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Original article

The bacterial aetiology of pleural empyema. A descriptive and comparative metagenomic study

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ABSTRACT

Objectives: The view of pleural empyema as a complication of bacterial pneumonia is changing because many patients lack evidence of underlying pneumonia. To further our understanding of pathophysiological mechanisms, we conducted in-depth microbiological characterization of empyemas in clinically well-characterized patients and investigated observed microbial parallels between pleural empyemas and brain abscesses.

Methods: Culture-positive and/or 16S rRNA gene PCR-positive pleural fluids were analysed using massive parallel sequencing of the 16S rRNA and *rpoB* genes. Clinical details were evaluated by medical record review. Comparative analysis with brain abscesses was performed using metagenomic data from a national Norwegian study.

Results: Sixty-four individuals with empyema were included. Thirty-seven had a well-defined microbial aetiology, while 27, all of whom had community-acquired infections, did not. In the latter subset, *Fusobacterium nucleatum* and/or *Streptococcus intermedius* was detected in 26 patients, of which 18 had additional facultative and/or anaerobic species in various combinations. For this group, there was 65.5% species overlap with brain abscesses; predisposing factors included dental infection, minor chest trauma, chronic obstructive pulmonary disease, drug abuse, alcoholism and diabetes mellitus. Altogether, massive parallel sequencing yielded 385 bacterial detections, whereas culture detected 38 (10%) and 16S rRNA gene PCR/Sanger-based sequencing detected 87 (23%).

Conclusions: A subgroup of pleural empyema appears to be caused by a set of bacteria not normally considered to be involved in pneumonia. Such empyemas appear to have a similar microbial profile to oral/sinus-derived brain abscesses, supporting spread from the oral cavity, potentially haematogenously. We suggest reserving the term 'primary empyema' for these infections. **R. Dyrhovden, Clin Microbiol Infect 2019;25:981**

Introduction

Empyema, defined as the presence of bacteria or pus in the pleural cavity, is a serious infection with high morbidity and mortality (15%–20%) and increasing incidence [1]. Predisposing conditions include bacterial pneumonia, surgery, trauma, oesophageal perforation, thoracentesis, subdiaphragmatic infection, spontaneous pneumothorax and septicaemia [2]. Traditionally, the predominant cause has been assumed to be bacterial pneumonia in which bacteria breach the visceral pleura to establish an infected parapneumonic effusion [1,2]. This concept has recently been challenged. Differences between the typical bacteriology of pneumonia and empyema have been highlighted, with many patients with empyema having no evidence of an underlying pneumonia [1,3,4]. Such observations suggest that pleural empyema and pneumonia should, in some cases, be considered separate conditions [1,3–5], and—furthermore—that the mechanisms and sources of bacterial invasion of the pleural cavity are poorly understood [1,4,5].

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In 2014, we conducted a nationwide Norwegian study using targeted 16S rRNA gene-based metagenomics on brain abscesses [6]. Three bacterial species (*Streptococcus intermedius*, *Fusobacterium nucleatum* and *Aggregatibacter aphrophilus*) were either sole pathogens or a dominant part of abscesses with an assumed oral or sinus origin. This led us to hypothesize that these three microbes may be involved in initial establishment of these infections, with the additional species detected representing later colonizers.

In our routine clinical practice, we more recently observed that many pleural empyema samples have a microbiome similar to that of brain abscesses. This suggested a new hypothesis that a small group of highly specialized bacteria in the oral cavity may, under appropriate conditions, spread to and establish purulent infections in highly oxygenated organs, including the brain and lung.

The aim of the present investigation was to conduct a thorough bacterial characterization of pleural empyemas from clinically well-characterized patients to further our understanding of pathophysiological mechanisms. This endeavour included a comparison between pleural empyemas and brain abscesses.

Materials and methods

We conducted a retrospective study at the Department of Microbiology, Haukeland University Hospital, Bergen, Norway. The study was approved by the regional ethical committee (2017/1095).

Study definition of pleural empyema

Pleural empyema was defined as the presence of bacteria in pleural fluid by Gram stain, culture or 16S rRNA gene PCR [1]. Since previous studies have not included detection of bacterial DNA in their definitions, all patients were also evaluated according to the traditional criteria for pleural empyema, defined as a positive Gram stain or bacterial culture of pleural fluid *or* macroscopic purulent pleural fluid *or* pleural pH < 7.2 combined with clinical evidence of infection [1,3,7].

