

Pyrosequencing reveals the complex polymicrobial nature of invasive pyogenic infections: microbial constituents of empyema, liver abscess, and intracerebral abscess

C. D. Sibley · D. L. Church · M. G. Surette ·
S. E. Dowd · M. D. Parkins

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Abstract The polymicrobial nature of invasive pyogenic infections may be underestimated by routine culture practices, due to the fastidious nature of many organisms and the loss of viability during transport or from prior antibacterials. Pyrosequencing was performed on brain and liver abscesses and pleural fluid and compared to routine culture data. Forty-seven invasive pyogenic infection samples from 44 patients [6 intracerebral abscess (ICA), 21 pyogenic liver abscess (PLA), and 18 pleural fluid (PF) samples] were assayed. Pyrosequencing identified an etiologic microorganism in 100 % of samples versus 45 % by culture, $p < 0.01$. Pyrosequencing was also more likely than traditional cultures to classify infections as polymicrobial, 91 % versus 17 %,

$p < 0.001$. The median number of genera identified by pyrosequencing compared to culture was 1 [interquartile range (IQR) 1–3] versus 0 (IQR 0–1) for ICA, 7 (IQR 1–15) versus 1 (IQR 0–1) for PLA, and 15 (IQR 9–19) versus 0 (IQR 0–1) for PF. Where organisms were cultured, they typically represented the numerically dominant species identified by pyrosequencing. Complex microbial communities are involved in invasive pyogenic infection of the lung, liver, and brain. Defining the polymicrobial nature of invasive pyogenic infections is the first step towards appreciating the clinical and diagnostic implications of these complex communities.

C. D. Sibley · M. G. Surette · M. D. Parkins
Department of Microbiology, Immunology and Infectious
Diseases, The University of Calgary,
3330 Hospital Drive NW, Calgary Laboratory Services,
Calgary, AB T2N 4N, Canada

M. D. Parkins (✉)
Department of Medicine, The University of Calgary,
3330 Hospital Drive NW, Calgary Laboratory Services,
Calgary, AB T2N 4N, Canada
e-mail: mdparkin@ucalgary.ca

D. L. Church
Department of Pathology and Laboratory Medicine,
The University of Calgary,
3330 Hospital Drive NW, Calgary Laboratory Services,
Calgary, AB T2N 4N, Canada

M. G. Surette
Department of Medicine, McMaster University,
Hamilton, ON, Canada

S. E. Dowd
Molecular Research LP (MR DNA),
Shallowater, TX, USA

Introduction

Invasive pyogenic infections are responsible for a large burden of disease in general populations [1]. Local tissue invasion by pathogenic bacteria and the resultant formation of a well-vascularized capsule and containment of infection through a focused host immune response characterize pyogenic infections. Abscesses can occur at any sterile site within the human body; however, the pathophysiologic mechanisms involved in each have unique features. The microbial pathogens classically associated with abscess formation have been derived from decades of traditional culture-based microbial practices and are relatively conserved between different abscess types. While the vast majority of invasive infections yield only a single pathogen (monomicrobial), polymicrobial infections have been demonstrated to occur in at least 11–18 % of intracerebral abscesses (ICAs) [2, 3], 11–49 % of pyogenic liver abscesses (PLAs) [4–6], and 10–15 % of empyemas [7]. This value is thought to under-represent the true diversity of polymicrobial infections owing to the limitations of traditional culture techniques,

effects of prior antibacterials, loss of organism viability during transport, and the presence of fastidious or uncultivable microorganisms. Furthermore, our understanding of bacterial infectious diseases is changing through the application of culture-independent technologies to complement traditional culture-based techniques. Recent work has shown that the diversity of bacterial species is much greater than originally thought in models of surgical site infections [8], diabetic foot infections [9, 10], brain abscesses [11, 12], and cystic fibrosis lower airways disease [13–16].

We hypothesized that traditional culture-based recovery of organisms present in invasive pyogenic collections likely underestimates the abundance and diversity of organisms present in abscesses. We sought to compare the microbial constituents identified in ICA, PLA, and empyema specimens by using culture-dependent and culture-independent approaches (pyrosequencing). The laboratory results were then used to determine if an expanded spectrum of causative microbial pathogens influenced patient outcomes.

Methods

Study population

Randomly selected samples of patients with suspected pyogenic infections during the period July 2009–October 2010 were identified from a centralized laboratory servicing four acute care hospitals within the Calgary Health Zone (CHZ). Clinical, demographic, microbiological, treatment, and outcome information for these patients was collected through a detailed chart review and diagnosis was confirmed. Approval was obtained from the Conjoint Health Research Ethics Board at the University of Calgary and the CHZ prior to commencement of this study.

Pyogenic samples

Clinical specimens were cultured aerobically and anaerobically. Aerobic culture was performed on blood agar, MacConkey agar, and colistin–nalidixic acid media at 35 °C in 5 % CO₂ for 48 h. Anaerobic culture was performed using brucella, kanamycin–vancomycin laked blood (KVLB), *Bacteroides* bile esculin (BBE), and phenylethyl alcohol (PEA) agar in the Anoxomat (Mart Microbiology, AJ Drachten, the Netherlands) anaerobic system environment at 35 °C for 96 h. Isolates were identified to the species level using standard biochemical methods using the automated Vitek 2 system (bioMérieux, Laval, Quebec, Canada), and partial sequencing of the 16S rRNA gene according to standard methods [17, 18]. Antibiotic susceptibility testing was performed on all isolates according to published guidelines [17, 19]. The remaining portion of

each pyogenic sample was stored at –80 °C for an extended period of time and then analyzed en masse to determine their molecular constituents.

DNA extraction

Pus/tissue was homogenized and 200 mg was aseptically suspended in 500 µl RLT buffer with β-mercaptoethanol (Qiagen, Valencia, CA). A sterile 5-mm steel bead (Qiagen, Valencia, CA) and 500-µl volume of sterile 0.1-mm glass beads (Scientific Industries, Inc., NY, USA) were used for complete bacterial lyses in a Qiagen TissueLyser (Qiagen, Valencia, CA) at 30 Hz for 5 min. Samples were centrifuged, and 100 µl of 100 % ethanol was added to a 100-µl aliquot of the sample supernatant. This mixture was added to a DNA spin column and DNA extraction protocols were followed (Qiagen, Valencia, CA). DNA was eluted from the column with 50 µl of sterile water and samples were diluted to a final concentration of 20 ng/µl. DNA samples were quantified using a NanoDrop spectrophotometer (Nyxor Biotech, Paris, France).

Massively parallel bTEFAP

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed as previously described using Gray28F 5'-TTTGATCNTGGCTCAG and Gray519r 5'-GTNTTACNGCGGCKGCTG [9, 10, 16, 20, 21]. Tag-encoded FLX amplicon pyrosequencing analyses utilized a Roche 454 FLX instrument with Titanium series reagents and procedures.

