

Original Article

Mixed group of *Rhizobiales* microbes in lung and blood of a patient with fatal pulmonary illness

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Abstract: We examined the microbial composition in the diseased lung and early-phase microbial cultures from the blood of a patient with a rapidly progressing fatal pulmonary illness. Although no microbes could be isolated from such cultures during the initial study, the HTS-microbiome study revealed the presence of a unique mixture of *alphaproteobacteria*, composed mainly of different families of *Rhizobiales* microbes. Microbial 16S rDNA sequences matching closely to *Afipia cberi* were identified mainly in the patient's diseased lung tissue, but only rarely in the early-phase blood cultures. Conversely, the high abundance of sequences found in early-phase blood cultures of different broth media matched closely with those of the families *Methylobacteriaceae*, *Phyllobacteriaceae* and *Sphingomonadaceae*. The two species that successfully adapted to grow in a laboratory culture system were *A. cberi* and *Mesorhizobium hominis*, which eventually were isolated from a previously cryopreserved blood culture of SP4 broth. Many other species, including members of the *Bradyrhizobiaceae* and *Phyllobacteriaceae* families, and all members of the *Methylobacteriaceae* and *Sphingomonadaceae* families identified by HTS remained non-cultivated. We developed specific PCR primers and FISH probes, which detected the target *Rhizobiales* microbes in former blood cultures and autopsy lung tissues. It is unclear what role these *Rhizobiales* microbes might have played in the patient's complex disease process. However, the above mentioned assays should help in rapidly detecting and identifying these previously unrecognized *Rhizobiales* microbes in patients.

Keywords: Microbiome, MPS, HTS, *Rhizobiales*, *alphaproteobacteria*, *Bradyrhizobiaceae*, *Phyllobacteriaceae*, *Methylobacteriaceae*, *Sphingomonadaceae*, *Afipia*, *Mesorhizobium*

Introduction

Non-biased massively parallel sequencing (MPS) studies using high-throughput sequencing (HTS) technology have provided powerful means for the sequence-based discovery and characterization of microbes. The new technique has been particularly useful for studying difficult-to-culture microbes [1, 2] and whole microbial communities in examined samples without the need for laboratory cultivation or isolation of individual species [3, 4]. The capabilities of HTS technology led us to re-examine some of our cryopreserved broths of blood cultures that were suspected of having low numbers of inactive unknown microbes, despite having negative microbial isolation results. We recently reported the discovery and isolation of

previously unknown *Rhizobiales* microbes from formerly cryopreserved blood sample cultures from three patients with chronic forms of poorly defined illnesses, as well as a patient who had an acute fatal pulmonary illness [5, 6]. In the process, genomic sequencing proved to be highly effective in the characterization of these novel microbes.

A blood sample was obtained shortly before a patient dying of an acute on-set of pulmonary illness and sent to the Armed Forces Institute of Pathology (AFIP) for diagnostic consultation in 1999. The AFIP laboratory set up cultures of the received blood sample in various broth media at the beginning of routine laboratory microbial work-ups to isolate and identify possible infectious microbial agents and could not isolate

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continuously growing microbial agents from any of these blood cultures in almost three months of careful follow-up [6]. As part of the infectious disease work-up, we obtained DNA from the early-phase blood cultures containing RPMI-1640, SP4 and BHI broth media supplemented with 5% irradiated fetal bovine serum (FBS) designated R5, SP4_F and BCF media. These DNA samples obtained from the first 1-3 weeks of blood cultures and DNA extracted from a small piece of this patient's frozen lung tissue obtained at autopsy were previously used for polymerase chain reaction (PCR) studies to investigate possible mycoplasmal and/or bacterial infections. However, PCR assays targeting *M. fermentans* insertion-like element sequences [7, 8] did not produce any positive signals. PCR amplification of 16S rRNA genes using universal bacterial primers [9, 10] and DNA sequencing of the amplicons using the Sanger method yielded mixed, uninterpretable sequencing results. The DNA specimens prepared from diseased lung tissue obtained at autopsy and early-phase blood cultures were archived and stored frozen.

Two different species of novel *Rhizobiales* microbes, *Affipia cberi* of the *Bradyrhizobiaceae* family and *Mesorhizobium hominis* of the *Phyllobacteriaceae* family, were recently isolated and characterized in our laboratory by re-cultivating a formerly cryopreserved SP4 broth culture of a blood sample from this same patient with acute fatal pulmonary disease using modified SP4 broth and culture condition [6]. To better understand the microbial compositions of these samples, we conducted microbiomic study using the stored DNA specimens prepared previously from the diseased lung tissue and the early-phase blood cultures. Microbial 16S rRNA gene sequences of ~440 bps covering the species-specific hyper-variable regions (V6, V7 and V8) were amplified from the DNA samples using broad-range PCR primers corresponding to the highly conserved eubacteria sequences [9, 10]. We examined the sequences of amplicons by both Sanger method and HTS technology [11, 12]. Polymorphisms of 16S DNA sequences across the three hyper-variable regions were used for taxonomic identification and classification of various microbial populations present in the examined samples. In this study, we designed

Bradyrhizobiaceae-specific and *Mesorhizobium hominis*-specific primers for PCR-based studies of the target *Rhizobiales* microbes in the diseased lung tissues and early-phase blood sample-derived cultures. In addition, we prepared a Cy3 labeled probe specific to the *Bradyrhizobiaceae* 16S rRNA for Fluorescence *in Situ* Hybridization (FISH) studies to directly identify *Bradyrhizobiaceae* microbes in the patient's formalin-fixed paraffin-embedded (FFPE) autopsy lung samples.