Clinical samples

The Department covers a population of ~500 000 people and also receives occasional samples from other regional hospitals. Upon clinical suspicion of infection, the laboratory routinely performs partial amplification and Sanger-based sequencing of a portion of the bacterial 16S rRNA genes directly from pleural fluid (see Supplementary material, Document S1). For the present investigation, we searched the laboratory information system for culture-positive and/or 16S rRNA gene PCR-positive pleural fluid samples obtained from patients >17 years of age, admitted from January 2016 through to December 2017. Remnant extracted DNA from these samples was collected from a Biobank for targeted metagenomic analysis. Samples from 64 unique patients were eligible for inclusion. Eighteen patients with culture-positive empyemas could not be included because 16S rRNA gene PCR had not been performed and no extracted DNA was available (see Supplementary material, Table S1). Pleural fluids from 11 patients with a low suspicion of infection, negative cultures and negative 16S rRNA gene PCR were included as a negative patient control group.

Clinical definition of pneumonia

Pneumonia was defined by fulfilment of the following criteria: (i) at least two of the following: fever, cough, sputum production and/or chest pain; and (ii) radiographic evidence of pneumonia, as interpreted by a radiologist [7].

Massive parallel sequencing of partial 16S rRNA and rpoB genes

Massive parallel sequencing was performed using the MiSeq system (Illumina, Redwood City, CA, USA). Some clinically important bacterial families and genera display too few variations in the 16S rRNA gene to reliably identify them to the species level [8]. Therefore, for selected groups of bacteria, we supplemented the analysis with massive parallel sequencing of parts of *rpoB* [9], which is more discriminatory than the 16S rRNA gene.

A detailed protocol for sequencing of all targets, including primers, description of negative controls, management of background DNA and sequence data analysis can be found in the Supplementary material (Document S1). For novel undescribed species we used the provisional HMT-taxonomy [10].

Statistical analysis

Statistical analysis was performed using SPSS 25 (IBM Corp). Differences between subgroups were analysed with Pearson's chi-squared test. Microbial β-diversity analysis for the empyemas, including unweighted and weighted UniFrac metrics [11,12], was performed in QIIME2. Comparison between a subset of the data from this study and previous data from the Norwegian brain abscess study [6], was undertaken using UniFrac and Principal Coordinate Analysis in QIIME2. EMPeror [13] was used to visualize Principal Coordinate Analysis plots. Venn-diagram analysis was performed using the web tool http://bioinformatics.psb.ugent.be/webtools/Venn/. For the Venn-diagram analysis, we included species that were found in at least two cases in one of the two groups.

Results

Description of study population

The mean age of the 64 patients was 62 years (median 68, range 18–93). Fifty (78%) patients were male. Twenty-one (32%) had no history of smoking, while 24 (38%) were current smokers and 19 (30%) were former smokers. The most frequent chronic diseases were hypertension (15 patients, 23%) and chronic obstructive pulmonary disease (11 patients, 17%). Nineteen patients (30%) had no chronic disease. Sixty (94%) patients fulfilled the traditional criteria [1,3,7] for pleural empyema. The four patients who did not (Patients 5, 28, 47 and 62) included two postoperative infections, one *Francisella tularensis* infection and one infection of poorly described aetiology.

Technical sequencing data

After removal of short reads (<250 base pairs), small clusters (<20 reads) and chimeras, the mean number of valid reads was 75 426 per sample (range 19 892 to 311 160, median 65 286).

Microbiological findings and correlations with clinical data

Out of 385 bacterial detections made by massive parallel sequencing, culture detected only 38 (10%) and Sanger-based 16S rRNA gene sequencing detected 87 (22.5%) (see Supplementary material, Table S2). Thirty-nine (61%) of the 16S rRNA gene PCR-positive samples were culture-negative. None of the 11 samples

in the negative patient control group contained bacterial DNA beyond that found in the negative controls.

By 16S rRNA and *rpoB* gene massive parallel sequencing, bacteria from 183 species were identified, of which 157 could be assigned to the species level, 13 to a species group level, and 8 to a genus level. Five Operational taxonomic units yielded no significant match with published sequences. *rpoB* sequencing was performed for 44 samples (69%) and provided identification at a higher taxonomic level than 16S rRNA gene sequencing for 25 species (see Supplementary material, Table S3).