Bacterial diversity analysis

Following sequencing, all failed sequence reads, products less than 350 bp in length, and reads of low-quality sequences were removed; tags and primers were removed. Sequence collections were depleted of any non-bacterial ribosome sequences and chimeras using B2C2 [22], as previously described [9, 10, 16, 20, 21]. To determine the identity of bacteria in the sequence collection, sequences were denoised, assembled into clusters, and queried with a distributed BLASTn .NET algorithm [23] against a database of high-quality bacterial 16S rRNA sequences from the NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Database sequences were characterized as high quality based upon similar criteria utilized by the Ribosomal Database Project (RDP) version 9 [24]. Using a .NET and C# analysis pipeline, the resulting BLASTn outputs were compiled, validated using taxonomic distance methods, and data reduction analysis was performed as described previously [9, 10, 16, 20, 21]. Inclusion criteria for further analysis included greater than 1,000 sequence reads per specimen.

Taxonomic determination

Based upon the BLASTn-derived sequence identity (percentage of the total length of the query sequence which aligns with a given database sequence) that was validated using taxonomic distance methods, bacteria were classified at the appropriate taxonomic levels based upon the following criteria. Sequences with identity scores to known or well characterized 16S rRNA sequences greater than 95 % were resolved to the genus level, 90–95 % to the family level, 85–90 % to the order level, 80–85 % to the class level, and 77–80 % to the level of phyla. After resolving based upon these parameters, the percentage of each bacterial ID was individually analyzed for each sample, providing relative abundance information within and among the individual samples. Evaluations presented at each taxonomic level, including percentage compilations, represent all sequences resolved to their primary identification or their closest relative, as has been previously described [16]. Organisms identified with sequence reads representing less than 1 % of the total population (i.e., <10 sequence reads per 1,000 total sequence reads) were excluded from the analysis.

Definitions

Abscesses were subcategorized according to their pathogenesis as identified by a detailed chart review [25]. ICAs were defined as being either odontogenic, sinus, otitis, penetrating/nosocomial, or hematogenous seeding in origin. PLAs were defined as being biliary, hepatic artery (i.e., hematogenous seeding), portal vein (intraabdominal/visceral/bowel in origin), direct extension from contiguous focus of infection, penetrating trauma, or amebic liver abscess in origin. Exudative effusions were classified as complex parapneumonic effusions or empyemas [26]. Bacteremia was defined by the isolation of an organism from one or more sets of aseptically obtained blood culture bottles [27].

Prior antibacterial therapy was classified based on the intended spectrum of activity against abscess pathogens [25]. Regimens containing metronidazole, clindamycin, carbapenem, or a β -lactam/ β -lactamase inhibitor combination were classified as having “anaerobic activity”. Regimens including a fluoroquinolone, macrolide, or tetracycline were described as having “atypical activity”. Regimens containing either a fluoroquinolone, aminoglycoside, a second-, third-, or fourth-generation cephalosporin, carbapenem, or β -lactam/ β -lactamase inhibitor combination were classified as having “Gram-negative activity”. Vancomycin was analyzed separately from other Gram-positive active drugs. “Gram-positive active” drugs were considered to be first-generation cephalosporins, ceftriaxone/cefotaxime, fourth-generation cephalosporins, carbapenems, β -lactam/ β -

lactamase inhibitor combinations, or a third- or fourth-generation fluoroquinolone.

In an analysis focused on bacterial DNA, antibiotic susceptibility testing was obviously not possible. For the purposes of this assessment, several assumptions were made regarding the susceptibility of organisms based on the genus-level identification [25, 28–30]. *Enterococcus* spp. were assumed to be resistant to all cephalosporin antibiotics. *Stenotrophomonas* spp. were assumed to be resistant to all cephalosporins and carbapenems. *Pseudomonas* spp. were assumed to be resistant to aminopenicillins, first- and second-generation cephalosporins, and ceftriaxone/cefotaxime. The tribe *Proteeae* were assumed to have resistance to first-, second-, and third-generation cephalosporins. Organisms that were obligate anaerobes were presumed to be resistant to first-generation cephalosporins. Atypical organisms (such as *Mycoplasma* and *Chlamydia*) were assumed to be resistant to cell wall-active agents.

Nosocomial-associated (NA) infections were those occurring ≥ 48 h after hospital admission or within 48 h of discharge from the hospital. Community-associated (CA) infections were those which were first identified <48 h after hospital admission or >48 h after discharge. Infections were defined as being healthcare-associated, community-associated (HCA-CA) if the patients had recent prior contact with the healthcare system, as previously described [27]. CA infections were those which were of community onset that were not healthcare associated.

Statistical analysis

Individual variables were assessed using histograms prior to analysis in order to identify their underlying distribution. Variables with normal or near-normal distributions were described with means and standard deviations (SDs) and compared using Student's *t*-test. Medians with interquartile ranges (IQRs) were used to describe non-normally distributed variables and compared using the Mann–Whitney *U*-test. Categorical variables were compared using Fisher's exact test. All statistical analyses were performed using Stata version 11.0 (Stata Corp., College Station, TX, USA).

Results

Patient characteristics

Over 15 months, 102 randomly selected clinical samples submitted for aerobic/anaerobic culture from 94 patients with presumptive invasive pyogenic infections were identified and immediately frozen at -80 °C. All of the study samples analyzed had been collected as part of each

patient's routine care. After detailed clinical review, only 57 samples from 52 patients collected over 12 months were confirmed to represent invasive pyogenic infection (other samples that were excluded included cancerous lesions or uninfected cysts, etc). Of these, 47 samples from 44 patients met the a priori criteria of yielding >1,000 sequence reads and were included in the final analysis.

Eight brain abscess samples were procured from six patients. The median age of the patients with ICAs was 36 years (IQR 4–50). Etiologies were odontogenic in four [right frontal (2), right fronto-temporal (1), left fronto-temporal (1)], sinus in one (right frontal), and hematogenous in one (thalamic). The co-morbidities of patients with ICAs are listed in Table 1. Three cases were CA and three were HCA-CA. Recognized risk factors for the acquisition of ICA included alcoholism in 3/6 cases and active chemotherapy for breast (1/6) and prostate cancer (1/6 cases).

Twenty-one liver abscess samples were procured from 20 patients. The median age of the patients was 61 years (IQR 51–72). Five cases were NA, three cases HCA-CA, and 12 cases were CA. Etiologies were infected cyst (1), biliary 4 (right lobe 3, left lobe 1), portal vein 13 (11 right lobe, 2 multiple focus), and one each of direct extension from a contiguous source of infection (right lobe) and hepatic artery (right lobe). Identified risk factors included chronic biliary disease 2/20 (10 %), alcoholism or substance abuse 4/20 (20 %), diabetes mellitus 4/20 (20 %), cancer 4/20 (20 %), and inflammatory bowel disease 3/20 (15 %).

Eighteen pleural samples were procured from 18 patients; five had complex parapneumonic effusions (four right-sided, one left-sided), and 13 had empyemas (nine left-sided, four right-sided). Seven cases were NA, four

cases were HCA-CA, and seven cases were CA. Identified co-morbidities for those with empyemas included chronic respiratory disease 9/18 (50 %), diabetes mellitus 7/18 (30 %), cancer 4/18 (22 %), and alcoholism and/or substance abuse 4/18 (22 %).

