of identical sequences from different patients. Case number is indicated in each circle. Cohort 1 and 3 sequences appear in *blue* and *yellow*, respectively. Environmental samples appear in *green. Red, green*, and *blue lines* surrounding the clusters refer to the colored wedges shown in the phylogeny in Fig. 10.