Thirty-seven patients had a well-defined aetiology underlying their pleural fluid infection (Table 1). Among these were 19 patients with monomicrobial infections caused by *Streptococcus pneumoniae* (8), *Staphylococcus aureus* (5), *Pseudomonas aeruginosa* (2), *Streptococcus pyogenes* (1), *Escherichia coli* (1), *Klebsiella pneumoniae* (1) or *Francisella tularensis* (1). *Streptococcus pneumoniae* was identified exclusively in monomicrobial samples, including seven cases of community-acquired pneumonia (CAP) and one case of postoperative empyema after lung resection. All identifications of *Staphylococcus aureus* and *Streptococcus pyogenes* were also made in monomicrobial samples, except for one patient with CAP who had a mixed infection with the two species. Empyemas resulting from surgical complications or spontaneous rupture of the oesophagus presented the highest microbial diversity. An overview of clinical data and identified microbes in each of these 37 individuals is provided in the Supplementary material (Table S4).

For the remaining 27 patients, the aetiology was less obvious. The Supplementary material (Table S5) summarizes the clinical history, computed tomography findings and identified bacteria in these individuals. All 27 infections were community-acquired. Several potential predisposing factors were identified. Six patients reported a minor blunt chest trauma before the onset of symptoms (such as blunt violence or fall accidents) and five of six patients in whom dental status was addressed had poor dental health. Other lung-related pathology among patients included chronic obstructive pulmonary disease (4) and computed tomography-diagnosed lung abscess (3). Only 12 out of 27 fulfilled the diagnostic criteria for CAP and all patients were negative for CAP-associated bacteria. Other possible predisposing comorbidities were drug abuse (4), alcoholism (3) and diabetes mellitus (3). Table 2 provides a comparison of demographics and clinical features between the 27 patients with empyema with poorly described aetiology versus the ten patients with classic post-pneumonia empyema.

In both the weighted and unweighted UniFrac analyses, most of the 27 samples with uncertain aetiology clustered in neighbouring branches, indicating significant similarities in microbial patterns (Fig. 1). Though they contained many of the species found in oesophageal ruptures and postoperative infections, they were distinguished from these by having lower microbial diversity and higher inter-sample consistency, both quantitatively and qualitatively. In total, they harboured 54 different species, of which 33 were identified in single patients. Among the 21 species found in multiple samples, *Streptococcus intermedius* and *Fusobacterium nucleatum* stood out both as being the most frequent bacteria detected on a patient-level and as being among the most abundant. Twenty-six (96%) of the 27 samples contained either *Streptococcus intermedius* ($n = 9$) or *F. nucleatum* ($n = 10$), or a combination of the two ($n = 7$). *Streptococcus intermedius* was the only detected species in eight samples, whereas *F. nucleatum* was detected alone in two. The single sample without either *Streptococcus intermedius* or *F. nucleatum* was a monomicrobial infection caused by *A. aphrophilus* in a patient with pulmonary sarcoidosis.

The microbial patterns observed in the empyema cases of uncertain aetiology had similarities with oral-type bacterial brain abscesses; Fig. 2 is a Venn diagram analysis for both infection types showing 19 (65.5%) shared species. The most frequent common species were *F. nucleatum*, *Streptococcus intermedius*, *Parvimonas micra* and *Eubacterium brachy*, all present in more than 30% of samples from both infection types. The most notable difference was observed for *A. aphrophilus*, found in 40% of brain abscesses but only in a single empyema (3.7%). In a comparative analysis of the two specimen types, Principal Coordinates Analysis plots based on UniFrac metrics showed no clear clustering into separate groups (Fig. 3).

Table 1
Patients with well-defined aetiologies of empyema

Postoperative[a]	12
Community-acquired pneumonia[b]	10
Metastatic cancer affecting the lung	4
Sepsis	4
Spontaneous rupture of the oesophagus	2
Hospital-acquired pneumonia	2
Lemierre syndrome	1
Francisella tularensis pneumonia	1
Post-traumatic	1
Sum	37

[a] Includes five cases occurring after upper gastrointestinal tract surgery, four cases after heart and lung surgery, and three cases after abdominal surgery.
[b] Fulfilment of diagnostic criteria for community-acquired pneumonia (CAP) and identification of bacteria known to cause CAP (*Streptococcus pneumoniae* (7), *Streptococcus pyogenes* (1), *Pseudomonas aeruginosa* (1), *Staphylococcus aureus* and *S. pyogenes* (1)).