Microbial constituents

Pyrosequencing yielded 269,303 total sequence reads from the 47 included pyogenic samples. The median number of sequence reads for ICAs was 7,540 (IQR 4,839–11,747), liver 6,555 (IQR 3,292–10,151), and lung 2,667 (IQR 1,364–6,336).

Routine culture practices were less likely to identify an etiologic microorganism [45 % of samples (21/47)] as compared to pyrosequencing [100 % of samples (47/47)], $p < 0.001$. Pyrosequencing was more likely than traditional cultures to identify polymicrobial infections [91 % (43/47) vs. 17 % (8/47)], $p < 0.001$.

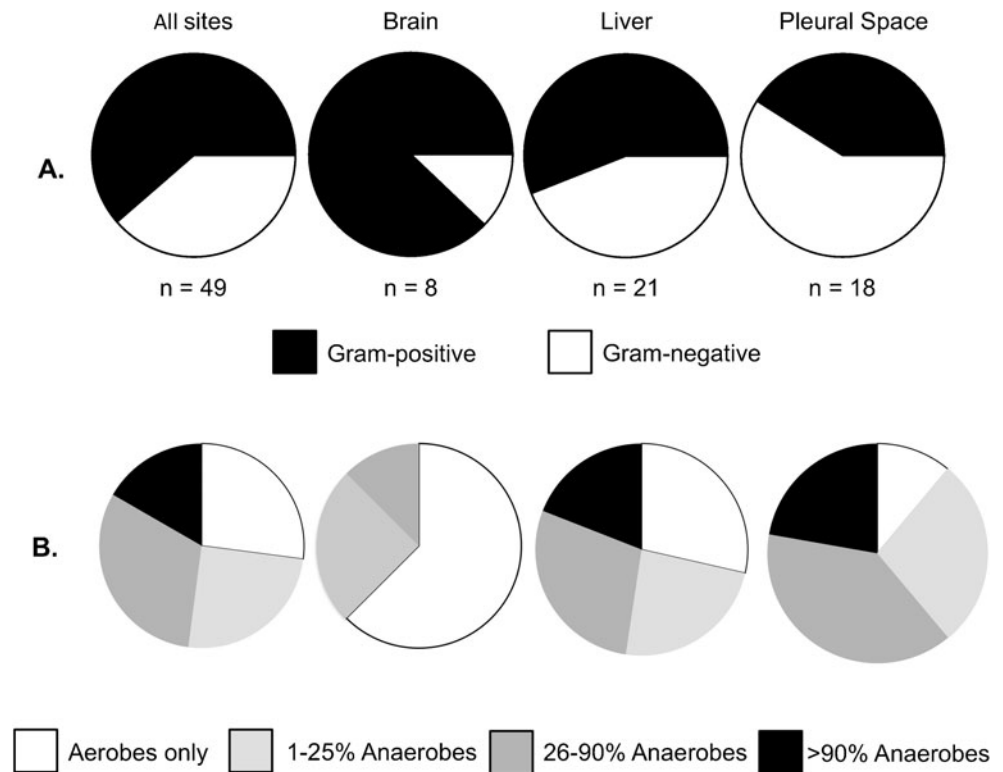
Pyrosequencing revealed that the majority of bacterial genera recovered from the brain were Gram-positive organisms, with obligate anaerobes being uncommon components of ICAs (Fig. 1). Within ICAs, four samples yielded an etiologic organism by culture, all growing monomicrobial *Streptococcus milleri* group (SMG) as the sole pathogen. Pyrosequencing confirmed three of these to be monomicrobial with organisms within the genus *Streptococcus*, accounting for >99.9 % of the total sequence reads. However, the fourth revealed the *Streptococcus* genus to account for 38.1 % of sequence reads, with significant concentrations of *Fusobacterium* spp. (53.2 %) and *Clostridium* spp. (8.0 %). Four samples failed to yield an organism through conventional culture-based approaches but had etiologic agents identified as a result of prior microbiologic testing [SMG bacteremia (2), and CONS and *Propionibacterium* spp. from abscess specimens from the same infection at a different point in time (2)]. Culture-independent analysis indicated that three of these specimens were polymicrobial (2, 7, and 27 genera identified) and one was monomicrobial. In those ICA samples with an organism identified by culture, the cultured organism accounted for the majority of sequences identified (97 %; range 38–100 %) (Fig. 2).

Eight PLA samples were culture-negative, 11 samples were monomicrobial (*E. coli* 4, *Proteus mirabilis* 3, and one each of *Candida albicans*, *Bacillus* spp., SMG *Fusobacterium* spp., and *Propionibacterium acnes*), two samples were polymicrobial [one with *E. coli*, *S. aureus*, SMG, and *Streptococcus viridans* group (SVG), and the other growing *Klebsiella pneumonia*, *Citrobacter koseri*, and SMG]. Pyrosequencing demonstrated a much broader range of organisms; a median

Table 1 Patient cohort characteristics as per the site of pyogenic infection

Co-morbidity	Brain, n=6 (%)	Liver, n=20 (%)	Pleural space, n=18 (%)
Cardiac disease	0/6 (0)	13/20 (65)	6/18 (33)
Chronic respiratory disease	2/6 (33)	5/20 (25)	9/18 (50)
Diabetes mellitus	0/6 (0)	4/20 (20)	7/18 (39)
Cancer	2/6 (33)	4/20 (20)	4/18 (22)
GERD/PUD	1/6 (17)	6/20 (30)	2/18 (11)
Crohn's/diverticulitis	0/6 (0)	3/20 (15)	2/18 (11)
Depression	2/6 (50)	1/20 (5)	2/18 (11)
Chronic hepatitis	0/6 (0)	1/20 (5)	4/18 (22)
Chronic hepatobiliary disease	0/6 (0)	2/20 (10)	0/18 (0)
Alcoholism or IVDU	3/6 (50)	4/20 (20)	4/18 (22)
Chronic renal disease	0/6 (0)	1/20 (5)	1/18 (6)
Chronic neurologic disease	0/6 (0)	1/20 (5)	2/18 (11)
Rheumatologic disease	0/6 (0)	1/20 (5)	1/18 (6)

Fig. 1 Microbial community composition of pyogenic specimens. **a** Classified as either predicted Gram-positive or Gram-negative bacteria. **b** Community members as determined by the presence of predicted obligate anaerobes (each individual member having to be present at a rate of >1 % of the total population)



of seven genera per sample (IQR 1–15, range 1–22) versus culture, which yielded one genus per sample (IQR 0–1, range 0–4). The majority of genera in PLA samples were Gram-positives (Figs. 1 and 3). The most commonly identified organisms from PLAs (identified at concentrations greater than 5 % in at least two samples) included: *Streptococcus*, *Lactobacillus*, *Stenotrophomonas*, *Enterobacter*, *Escherichia*, and *Clostridium* (Fig. 4). Culture failed to identify low-abundance organisms and anaerobes/nutritionally fastidious organisms not previously described in PLAs (Fig. 2). In PLAs where an organism(s) was identified by culture, it accounted for a median of 86 % of sequence reads (IQR 13–89 %; range 1–99 %), indicating that culture-based approaches are effective at recovering numerically dominant organisms. Anaerobes were found in 71 % of samples (Fig. 1) and often comprised the dominant community members (Figs. 3 and 4).