Materials and methods

Ethics

Studying previously frozen DNA samples from cultures of the blood samples were conducted under the FDA Research Involving Human Subjects Committee (RIHSC) protocol #10-008B entitled "Detection of Infectious Agents in Previously Frozen Blood Samples from Patients with Various Illnesses and Healthy Blood Donors". For this study, Dr. Zucker provided the patient's original clinical information and Dr. Olesnick provided the preserved lung sections from an autopsy performed in 1999. The IRB of Newark Beth Israel Medical Center (NBIMC) and Bamabas Health Corporate legal representatives reviewed the activity and determined that it did not meet the definition of human subject research that requires written informed consent from the patient. The clinical case was described in the study without revealing the patient's identity.

DNA preparation from the original microbial cultures of blood samples in diagnostic studies

The laboratory received 600 to 700 µL of partially hemolyzed whole blood (WB) for the diagnostic study in 1999. The details of the original microbial cultures used for diagnostic studies were previously described [6]. In the original microbial study, 100 µL aliquots of WB were inoculated into each of the four tube cultures. Each culture tube contained 5 mL of 1) SP4 broth medium supplemented with 5% fetal bovine serum (FBS) (SP4_F), 2) RPMI-1640 broth medium supplemented with 5% FBS (R5), 3) BHI broth medium (BD Diagnostics, Circle Sparks, MD) and 4) BHI broth medium supplemented with 5% FBS (BCF). The cultures were kept at 32°C under aerobic conditions. When

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none of the microbial cultures (cultures kept at different temperatures under aerobic conditions and in GasPack) in the study showed evidence of microbial growth after one week of incubation, DNA was extracted from 1 mL of the cultures of SP4_F and R5 broth media using the DNeasy Blood & Tissue Kit (Qiagen). Similarly, DNA was extracted from 1 mL of the cultures of BHI and BCF broth media at week three, when no microbial growth was identified in the study. These DNA specimens were used for PCR to detect target mycoplasma DNA [7, 8] and amplification of microbial 16S rRNA sequences [9, 10] for Sanger sequencing before being stored at a temperature of -80°C. In the study, DNA was prepared from an equal amount of each control culture medium and water using the same Qiagen kit. As previously described [6], we continuously followed the microbial cultures for three months and routinely streaked their broth on SP4 agar plates and BHI agar plates without successful isolation of any microbes.

DNA preparation from autopsy lung tissue

DNA was extracted from a small piece of lung tissue obtained at autopsy using the DNeasy Blood & Tissue Kit (Qiagen) and stored at -80°C.

Amplification of 16S rRNA gene sequences and BAMQ5 and MSMQ8 target sequences

Microbial 16S rRNA genes were amplified from tested DNA samples using broad-range (pan-bacteria) primers p91F (5'-CAAATGAATGACGGGGGC-3') and p13B (5'-AGCCCCGGAACGTATTAC-3') which were adapted with minor modifications from previous studies [9, 10]. The region of the microbial 16S rRNA gene sequences (corresponding to positions 911 to 1390 of the *E. coli* sequence) that were amplified covers three species-specific hypervariable regions (V6, V7 and V8). To amplify the *Bradyrhizobiaceae*-specific BAMQ#8 primers-targeting gene, the BAMQ#8F (5'-GGMCCGGTGATCTACACCTT-3') and the BAMQ#8R (5'-AGCGTGGTCCAGGTCTTCTG-3') primer set was used. The *M. hominis*-specific MSMQ#5 primers-targeting gene was amplified by the MSMQ8F (5'-CAATGCCG-CAGATCTGAAT-3') and the MSMQ8R (5'-AC-

TCCGCAATGCCTCTCTTA-3') primer set. PCR was conducted by an initial denaturation at 95°C for 5 min, 40 cycles at 95°C for 30 sec; 55°C for 30 sec; 72°C for 1 min and a final extension at 72°C for 7 min using the Hotstar Taq-Plus DNA polymerase (Qiagen).

Amplification of 16S rRNA gene and BAMQ#8 target gene amplicons with Nextera XT indexes

The amplicons of the 16S rRNA gene and BAMQ#8 primers-targeting region were amplified for 10 PCR cycles using the primer set p91F-XT (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAAATGAATTGACGGGGGC-3') and p13B-XT (5'-GTCTCGTGGGCTCGGAGATGTGTA-TAAGAGACAGAGCCCCGGAACGTATTAC-3'), and the primer set BAMQ#8F-XT (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGMCCGGTGATCTACACCTT-3') and BAMQ#8R-XT (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGCGTGGTCCAGGTCTTCTG-3'), respectively. The second-step PCR for adding XT tags to the tails of the respective amplicons was conducted using HotStart-I_t FidelityTaq master mix (Affymetrix). Amplicons of the second-step PCR were purified using the Agencourt AMPure XP system (Beckman Coulter) and indexed by PCR using indexing primers. The amplification system included in the Nextera XT kit (Illumina) was used to construct amplicon libraries. These libraries were purified using the Agencourt AMPure XP system before being applied to the MiSeq sequencer (Illumina, San Diego, CA).

High-throughput sequencing

We previously described conducting 2×250 bp pair-end sequencing using the MiSeq sequencer [5, 6]. To optimize the sequencing quality of the amplicons, 3-4 multiplexed amplicon DNA libraries with proper indexing were sequenced together with DNA samples from whole bacterial genomes to increase the overall complexity of sequencing reads in each run. The FASTQ file for each amplicon library was de-multiplexed according to the unique index present in the library construct.