Table 2
Demographic and clinical characteristics of empyemas with poorly described aetiology (PDE) versus those occurring after classic community-acquired pneumonia[a] (CAP)

	PDE	CAP	p-value[b]
Number of patients	27	10	
Male, *n*	24	5	0.01
Mean age, years (SD)	56 (18)	65 (18)	0.17
Smoker, *n*	11	4	0.97
Fever, *n*	21	8	0.88
Chest pain, *n*	25	5	0.003
Dyspnoea, *n*	24	9	0.92
Cough, *n*	18	8	0.43
Purulent sputum, *n*	8	3	0.98
Mean CRP[c] (SD)	230 (107)	313 (83)	0.03
Mean leucocytes[d] (SD)	19.3 (8.2)	21.0 (11.8)	0.60
Purulent pleural fluid, *n*	19	9	0.21
Mean pH of pleural fluid (SD)	7.0 (0.6)	7.1 (0.3)	0.69
Mean glucose[e] (SD)	2.2 (2.8)	1.0 (2.2)	0.41
Intensive care, *n*[f]	2	2	0.27
Death, *n*[g]	0	1	0.10

[a] Fulfilment of diagnostic criteria for CAP and identification of bacteria known to cause CAP: *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes*.
[b] Pearson's chi-squared test for categorical variables. Students *t*-test for continuous variables.
[c] C-reactive protein (mg/L) at admission.
[d] Leucocytes (10^9/L) at admission.
[e] Mean glucose level (mmol/L) in pleural fluid.
[f] Admitted to intensive care unit during the hospital stay.
[g] Death during the hospital stay.

Discussion

In this study, we provide a clinical and microbiological characterization of 64 patients with empyema. To the best of our knowledge, this is the first investigation in which such a large

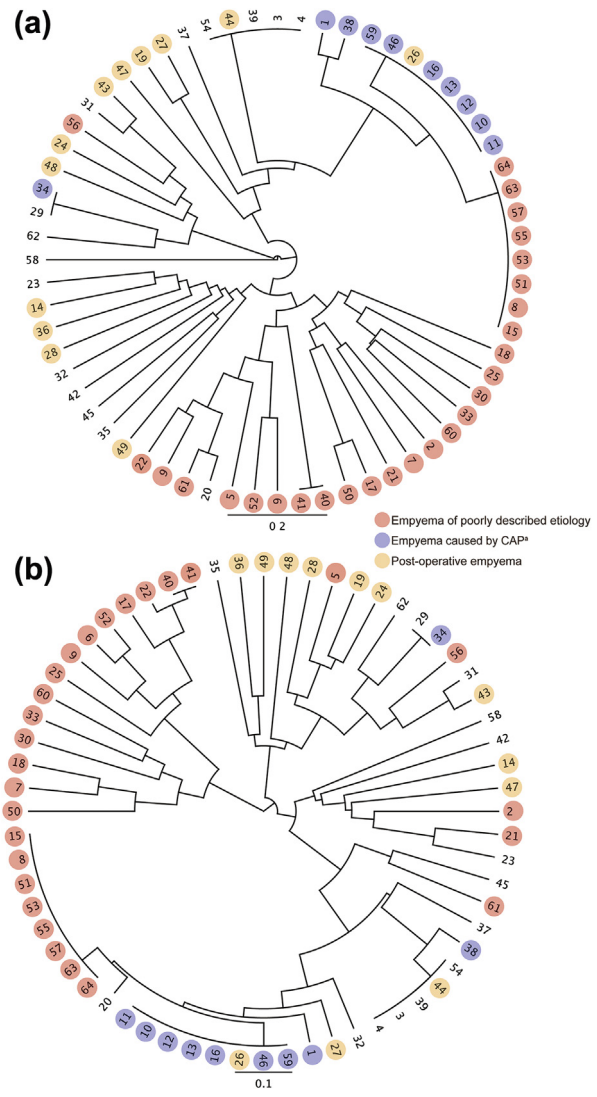


Fig. 1. UniFrac-analysis of empyemas. Phylogenetic tree of unweighted (a) and weighted (b) UniFrac-analysis of all included pleural empyemas. Each number represents one sample. CAP, fulfilment of diagnostic criteria for community-acquired pneumonia (CAP) and identification of bacteria known to cause CAP: *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes*.

sample of pleural empyemas has been characterized using a targeted metagenomics analysis.