In 18 empyema/parapneumonic effusion cultures, there were three culture-positive samples; one monomicrobial (CONS) and two polymicrobial (methicillin-resistant *Staphylococcus aureus* and CONS, and *S. pneumoniae* and *Peptostreptococcus* spp.). These organisms accounted for a median of 64 % (IQR 27–80 %) of sequences identified from those culture-positive samples. Considerably greater diversity was identified in microbial constituents by using a culture-independent approach; a median of 15 genera by pyrosequencing (IQR 9–19, range 3–27) versus a median of zero

genera by culture (IQR 0–0, range 0–2). Specimens from the pleural cavity were the only sample type where it was more common to detect Gram-negatives than Gram-positives (Fig. 1). The vast majority (89 %) of these samples contained obligate anaerobes, often as the dominant community member (Figs. 3 and 4).

Bacteremia was more likely to accompany specific infections; 45 % (9/20) PLA, 17 % (1/6) ICA cases, and 17 % (3/18) respiratory pleural infections, $p=0.05$. Bacteremias were monomicrobial in 91 % (10/11) of episodes and polymicrobial in 9 % (1/11). The most frequent bacteremic isolates were: SMG (1 ICA, 3 PLA), followed by SVG (2 PLA), *E. coli* (2 PLA), and one of each *C. perfringens* (PLA), *Corynebacterium amycolatum* (empyema), methicillin-resistant *Staphylococcus aureus* (empyema), *Enterococcus faecium* (PLA), and *Enterobacter cloacae* (PLA). In bacteremic patients, the organism recovered by the blood culture was also the most abundant organism identified by pyrosequencing in 91 % (10/11) of episodes, accounting for a median of 78 % of the total sequence reads (IQR 39–97 %).

Taxonomic classification at the family level revealed that 10 % (2/20), 16.3 % (7/43), and 38.1 % (24/64) of the organisms recovered from brain, liver, and lung samples were unique to that specific specimen type, respectively (Fig. 5). A detailed list of these families is provided in Table 2. Eleven bacterial families were detected in all pyogenic specimens (from at least two samples).

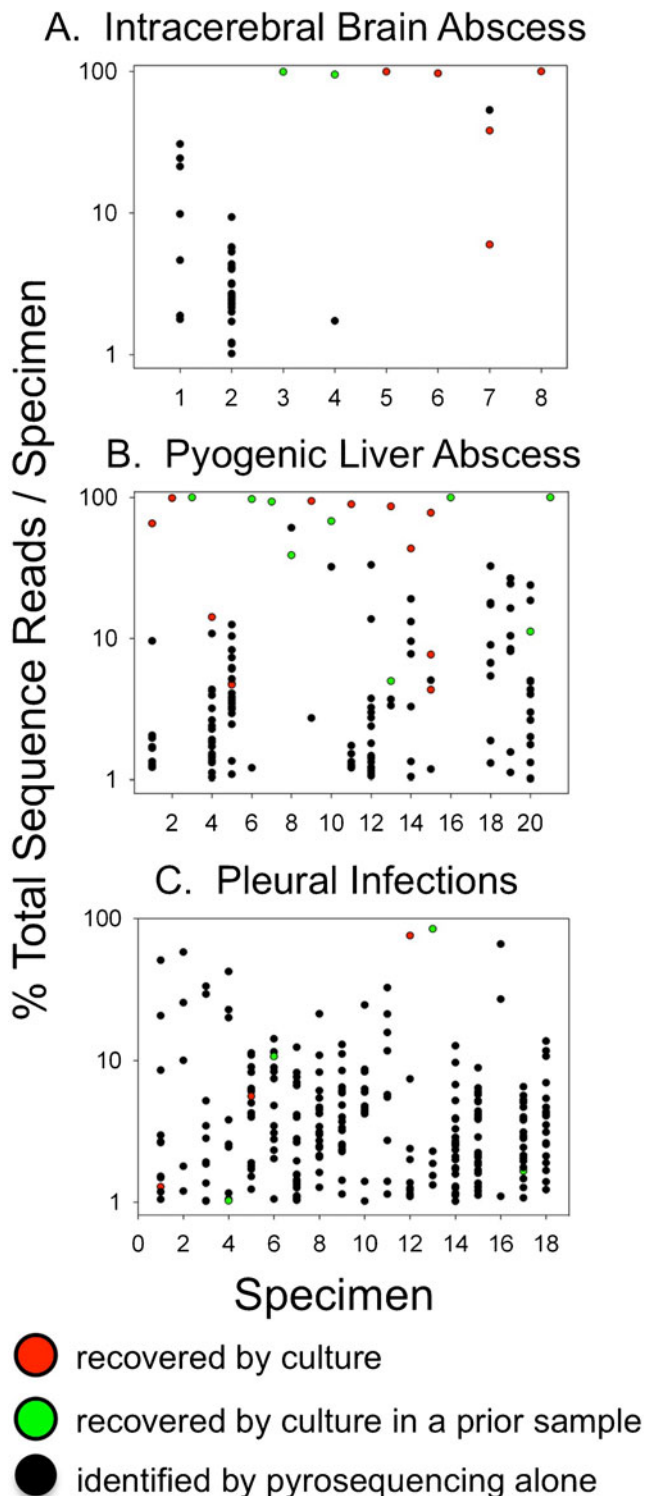


Fig. 2 Identified constituent organisms of pyogenic specimens as a function of relative abundance within the community, as measured by pyrosequencing. **a** Intracerebral brain abscess. **b** Pyogenic liver abscess. **c** Pulmonary empyema/complicated pleural effusion. Those organisms identified molecularly that were also recovered using culture-based approaches are illustrated within the accompanying legend

Effects of prior empiric antibiotics

Empiric antibacterial therapies were provided before culturing in 44/47 samples (94 %). The median duration of antibiotic exposure before sampling was 6 days (IQR 1.2–11 days; range 0–53 days). Antibiotic regimens administered to the six patients with ICAs before aspiration had broad-spectrum Gram-positive [83 % (5/6)] and Gram-negative activity [83 % (5/6)], and were supplemented with vancomycin [83 % (5/6)] and occurred for a median of 4 days (IQR 0.1–53 days). Anaerobic treatment was provided in 83 % (5/6). No ICAs were treated with antibiotics with atypical organism coverage. Empiric regimens provided before the sampling of PLAs in 20 patients contained broad-spectrum Gram-positive [75 % (15/20)] and Gram-negative activity [95 % (19/20)], and were supplemented with vancomycin [15 % (3/20)] and occurred for a median of 8 days (IQR 1–12 days) before aspiration. These regimens covered for anaerobic microorganisms or atypical organisms in 90 % (18/20) and 30 % (6/20) of cases, respectively. Empyema cases were treated for a median of 5 days (IQR 2.2–9.5 days) before thoracentesis and contained Gram-positive [78 % (14/18)] and Gram-negative [89 % (16/18)] activity supplemented with vancomycin in 33 % (6/18) of cases. The regimens had anaerobic and atypical activity in 61 % (11/18) and 78 % (14/18) of cases, respectively.