Bioinformatic and metagenomic analyses

Total raw reads obtained from amplicons of each examined sample by MiSeq sequencing were first qualified by removing poor quality reads and trimming out the sequences of PCR

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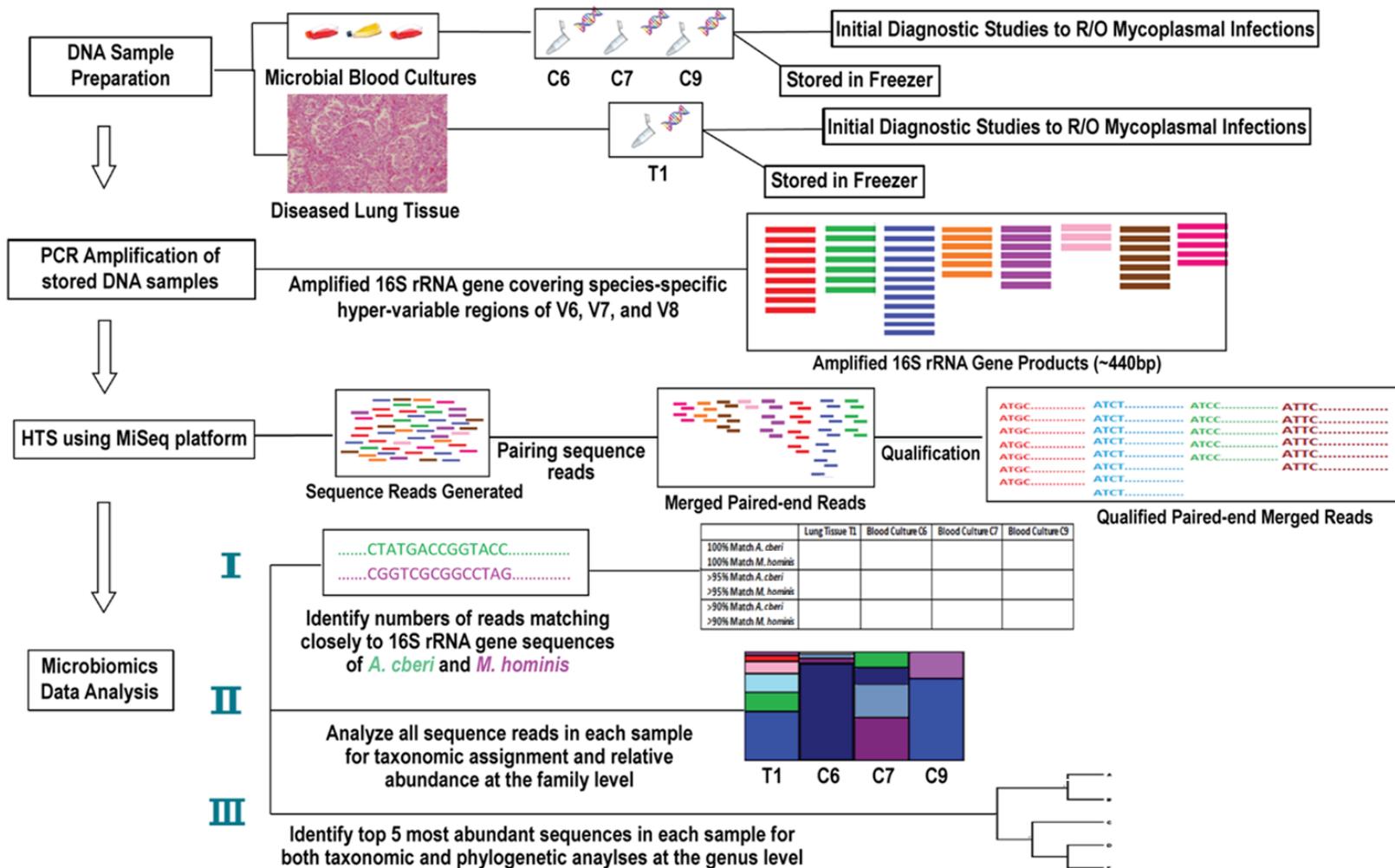


Figure 1. Schematic workflow to study microbial populations of the stored frozen DNA samples from diseased lung tissue and early-phase (the first 1-3 weeks) cultures of the patient's blood sample involving different broth media by HTS.

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Table 1. Datasets generated by MPS for microbiomics analyses

DNA Samples	T1	C6	C7	C9	Culture Broth Control	Culture Broth Control	Culture Broth Control	Blank/Reagent Control
Prepared from	Diseased lung tissue	Culture of R5 medium	Culture of SP4_F medium	Culture of BCF medium	R5 medium	SP4_F medium	BCF medium	H ₂ O
Time of Culture	NA	Week 1	Week 1	Week 3	NA	NA	NA	NA
Qualified raw reads	5,573,804	4,692,312	6,988,910	3,581,211	626	2,453,560	771,844	538,744
No. of reads with paired-ends	3,933,870 (70.61%*)	3,558,936 (75.86%*)	3,933,342 (56.3%*)	981,934 (27.4%*)	12 (1.9%)	704 (0.03%)	968 (0.13%)	182 (0.03%)
No. of paired-end reads with 440 bp joint sequences	1,965,819	1,778,015	1,965,500	490,967	0	1	0	0
Numbers of reads matching:								
1) 100% to sequences of								
<i>A. cberi</i>	95,247 (4.84% ^{**})	15	7	0	0	0	0	0
<i>M. hominis</i>	0	0	0	2,164 (0.45% ^{**})	0	0	0	0
2) >95% to sequences of								
<i>A. cberi</i>	356,393 (18.13% ^{**})	243	599	0	0	0	0	0
<i>M. hominis</i>	0	21	12	129,667 (26.41% ^{**})	0	0	0	0
3) >90% to sequence of								
<i>A. cberi</i>	392,413 (19.96% ^{**})	22,902 (1.29% ^{**})	207,281 (10.55% ^{**})	30	0	0	0	0
<i>M. hominis</i>	7	12,469 (0.70% ^{**})	31,506 (1.60% ^{**})	142,745 (29.08% ^{**})	0	0	0	0