We identified a subgroup of 27 patients with community-acquired infections with unclear explanation as to how the bacteria had reached the pleural cavity. All empyemas harbored oral bacteria not normally associated with pneumonia, and shared distinct microbial patterns overlapping with those previously described for oral/sinus-type brain abscesses. Samples from 26 of 27 patients contained either *Streptococcus intermedius* or *F. nucleatum* or a combination of the two, and these were also, together with *A. aphrophilus*, the only bacteria detected in monomicrobial infections. We therefore hypothesize that *F. nucleatum* and *Streptococcus intermedius* are possible key pathogens for establishing these empyemas, much like previously suggested for bacterial brain abscesses [6]. *Aggregatibacter aphrophilus*, found in a single monomicrobial empyema, does not seem to be very common in pleural empyemas, though it was found in 40% of brain abscesses.

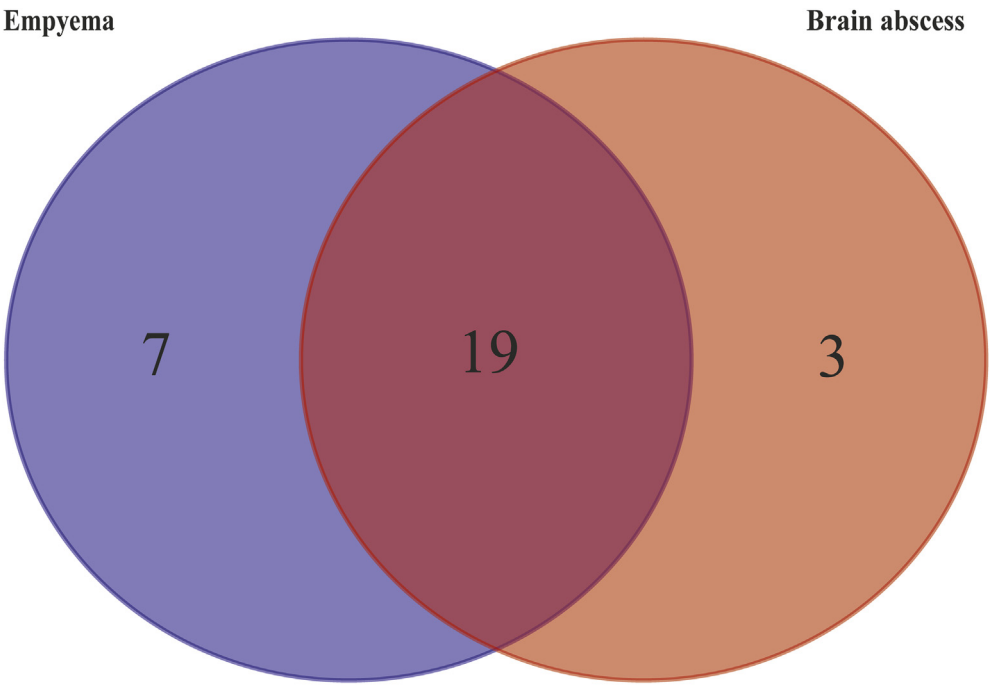
Fusobacterium nucleatum was found in all empyemas harbouring strict anaerobic bacteria, except for one containing *Fusobacterium gonidiaformans*. Although traditionally thought of as a strict anaerobe, *F. nucleatum* is a moderate anaerobe with capacity for oxygen adaption [14,15]. In periodontitis research, it is considered a key organism in the transition between early facultative colonizers and later obligate anaerobes [16].

In patients with pneumonia, pathogens may overcome pulmonary defence mechanisms and reach the pleural cavity by direct transpleural spread from the respiratory alveoli. The capacity of *Streptococcus pneumoniae* to do this is well-described [1], but for *Streptococcus intermedius* and *F. nucleatum*, we have found no evidence of a similar ability. For anaerobic bacteria this mode of dissemination would be halted by the high oxygen tension in the respiratory tract. When examining the clinical characteristics for the 27 patients with empyemas of uncertain pathogenesis, only 12 had possible pneumonia. However, since diagnostic criteria for CAP are based on clinical features with low diagnostic specificity and sensitivity [7,17] and display considerable overlap with the most common signs and symptoms of pleural empyema, we believe the actual number of pneumonias may have been even lower than reported [7].