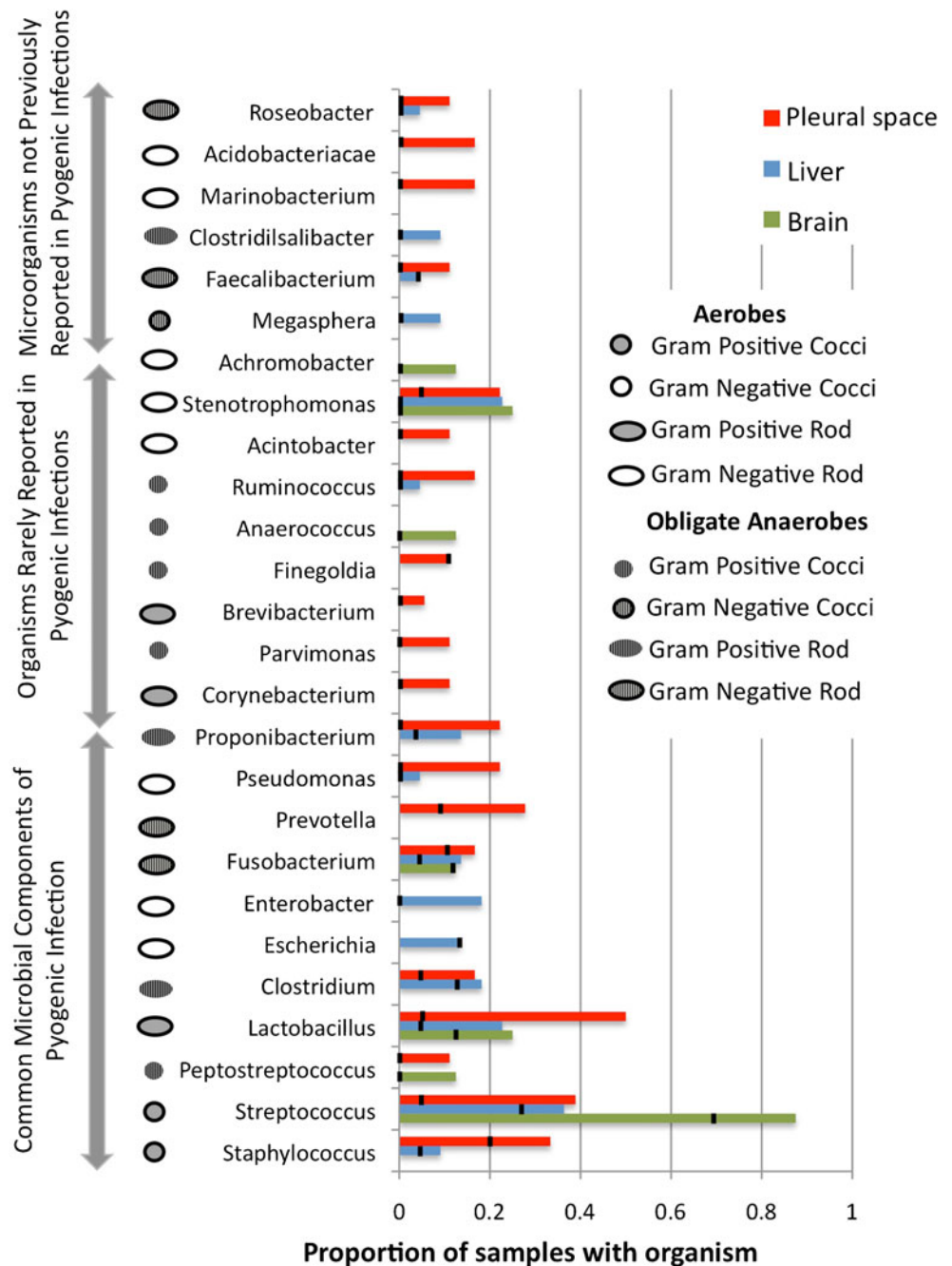
The number of organisms recovered from culture-based approaches decreased with antibiotic exposures beyond one day; median one versus zero, $p=0.001$. However, bacterial nucleic acid could be identified in all samples. As expected, the number of bacterial organisms detected by culture-independent processes did not change as a result of empiric therapy stratified by more or less than 1 day (mean 10.4 vs. 10.2 genera/sample, $p=0.91$), or more or less than 5 days (median 9.6 vs. 9.2 genera/sample, $p=0.61$). The emergence of bacteria intrinsically resistant to antibacterial agents was not observed with a longer duration of empiric antibacterial therapies when comparing the results of samples taken before or after 1 and 5 days of therapy (Table 3).

Treatment and outcomes

All patients were treated for invasive pyogenic infections. The median treatment duration for ICAs was 66 days (IQR 61–71 days), 50 days for PLAs (IQR 34–81 days), and 44.5 days (IQR 25–49 days) for empyema. Intensive care unit support was required for 3/6 (50 %) cases of ICA, 1/20 (5 %) cases of PLA, and 9/18 (50 %) cases of empyema. All patients initially received therapy with more than one antibacterial agent. Only 11 % (5/44) of the patients had their initial empiric regimen narrowed to account for only those organisms identified in culture.

The all-cause in-hospital mortality was 0/6 (0 %) for ICAs, 2/20 (10 %) for PLAs, and 3/18 (17 %) for

Fig. 3 Relative frequency of microorganisms identified in pyogenic infections. Only organisms which account for $\geq 5\%$ of sequences within an individual sample are included for ease of visualization. The relative distance between each individual black line and the total abundance of each organism indicates the proportion of samples in which this organism was a numerically dominant member of the community



empyemas, $p=0.55$. Patients who died were no more likely to have positive abscess clinical cultures: 60 % (3/5) versus 44 % (17/39), $p=0.64$. Pyogenic samples with organisms presumed to be intrinsically resistant to specific classes of antibacterials were no more likely to require a prolonged course of antibiotics and did not have an increased risk of death (Table 4). In those patients whose treatment regimen lacked adequate antimicrobial activity against intrinsically resistant abscess component flora, there were no differences noted in either their treatment duration or outcome compared to those on appropriate therapy (Table 5).

Discussion

Several groups have explored the use of pyrosequencing to determine the richness of bacterial communities of various human sites. Whereas studies of the microbiome of the gastrointestinal tract [31, 32], superficial wounds [8, 10, 33–35], and lower airways of patients with chronic respiratory disease [14, 16, 36] must contend with the issue of external environmental contamination of samples, pyogenic infections represent invasion into normally sterile sites. As such, it can be assumed that any bacterial DNA present within an abscess is foreign in nature.