(*): Percentage of the number of reads with qualified paired-ends out of the numbers of qualified raw reads. (**): Percentage of the number of reads out of the numbers of 440 by merged sequences derived from paired-ends reads. The percentage of <0.1% is not marked.

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primers using CLC Genomics Workbench (version 7.0.3) (www.clcbio.com). The trimmed pair-reads were then merged using the CLC "Merge Overlapping Pairs" function with the default parameters. The qualified merged reads (~440 bp) from each examined sample were then aligned with the reference sequences of *A. cberi* or *M. hominis*. The numbers of reads with 100%, 95% and 90% sequence similarity to the reference sequences were identified. Sequences of all of the qualified merged-reads from each sample were then blast searched against the 16S ribosomal RNA sequences in the NCBI database using Easyblast on NIH Biowulf (<http://biowulf.nih.gov>). The BlastN results were then imported into the MEGAN program (version 4.70.4) [11]. The distribution of the taxonomic classifications for all sequence reads was set at the family level, sorted by abundance and displayed as a bar graph. The qualified merged reads with identical sequences from each sample were calculated and consolidated as one read using the Fastx Collapser program available on EMBOSS [13]. A phylogenetic tree for the top number reads that match with *Rhizobiales* and their neighboring species was constructed using MEGA4 [14].

Retrieval of DNA from FFPE tissue sections

DNAs were retrieved from a de-waxed and rehydrated FFPE autopsy tissue section using the on-slide non-destructive molecule extraction method (NDME) as previously described [15].

Infection of A549 lung cell culture with *A. cberi* and *M. hominis*

The human lung carcinoma A549 cell line (ATCC CCL-185) was cultured in 75 cm² flasks using RPMI-1640 broth medium supplemented with 10% of FBS. Cultures at 80-90% confluence were infected using ~10⁸ *A. cberi* or *M. hominis* at 32°C for 30 min. The infected A549 cell cultures and the non-infected control cultures were then incubated in a CO₂ incubator at 35°C for 36 hrs. The cells were harvested from culture flasks by scraping and pelleted by centrifugation. The cell pellets were fixed with 4% paraformaldehyde in PBS for 24 hrs and then processed in centrifuge tubes and embedded in paraffin blocks. The FFPE A549 cell pellets were sectioned and processed for FISH studies as were the FFPE autopsy lung tissues.

Fluorescence *in situ* hybridization

The 16S sequences of all of the different species of *Afipia* bacteria and *B. japonicum* were aligned using MEGA4 [14] against those of *M. hominis*, *E. coli*, *P. aeruginosa*, *Klebsiella pneumoniae*, *S. pyogenes* and *S. aureus*. Regions that displayed high conservation among *Bradyrhizobiaceae* microbes and maximum sequence heterogeneity from non-*Afipia* bacteria including *M. hominis* were subjected to computational evaluation using an RNA-targeted FISH probe design using mathFISH [16]. The P1-2 FISH probe (5'-Cy3/ATCTCTGG-TACCGTCAT-3') was selected based on its overall predicted lowest free energy in hybridization against the target 16S rRNA sequence. The P1-1 FISH probe (5'-Cy3/ATGACCGTACCAGAGAT-3') with reverse polarity to P1-2 was prepared and used as a negative control. Cy-3 labelling of the P1-2 probe and *in situ* hybridization were conducted as described [17]. Briefly, FFPE autopsy lung tissues and culture cell pellet sections were deparaffinized using five changes of xylene, followed by three changes of 100% ethanol and two changes of 95% ethanol. The de-waxed sections were incubated with 1 mg/mL lysozyme for 15 minutes at 37°C. Probes were pre-diluted in ddH₂O to a concentration of 50 ng/mL. Half microliter (0.5 µL) of the probe was added to 50 µL of hybridization buffer (0.88 M NaCl, 20 mM Tris HCl, pH 7.4, 0.05% SDS) containing 1% formamide and applied onto each slide. The samples were incubated with the probe solution for two hours in a 50°C water bath. Then, the slides were washed in a pre-warmed wash buffer (900 mM NaCl, 100 mM Tris HCl, pH 7.4, 0.03% SDS) for six minutes at 50°C in a shaking water bath. The slides were washed with ddH₂O and mounted using Prolong Gold antifade with DAPI (Invitrogen, Carlsbad, CA) before microscopic examination.

Results

A schematic of the workflow to study microbial populations by HTS and taxonomical analysis of 16S rRNA gene sequence reads PCR-amplified from formerly stored frozen DNA samples of diseased lung tissue obtained at autopsy and early-phase (the first 1-3 weeks) cultures of the patient's blood sample involving different broth media is shown in **Figure 1**.