For brain abscesses, an important route of infection is dissemination of bacteria via the haematogenous route from primary sinusitis or an odontogenic focus [18–20]. Bacteria are thought to reach the brain parenchyma by transit trough valveless emissary and diploic veins that drain through the skull bone and into the venous system of the brain [19]. This mode of transmission is supported by the observation that abscesses formed by oral pathogens are the dominant type of abscess in all regions of the brain, not just in the frontal lobe [6]. The lung parenchyma and the visceral pleura are therefore natural sites along the same route of infection. Infected venous blood follows the venous draining system to the right ventricle of the heart and is pumped into the pulmonary arteries, ending up in the capillary network of alveoli and parts of the visceral pleura. Indeed, several studies and case reports describe the simultaneous occurrence of brain abscess and lung abscess or pleural empyema [21–24], both in general and for *F. nucleatum* and *Streptococcus intermedius*, specifically.

We therefore suggest, based on the findings in this study, that facultative and anaerobic oral bacteria, able to spread via deoxygenated venous blood to establish purulent infections in brain tissue, are also capable of reaching and establishing pyogenic infections in the lung parenchyma or pleural cavity, and that right-sided haematogenous spread is a plausible route of infection in oral-type bacterial pleural empyema.

We found a remarkably high involvement of males in oral-type bacterial empyema that significantly differed from the even gender distribution of classic post-pneumonia empyema (Table 2). The same uneven gender distribution has been observed in other studies on pleural empyema, and specifically among those caused by the *Streptococcus anginosus* group [25–27]. Odontogenic infections have been identified as a potential risk factor for pleural empyema [27] and one explanation of the male predominance might be a higher frequency of serious odontogenic infections in men [28]. Except for dental caries, we found the most common comorbidities to be hypertension, chronic obstructive pulmonary disease, injection drug use, alcoholism and diabetes mellitus (see Supplementary material, Table S5). This correlates with findings from previous studies [27,29]. Male gender, odontogenic infections and injection drug use are also definite risk factors for brain abscesses [19,20]. Another finding that might represent a hitherto unappreciated predisposing factor, is that six patients had a history of a minor blunt chest trauma just before symptom onset. In brain



Species found only in empyema:
Catonella morbi (3)
Gemella bergeriae (2)
Peptostreptococcaceae sp. HMT 369(2)
Treponema maltophilum (2)
Dialister pneumosintes (2)
Mycoplasma salivarium (2)
Prevotella buccae (2)

Species found in both infections:
Fusobacterium nucleatum (17/18)
Streptococcus intermedius (16/19)
Parvimonas micra (11/17)
Eubacterium brachy (8/9)
Campylobacter rectus (5/4)
Prevotella oris (4/3)
Streptococcus constellatus (4/2)
Porphyromonas endodontalis (4/1)
Actinomyces meyeri (3/12)
Prevotella pleuritidis (3/3)
Campylobacter gracilis (2/7)
Eikenella corrodens (2/6)
Prevotella sp. HMT 317 (2/3)
Alloprevotella tannerae (2/1)
Aggregatibacter aphrophilus (1/10)
Eubacterium yurii (1/2)
Filifactor alocis (1/2)
Gemella morbillorum (1/2)
Tannerella forsythia (1/2)

Species found only in brain abscess:
Actinomyces georgiae (2)
Anaeroglobus geminatus (2)
Capnocytophaga sp. HMT 338 (2)

Fig. 2. Venn diagram analysis comparing species found in 27 empyemas of poorly described aetiology and 25 brain abscesses with assumed oral/sinus origin. The numbers in parenthesis are the total number of species found in each group. In the middle column, the first number in parenthesis represents empyemas and the second brain abscesses.

abscesses one of the main predisposing pathogenic factors is the presence of ischaemic or devitalized brain tissue occurring after incidents such as trauma or cerebrovascular accidents [20]. Blunt chest trauma may cause damage to the visceral pleura or adjacent lung parenchyma, leading to the formation of a poorer oxygenated *locus minores resistentiae* (e.g. a haematoma or atelectasis) facilitating colonization by blood-borne oral microbes.