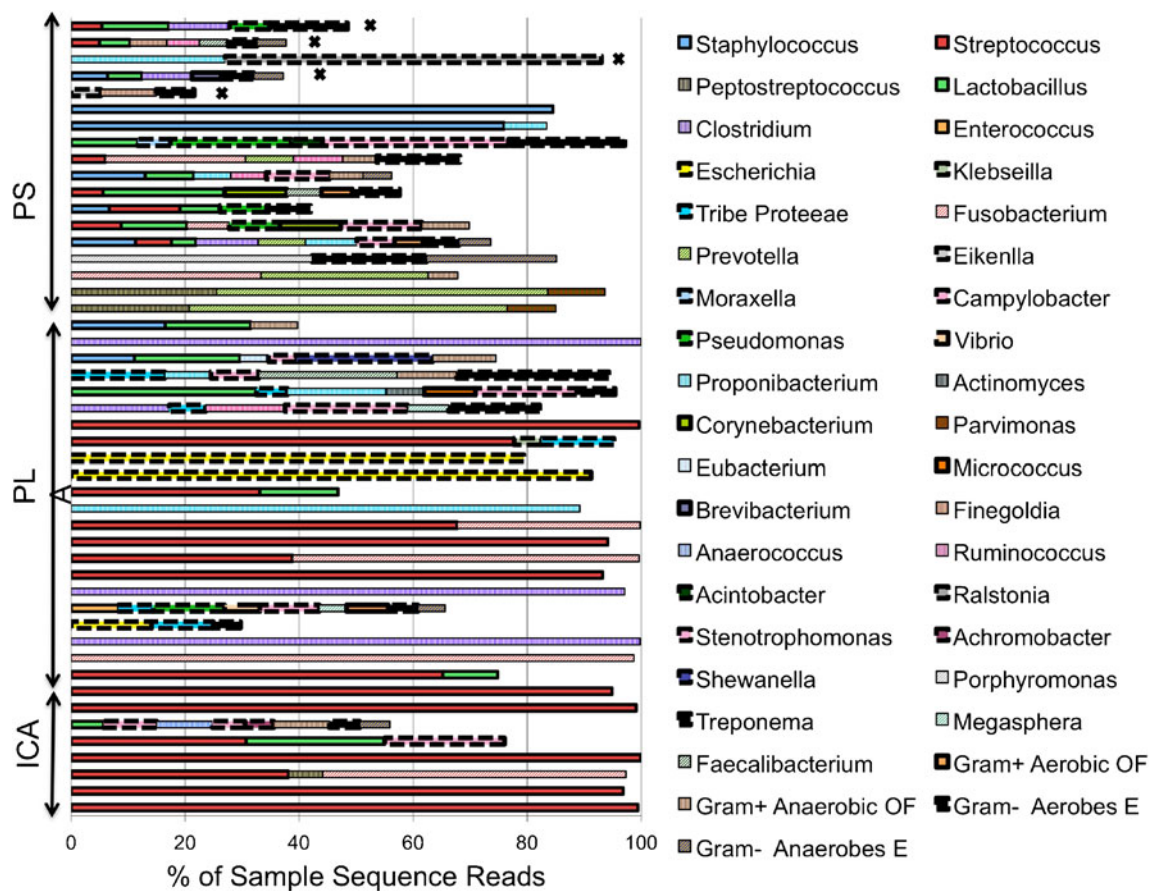


Fig. 4 Microbial composition of individual abscesses. Only organisms that account for $\geq 5\%$ of the total number of sequences within an individual sample are included for ease of visualization. The *solid black lines* around each respective color identify Gram-positive aerobes. Gram-positive obligate anaerobes are identifiable by *striped vertical bars*. The *dashed black lines* around each respective color indicate Gram-negative aerobes. The *angled bars* indicate Gram-negative anaerobes. Parapneumonic effusion are indicated by an *X*, *OF* denotes oropharyngeal flora, and *E* represents (classically) environmental isolates. *Several genera are grouped; Gram-negative environmental anaerobic organisms

included: *Leptotrichia*, *Tannerella*, *Merismopedia*, *Levilinea*, and *Roseobacter*. Gram-negative environmental aerobic organisms included: *Marinobacterium*, *Acidobacteriaceae*, *Hyphomonadaceae*, *Comamonadaceae*, *Pelagibacter*, *Rubellimicrobium*, *Agarivorans*, *Methylophilales*, *Nautella*, *Acidithiobacillus*, *Sphingobacterium*, and *Sulfitobacter*. Gram-positive anaerobic organisms included: *Peptoniphilus*, *Lachnospiraceae*, *Oscillospira*, *Ferrovum*, *Anaerococcus*, *Micrococccineae*, and *Clostridiisalibacter*. Gram-positive aerobic organisms included: *Janibacter*, *Conexibacter*, and *Arthromitus*. The tribe *Proteeae* includes *Enterobacter*, *Citrobacter*, and *Proteus*

A comprehensive understanding of the microbial constituents is fundamental to understanding the pathophysiology of pyogenic infections. Communication between bacterial community members have been shown to alter the virulence factor production of pathogens [37]. Therefore, the microbial community context in which pathogens exist during infection may be important in understanding clinical outcomes. In animal models of infection, even presumed benign commensal organisms have the ability to increase the pathogenicity of traditionally accepted pathogens [38, 39]. Community diversity may enable the development of a microniche, whereby different community members modify local environments to be more or less favorable to other community members [40, 41]. The impairment of host immune response may similarly be afforded through intraspecies phagocytic inhibition [40]. Finally, minor community members may have the ability to

limit the effects of clinical intervention through the production of secreted antibiotic-modifying enzymes which may serve to protect the entire community [42–44].

The data herein have demonstrated that the distribution of organisms involved in pyogenic infection to be more diverse than that recovered through routine clinical culture practice. Indeed, polymicrobial infection is the norm, as opposed to the exception in PLAs and empyemas. SMG was the most commonly cultivated pathogen in brain and liver abscess samples. Indeed, this organism is often reported as the most common organism from a variety of pyogenic infections, including: brain abscess [45–47], liver abscess [48, 49], and empyema [50, 51]. We found streptococcal DNA to be present in high abundance in all specimen types.

Only one other group has explored pyrosequencing to examine the polymicrobial community structure of

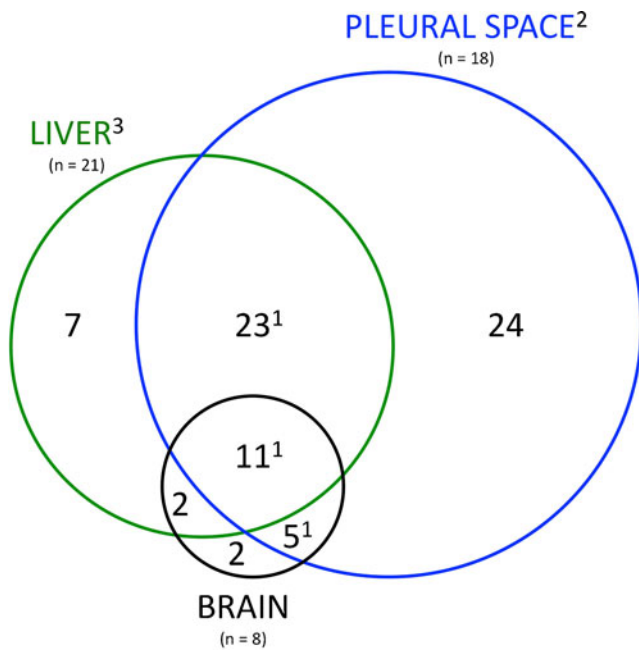


Fig. 5 Venn diagram displaying abscess microbial constituents organized into bacterial families. To be included, each organism had to be identified in >1 % of at least two independent samples. The *superscripts* indicate the number of order-level-only classifications within each category. A detailed breakdown is available in Table 2

pyogenic samples [11, 12]. In their initial study, Al Masalma et al. compared culture to pyrosequencing in patients with ICAs and found that an etiologic agent could be identified in all cases, that one-quarter of the presumed monomicrobial infections were indeed polymicrobial, and that the diversity of organisms in polymicrobial specimens was under-represented by culture [11]. Our results are in accord with these findings. In their most recent publication which included a much larger number of patient samples, they were even able to identify that the etiology of ICAs correlated with the microbiologic constituents (otitis media-associated cases were often monomicrobial, whereas dental- and sinusitis-associated cases were polymicrobial) [12].