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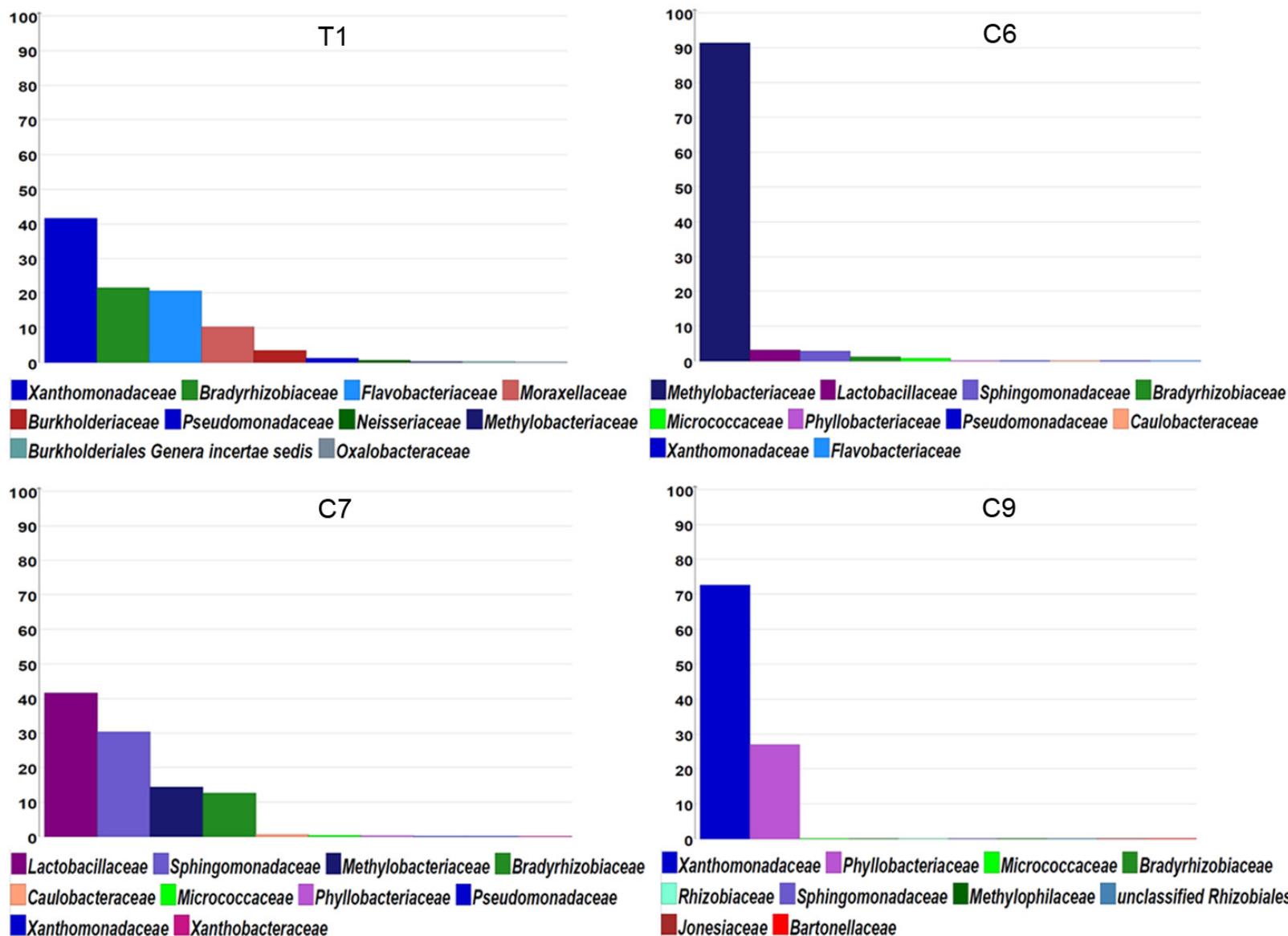


Figure 2. The distribution of taxonomic classification at the level of Families for all qualified merged paired reads of 16S rRNA sequences amplified from the diseased lung tissue and early-stage blood cultures set up with R5 medium (C6), SP4_F medium (C7) and BCF medium (C9). Only the top 10 families for each sample are shown in the MEGAN display.

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Table 2. Identification of the 5 most abundant sequences reads in each examined sample for taxonomic classification

DNA samples studied by MPS	Top 5 most abundant sequences	Percentage reads of total qualified 440 bp joint sequences	Taxonomic classification of the 16S rRNA gene sequences
Diseased Lung Tissue (T1 Sample)	TI_1	8.46%	<i>Pseudomonas</i> genus of <i>Pseudomonadaceae</i>
	TI_2	4.84%	<i>Afiplia</i> genus of <i>Bradyrhizobiaceae</i>
	TI_3	3.74%	<i>Pseudomonas</i> genus of <i>Pseudomonadaceae</i>
	TI_4	2.57%	<i>Flavobacteriaceae</i>
	TI_5	2.48%	<i>Flavobacteriaceae</i>
Blood Culture of R5 Medium (C6 Sample)	C6_1	28.47%	<i>Methylobacterium</i> genus of <i>Methylobacteriaceae</i>
	C6_2	2.19%	<i>Methylobacterium</i> genus of <i>Methylobacteriaceae</i>
	C6_3	0.47%	<i>Sphingomonas</i> genus of <i>Sphingomonadaceae</i>
	C6_4	0.31%	<i>Lactobacillaceae</i>
	C6_5	0.18%	<i>Methylobacterium</i> genus of <i>Methylobacteriaceae</i>
Blood Culture of SP4_F Medium (C7 sample)	C7_1	10.30%	<i>Sphingomonas</i> genus of <i>Sphingomonadaceae</i>
	C7_2	5.58%	<i>Lactobacillaceae</i>
	C7_3	2.77%	<i>Lactobacillaceae</i>
	C7_4	2.77%	<i>Methylobacterium</i> genus of <i>Methylobacteriaceae</i>
	C7_5	2.32%	<i>Bradyrhizobium</i> genus of <i>Bradyrhizobiaceae</i>
Blood Culture of BCF Medium (C9 Sample)	C9_1	27.48%	<i>Pseudomonas</i> genus of <i>Pseudomonadaceae</i>
	C9_2	12.09%	<i>Mesorhizobium</i> genus of <i>Phyllobacteriaceae</i>
	C9_3	3.73%	<i>Pseudomonas</i> genus of <i>Pseudomonadaceae</i>
	C9_4	0.72%	<i>Pseudomonas</i> genus of <i>Pseudomonadaceae</i>
	C9_5	0.52%	<i>Pseudomonas</i> genus of <i>Pseudomonadaceae</i>