The retrospective design is a weakness of our study; 18 culture-positive samples were lost due to lack of sample availability (see Supplementary material, Table S1). Information about dental status was available from only six patients, and information about preceding minor trauma was not systematically collected. Another limitation is that this is a case study from one particular area of the world. The oral microbiome varies between geographical areas [30] and this may influence the bacterial composition of pleural empyema as well. The findings and hypotheses proposed in this article should clearly be challenged in future, prospective studies.

We have shown that a large subgroup of community-acquired pleural empyemas is caused by a limited set of oral bacteria not normally involved in pneumonia. We provide microbiological, anatomical and epidemiological arguments to support that these pleural empyemas and oral/sinus-derived brain abscesses might be two sides of the same coin sharing microbial composition, haematogenous routes of infection and risk factors. We suggest that the term 'primary empyema' should be reserved for this type of infection to distinguish it from pleural empyema secondary to other lung conditions, including classic post-pneumonia empyema and post-operative empyema. Our finding also suggest that traditional culture-based methods and even Sanger-based 16S rRNA gene PCR/sequencing may be insufficient in characterizing the microbial spectrum of primary empyemas.

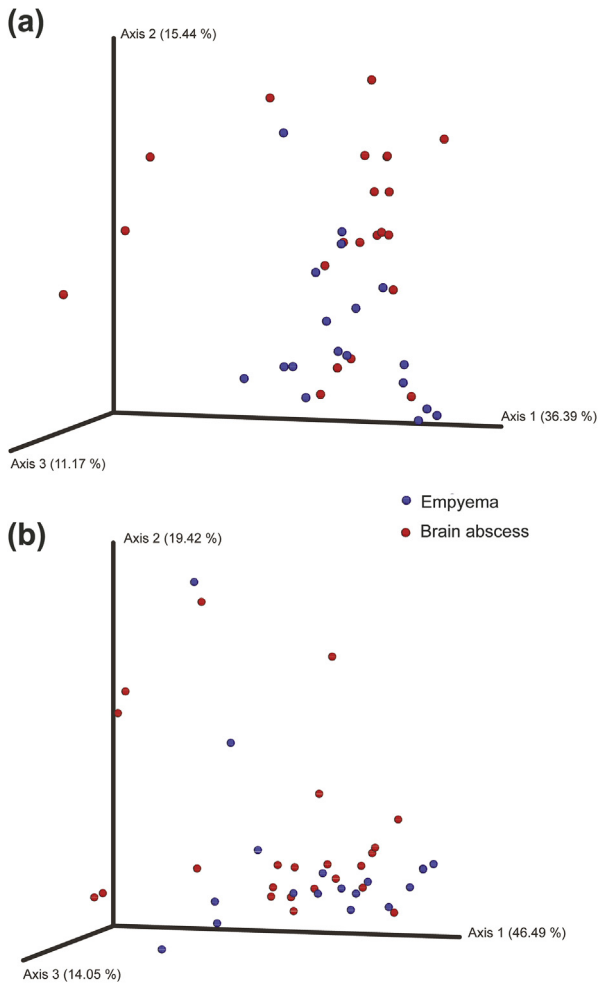


Fig. 3. Comparative UniFrac-analysis of empyemas and brain abscesses. Principal Coordinates Analysis of unweighted (a) and weighted (b) UniFrac-analysis of the 27 empyemas of poorly described aetiology and 25 oral/sinus-derived brain abscesses. The figure shows no clear clustering into separate groups. Each dot represents one sample.

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Transparency declaration

Dr. Patel reports grants from CD Diagnostics, BioFire, Curetis, Merck, Contrafect, Hutchison Biofilm Medical Solutions, Accelerate Diagnostics, Allergan, and The Medicines Company. Dr. Patel is or has been a consultant to Curetis, Specific Technologies, Selux Dx, GenMark Diagnostics, PathoQuest, Heraeus Medical, and Qvella; monies are paid to Mayo Clinic. In addition, Dr. Patel has a patent on *Bordetella pertussis/parapertussis* PCR issued, a patent on a device/method for sonication with royalties paid by Samsung to Mayo Clinic, and a patent on an anti-biofilm substance issued. Dr. Patel receives travel reimbursement from ASM and IDSA and an editor's stipend from ASM and IDSA, and honoraria from the NBME, Up-to-Date and the Infectious Diseases Board Review Course.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2018.11.030.

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