The most frequently cultured organisms from PLAs in published series include *Enterobacteriaceae*, such as *E. coli* and *Klebsiella* spp., and SMG [5, 6, 52]. These genera were commonly identified in our pyrosequencing studies and, furthermore, represented the numerically abundant organisms in those samples that were culture-positive. However, a much greater diversity of minor community members was observed using pyrosequencing, with a seven-fold increase in the number of community members identified relative to traditional culture. Most notably, anaerobes were identified in more than three-quarters of samples as compared to published series, where these are found in less than 20 % of samples [5, 53, 54].

Most intriguingly was the information from the pleural infection samples. Whereas organisms are recovered from

Table 2 Bacterial families detected through the pyrosequencing of pyogenic specimens

Category	Family name	
All categories	<i>Alcaligenaceae</i>	
	<i>Campylobacteraceae</i>	
	<i>Comamonadaceae</i>	
	<i>Corynebacteriaceae</i>	
	<i>Enterobacteriaceae</i>	
	<i>Fusobacteriaceae</i>	
	<i>Lachnospiraceae</i>	
	<i>Rhodobacteraceae</i>	
	<i>Streptococcaceae</i>	
	<i>Vibrionaceae</i>	
	<i>Xanthomonadaceae</i>	
	Brain only	<i>Flavobacteriaceae</i>
		<i>Thermodesulfobiaceae</i>
		Liver only ^a
<i>Anaerolineaceae</i>		
<i>Caldilineaceae</i>		
<i>Flavobacteriaceae</i>		
<i>Hydrogenophilaceae</i>		
<i>Nitrospiraceae</i>		
<i>Shewanellaceae</i>		
Pleural space only ^a	<i>Acidimicrobiaceae</i>	
	<i>Acidobacteriaceae</i>	
	<i>Bacteriovoraceae</i>	
	<i>Bacteroidaceae</i>	
	<i>Caulobacteraceae</i>	
	<i>Desulfobacteraceae</i>	
	<i>Desulfuromonadaceae</i>	
	<i>Erysipelotrichaceae</i>	
	<i>Gemmatimonadaceae</i>	
	<i>Oxalobacteraceae</i>	
	<i>Intrasporangiaceae</i>	
	<i>Methylobacteriaceae</i>	
	<i>Microbacteriaceae</i>	
	<i>Neisseriaceae</i>	
	<i>Patulibacteraceae</i>	
	<i>Pelobacteraceae</i>	
	<i>Peptostreptococcaceae</i>	
	<i>Planctomycetaceae</i>	
	<i>Prevotellaceae</i>	
	<i>Pseudoalteromonadaceae</i>	
<i>Rhizobiaceae</i>		
<i>Rickettsiaceae</i>		
<i>Streptosporangiaceae</i>		
<i>Wolbachieae</i>		
Brain and liver	<i>Alteromonadaceae</i>	
	<i>Bradyrhizobiaceae</i>	
Liver and pleural space ^a	<i>Anaerolineaceae</i>	
	<i>Bacillaceae</i>	

Table 2 (continued)

Category	Family name
	<i>Brevibacteriaceae</i>
	<i>Burkholderiaceae</i>
	<i>Clostridiaceae</i>
	<i>Conexibacteraceae</i>
	<i>Desulfobulbaceae</i>
	<i>Enterococcaceae</i>
	<i>Erythrobacteraceae</i>
	<i>Eubacteriaceae</i>
	<i>Helicobacteraceae</i>
	<i>Micrococcaceae</i>
	<i>Moraxellaceae</i>
	<i>Pasteurellaceae</i>
	<i>Porphyromonadaceae</i>
	<i>Propionibacteriaceae</i>
	<i>Pseudomonadaceae</i>
	<i>Rhodocyclaceae</i>
	<i>Ruminococcaceae</i>
	<i>Sphingobacteriaceae</i>
	<i>Spirochaetaceae</i>
	<i>Staphylococcaceae</i>
	<i>Veillonellaceae</i>
Pleural space and brain ^a	<i>Clostridiales Family XI. Incertae Sedis</i>
	<i>Cytophagaceae</i>
	<i>Hyphomonadaceae</i>
	<i>Prochlorococcaceae</i>
	<i>Synergistaceae</i>

^a Categories with 16S rRNA sequence reads corresponding to organisms that could only be taxonomically classified to the order level. Sequence reads corresponding to organisms within the following orders were identified: *Clostridiales* in all specimen types; *Actinomycetales*, *Pleurocapsales*, and *Pseudomonadales* only in liver specimens; *Bacillales* and *Chroococcales* in lung specimens only; *Rickettsiales* in both liver and lung specimens; and *Methylophilales* in both lung and brain specimens

traditional culture in less than 15 % of empyema samples [50, 51], pyrosequencing detected bacteria in 100 % of samples. Furthermore, the greatest microbial diversity from each of the pyogenic infections was found in pleural

samples. Anaerobic microorganisms were found to predominate pleural samples in both the number of genera and the total relative abundance, with many of these anaerobes being common members of the oropharynx [55]. A great number of organisms classically described only in marine and freshwater environments were also identified and likely represent minor components of the oropharynx [55, 56]. Within normal healthy hosts, it has been demonstrated that the respiratory microbiome constituents are highly distinct from individual to individual [56, 57] and, as such, partially explains the diversity in the pleural samples assessed. Finally, no difference between complicated parapneumonic effusion samples and those of empyemas were observed, suggesting that these processes exist along a continuum.

In order to improve patient outcomes, empiric broad-spectrum anti-bacterial therapy is recommended for the management of deep-tissue pyogenic infection once infection is suspected, regardless of the microbiologic sampling. Prior therapy is associated with lower rates of pathogen identification by culture [58, 59]. Remarkably, pyrosequencing can identify bacterial DNA from pyogenic collections, even after more than 7 weeks of antibacterial therapy.