Identification of microbial 16S rRNA gene sequences in diseased lung tissue and early-phase blood cultures matching closely to those of A. cberii and M. hominis

Table 1 reveals the sequencing data sets generated using the MiSeq sequencer for the 16S rRNA gene sequences amplified using pan-bacteria PCR primers [10] from the DNA samples prepared from the diseased lung tissue (T1 sample) and the early-phase blood cultures involving RPMI-1640 with 5% FBS (R5, C6 sample), SP4 with 5% FBS (SP4_F, C7 sample) and BHI with 5% FBS (BCF, C9 sample) broth media. The sequence reads matching closely to those of *A. cberii* and *M. hominis*, the two novel *Rhizobiales* microbes isolated from our re-initiated SP4 broth culture of the blood sample [6], were first sought in each of these examined samples using CLC Genomics Workbench (**Table 1**). Of the nearly two million total qualified 440 bp sequence reads identified in the T1 sample, ~4.8% of the sequences had a 100% match and ~18% of the sequences had a high similarity (>95% matching) to the *A. cberii* sequence. On the other hand, only a very small fraction (less than 0.03% of total reads) of sequences with close similarity (>95% match)

to that of *A. cberii* were found in the early phase of blood cultures in the C6 or C7 samples (**Table 1**). There were few sequences with close similarity (>95%) to that of *M. hominis* in the T1 sample. The numbers of reads with sequences closely matching (>95%) to those of *M. hominis* were also low in blood cultures of either the C6 or C7 samples. However, nearly 27% of total qualified reads in the early-phase blood culture of the C9 sample had sequences with high similarity (>95% matching) to the 16S rRNA sequence of *M. hominis*; 0.45% had the exact (100% match) sequence of *M. hominis*.

The concurrent 16S rRNA PCR products amplified in parallel from DNA preparations of R5, SP4_F and BCF culture broths as well as water control using primers p91F/p13B were minimal, barely detectable in gel electrophoresis. In the study, sequencing libraries were similarly prepared for the retrieved amplicon DNA of these control samples. Sequencing of these samples by MiSeq yielded very low numbers of paired reads (**Table 1**). Blast analysis revealed the low numbers of sequence reads with paired-ends as well as the unpaired raw sequence reads obtained from the culture broth and water controls were best-matched to

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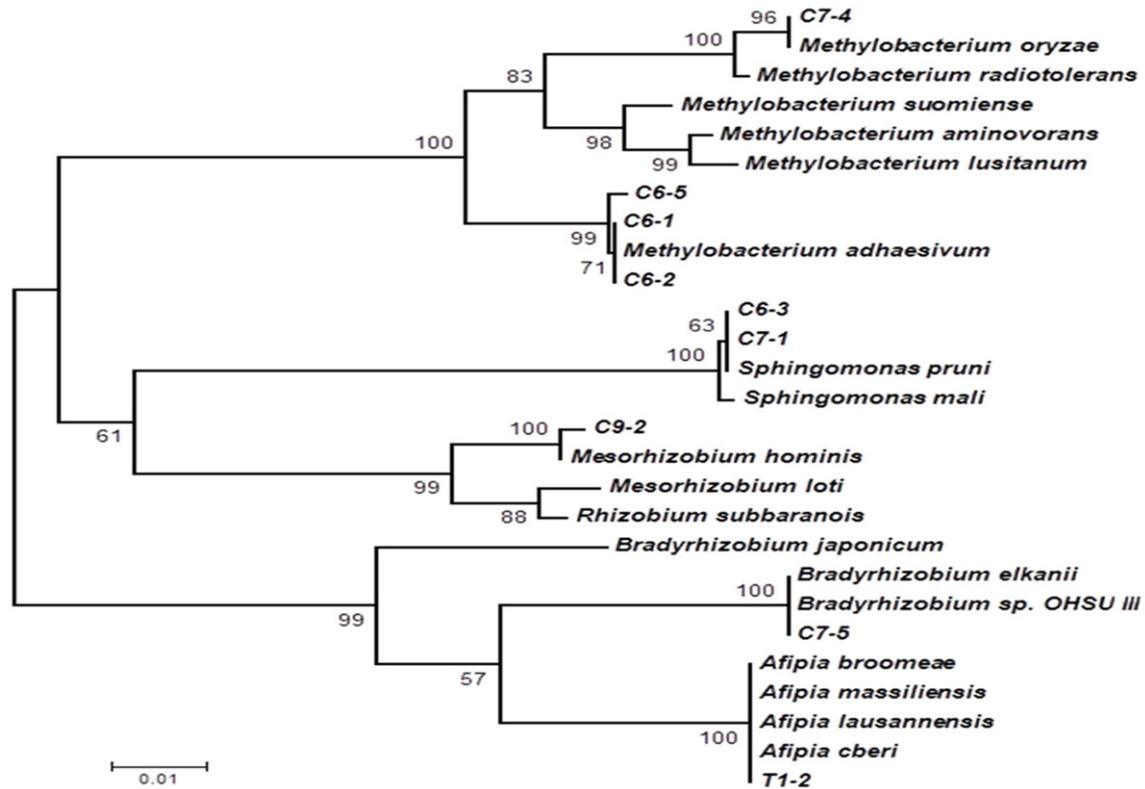