Many of the infections from our cohort were associated with minor component members that were inherently resistant to the antibacterial agents to which they were treated. However, this cross-sectional data suggests that infections with organisms with intrinsically resistant organisms were no more likely to result in protracted therapy or increase the risk of death even when these therapies were not provided. This is in accord with the clinical data from a number of situations, including diabetic foot infections [60, 61] and intraabdominal abscess [62]. Notably, in intraabdominal infections, despite their association with worse clinical outcomes, therapies with anti-enterococcal coverage do not result in improved outcomes relative to those that are enterococcal-sparing [62–64]. However, clinicians treat invasive pyogenic infections as polymicrobial, with broad-spectrum antibiotics for a pre-specified time periods, often without narrowing their regimen based on culture results, acknowledging the inherent limitations of culture results. As such, retrospective studies evaluating treatment duration may be too insensitive to allow assessing the influence of

Table 3 Constituents of invasive pyogenic collections as determined by pyrosequencing do not change according to the duration of prior empiric antibacterial therapy

Organism	Treatment duration			Treatment Duration		
	<1 day (%)	≥1 day (%)	<i>p</i> -value	<5 days (%)	≥5 days (%)	<i>p</i> -value
Any obligate anaerobe	9/12 (75)	30/36 (83)	0.40	15/20 (75)	24/28 (86)	0.29
<i>Pseudomonas</i>	3/12 (25)	10/36 (28)	0.58	6/20 (30)	7/28 (25)	0.47
<i>Stenotrophomonas</i>	2/12 (17)	13/36 (36)	0.19	5/20 (25)	10/28 (36)	0.32
<i>Enterococcus</i>	2/12 (17)	5/36 (14)	0.57	3/20 (15)	4/28 (14)	0.63
Tribe <i>Proteeae</i>	3/12 (25)	8/36 (22)	0.56	4/20 (20)	7/28 (25)	0.48

Table 4 Clinical outcome of invasive pyogenic infections defined by microbial constituents identified by retrospective pyrosequencing

Samples with >1 % of sequence reads	Treatment duration			Mortality		
	Yes (days)	No (days)	<i>p</i> -value	With factor (%)	Without (%)	<i>p</i> -value
Anaerobes	55	60.3	0.46	3/39 (8)	2/9 (22)	0.23
Atypicals	n/a	n/a	n/a	0/0	5/48 (10)	1
<i>Pseudomonas</i>	45.1	56.6	0.17	3/13 (23)	2/35 (6)	0.11
<i>Stenotrophomonas</i>	57.3	52.1	0.61	1/15 (7)	4/43 (9)	1
<i>Enterococcus</i>	49.5	54.4	0.52	0/7	5/41 (12)	0.44
Tribe <i>Proteaeae</i>	59.3	52.2	0.49	1/11 (9)	4/37 (11)	0.67

the polymicrobial community structure on clinical outcomes. It is possible that pyrosequencing may provide clinicians the confidence to offer narrow-spectrum antibiotics for shorter periods of time, thus, reducing antibiotic consumption and reducing the risk of emerging resistance. Prospective comparative studies evaluating pyrosequencing-supplemented culture results to the standard of care are required.

It is unlikely that all microorganisms identified within an abscess must be specifically targeted with antibacterial therapies. What is not clear, however, is which microorganisms do require targeted therapy. Is it sufficient to merely target the dominant community members—those identified by routine anaerobic culture practice? Could focused therapy against a particular minor but crucial community member be sufficient to enable cure? Given the potential of organisms at low concentrations to alter the pathogenicity of the entire community, targeted therapy towards such constituents may generate clinically relevant ecological disturbances.

The advantage of characterizing microbial communities using culture-independent molecular methodologies (based on the universally present 16S rRNA gene) include the ability to assess for those organisms that have been killed as a result of prior antibacterial therapy or during transportation/sampling and to identify those organisms that are fastidious or are uncultivable using routine microbiologic practices. However, inherent to such approaches is the fact that they identify nucleic acid belonging to an organism and cannot predict viability at the time of sampling. Therefore, the biological interactions that contribute to community

pathogenesis are not always clear. Novel approaches are increasingly being used to more effectively discern the composition of live organisms during microbial profiling [65, 66]; application of these towards the future characterization of the pyogenic infections will undoubtedly generate valuable data. Additionally, without the ability to grow and pass bacteria in the presence of antibacterial agents, vital antibiogram data are not available for clinical use.

There are a number of limitations of our study. This study was meant as an initial foray into the area of polymicrobial infections and, as such, we chose to sample three distinct pyogenic infections, each with a relatively small number of samples. This small sample size increases the risk of bias. The clinical significance of the polymicrobial nature of infections at these sites requires further investigation. Low-level bacterial DNA contamination has been demonstrated to exist in a number of the reagents for pyrosequencing [67, 68]. As such, sterile samples, when subjected to deep sequencing, have often identified low levels of bacterial DNA [56]. However, in clinical situations whereby high bacterial concentrations are expected, such as pus, low-level contamination would not be a significant issue and is minimized by using stringent criteria such as the analysis of only specimens with at least 1,000 sequence reads and the inclusion of only those organisms accounting for >1 % of the total sequence reads. Our assumptions of intrinsic resistance based on genus identification, while relevant for most members of a genus/family, have several notable exceptions (for example, *Proteus mirabilis*, unlike other members of the

Table 5 Clinical outcome of pyogenic infections as a function of empiric antibacterial therapy

Antibacterial spectrum of interest		Treatment duration			Mortality		
		Yes (days)	No (days)	<i>p</i> -value	With agent	Without agent	<i>p</i> -value
Any obligate anaerobes (<i>n</i> =39)	Anaerobic coverage	49	41	0.46	3/37	0/2	0.85
<i>Pseudomonas</i> (<i>n</i> =13)	Pseudomonadal coverage	54.5	53.4	0.59	3/12	0/1	0.77
<i>Stenotrophomonas</i> (<i>n</i> =15)	Stenotrophomonadal coverage	47.5	50	0.32	1/10	0/5	0.67
<i>Enterococcus</i> (<i>n</i> =7)	Enterococcal coverage	61	30	0.24	0/5	0/2	–
Tribe <i>Proteaeae</i> (<i>n</i> =11)	Tribe <i>Proteaeae</i> coverage	49	61	0.75	1/10	0/1	0.91

tribe *Proteaeae*, does not produce an inducible AmpC β -lactamase). Furthermore, the relatively small number of organisms intrinsically resistant to therapies and the relatively small sample size for each cohort makes small-effect observations impossible, particularly with cross-sectional data. Most importantly, acquired resistance mechanisms, which account for the bulk of bacterial resistant determinants [69–71], are not assessed by the approaches that we have utilized.

From this data, we can suggest that molecular-based technologies yield a much more complex perspective on the microbial etiology of pyogenic infections. Clearly, traditional aerobic and anaerobic microbial culture-based approaches underestimate the true diversity of organisms in these infections. However, our data suggests that, in the majority of cases, the numerically dominant organism is cultivated and correctly identified by routine microbiologic practices. The additional information provided by the application of advanced culture-independent technologies affords an invaluable perspective on the invasive nature of microbial communities. Characterizing the dynamic changes in microbial constituents, microbe–microbe interactions, and the interplay of the immune system within these communities over time represents a promising area for future research.

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