Figure 3. Phylogenetic analysis based on 16S rRNA gene sequences of the top-number reads amplified from the diseased lung tissue and early-stage blood cultures using the neighboring-joining method. Some of the neighboring *Rhizobiales*-related species: *Methylobacterium oryzae* (NR_043104.1), *Methylobacterium radiotolerans* (NR_112235.1), *Methylobacterium suomiense* (NR_041030.1), *Methylobacterium aminovorans* (NR_041025.1), *Methylobacterium lusitanum* (NR_112233.1), *Methylobacterium adhaesivum* (AB698708), *Sphingomonas pruni* (NR_113760.1), *Sphingomonas mali* (NR_113762.1), *Mesorhizobium hominis* (KF040403), *Mesorhizobium loti* (NC_002678.2), *Rhizobium subbaranois* (NR_108508.1), *Bradyrhizobium japonicum* (NC_004463.1), *Bradyrhizobium elkanii* (AB931148), *Bradyrhizobium* sp. OHSU_III (KC677617), *Afipia broomeae* (NR_029200.1), *Afipia massiliensis* (NR_025646.1), *Afipia lausannensis* (DQ123622.1) and *Afipia cberi* (KF040404) are included as references. The scale bar unit represents the number of substitutions per 100 sites. Numerals indicate bootstrap percentages over 50 after 500 replications.

some gamma-proteobacteria and *Bacteroides*. No sequence reads matching with those of *Rhizobiales* microbes were found.

Taxonomic analyses of all the sequence reads obtained in each examined sample for relative abundance at the family level

Figure 2 reveals the MEGAN display for relative abundance of 16S rRNA gene sequences with taxonomic classification at the level of microbial families based on BlastN results of all the qualified sequence reads from each of the samples. In the T1 sample, ~40% of the total 16S rRNA gene sequence reads were found to correspond to those of *Pseudomonadaceae*/*Xanthomonadaceae* microbes. There was no significant difference of rRNA gene sequences

in the amplified region for the two families of bacteria. However, more than 20% of the total qualified sequence reads (near two million) were classified as those of *Bradyrhizobiaceae* microbes (**Figure 2**), with ~4.8% of the reads with the sequence matching 100% with that of *A. cberi* (**Table 1**). In the C6 sample, ~90% of the total 16S rRNA gene sequence reads obtained were those from *Methylobacteriaceae* microbes. The second and third highest abundant sequence reads found in the C6 sample were those from *Lactobacillaceae* (~3%) and *Sphingomonadaceae* (~2%) microbes. In the C7 sample, most of the sequence reads found were those of *Lactobacillaceae* (~40%), *Sphingomonadaceae* (~30%), *Methylobacteriaceae* (~13%) and *Bradyrhizobiaceae* (~12%) microbes. In the C9 sample, similar to

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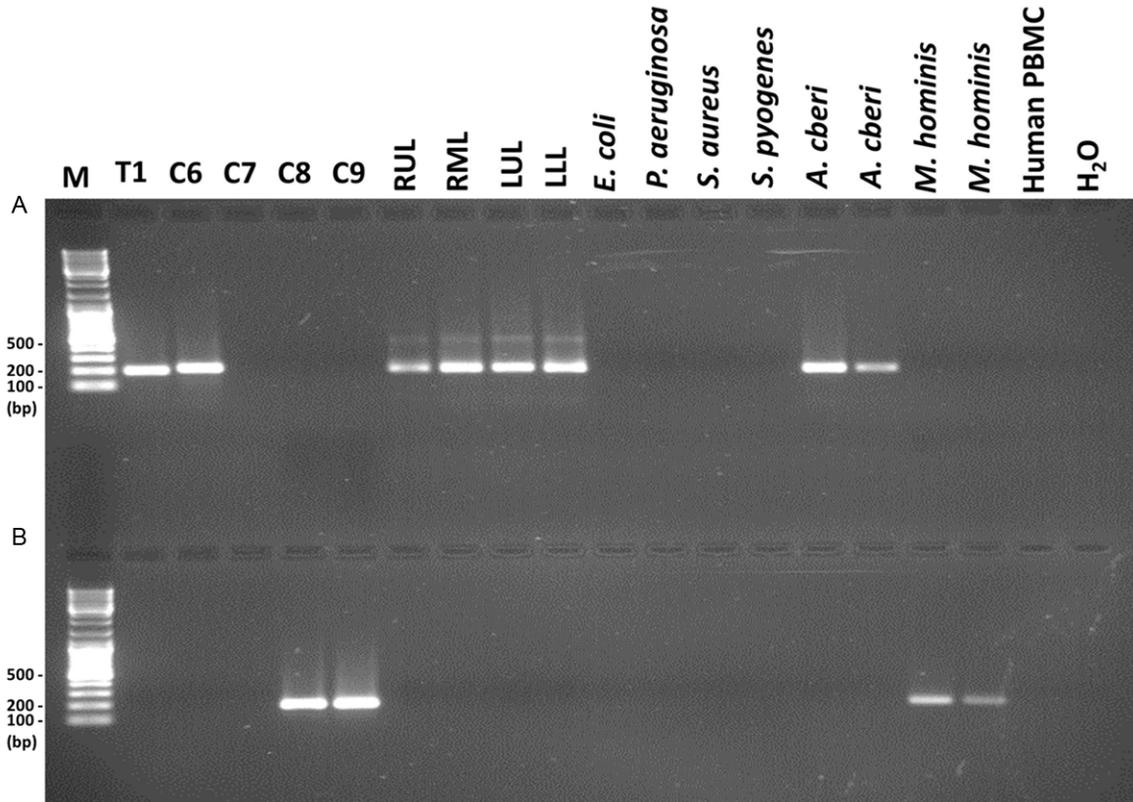


Figure 4. PCR studies of *Bradyrhizobiaceae* microbes and *M. hominis* in the patient's autopsy lung tissues and early-phase cultures of the blood sample. The DNA samples from diseased lung tissue obtained at autopsy (T1) and early-phase blood cultures involving broth media R5 (C6), SP4 (C7), BHI (C8) and BCF (C9) as well as FFPE autopsy lung tissues right upper lobe, right middle lobe, left upper lobe and left lower lobe (RUL, RML, LUL and LLL) were PCR amplified using the BAMQ#8 primer set (upper panel, A) and the MSMQ#5 primer set (lower panel, B). Genomic DNAs of *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes* and human PBMC (500 ng per reaction) were included in the negative control for both panels. In the upper panel, *M. hominis* DNAs (500 ng, duplicated) were used as negative controls and *A. cberi* DNAs (50 fg and 10 fg, respectively) as positive controls. In the lower panel, *A. cberi* DNAs (500 ng, duplicated) were used as negative controls and *M. hominis* DNAs (50 fg and 10 fg, respectively) as positive controls.

the finding with the T1 sample, the sequences of the most abundant reads (nearly 70% of total reads) belonged to those of *Pseudomonadaceae/Xanthomonadaceae* microbes. However, there were ~30% of reads with sequences belonging to *Phyllobacteriaceae* microbes (**Figure 1**) with a large majority of these reads (~27%) having sequences with very close similarity (>95%) to that of *M. hominis* (**Table 1**).

Taxonomic classification of the five most abundant sequences identified in each examined sample at the genus level for phylogenetic analysis

The individual sequences of the five top-number reads in each examined sample were spe-

cifically identified and taxonomically classified at the level of microbial genus through BlastN against the NCBI 16S bacteria ribosomal sequence database (**Table 2**). The sequence with the most abundant reads in the T1 sample (T1_1, ~12% of total sequence reads) was that of *Pseudomonas* genus microbes of the *Pseudomonadaceae/Xanthomonadaceae* family. The sequence with the second highest abundance of reads (T1_2, 4.86% of total reads) was that of *Afipia* genus microbes of the *Bradyrhizobiaceae* family, matching 100% to that of *A. cberi* (**Table 1**). The sequences with the most abundant reads in the early-phase blood cultures involving various media were those of genera in the *Methylobacteriaceae* (C6_1 and C6_2, *Methylobacterium*), *Sphingomonadaceae* (C7_1, *Sphingomonas*)

and *Phyllobacteriaceae* (C9_2, *Mesorhizobium*) families. However, no microbes of the *Methylobacteriaceae* (C6_1 or C6_2) and *Sphingomonadaceae* families (C7_1) were isolated by culture. The high abundant read sequence of C9_2 *Mesorhizobium* microbes with 12.1% of the total reads amplified from the culture of BCF media had only one nucleotide mismatch with that of *M. hominis* isolated subsequently from the re-initiated cryopreserved SP4 broth culture. **Figure 3** shows the phylogenetic relationship among *Rhizobiales*-related 16S rRNA gene sequences identified as the top-number reads in the diseased lung tissue and the early-phase cultures of blood samples. The phylogenetic tree also includes the related *Rhizobiales* microbes with their 16S rRNA genes sequences available in the NCBI database as reference species.

PCR detection of Bradyrhizobiaceae microbes and M. hominis in the diseased lung tissues from autopsy samples and early-phase blood cultures

Specific PCR primers were developed for the detection of *Bradyrhizobiaceae* microbes in the diseased lung tissues and in early-phase blood cultures. The *Bradyrhizobiaceae*-specific primer set (BAMQ#8) corresponds to highly conserved sequences in the genomes of microbes in the *Bradyrhizobiaceae* family, including all *Afipia* sp., *B. japonicum* and *B. elkanii*. However, the intervening sequences of the amplicons amplified using the BAMQ#8 primer set varied among different microbes of the *Bradyrhizobiaceae* family. As predicted from the genomic sequence datasets, BAMQ#8 amplified DNA sequences from *Bradyrhizobiaceae*, *A. felis* and *B. japonicum* with high sensitivity, but not DNAs from *M. loti* of the family of *Phyllobacteriaceae*, or non-alphaproteobacteria species. **Figure 4A** shows that primer set BAMQ#8 amplified specific sequences using DNA of *A. cberii*, with high sensitivity using 10 fg per PCR reaction, but not using genomic DNA from non-target *M. hominis*, *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes* or human PBMC at more than 10 million-fold higher concentration of 500 ng per reaction. As shown in part (**Figure 4A**), primer set BAMQ#8 would not amplify genomic DNAs from 20 different non-target species of commonly-encountered Gram positive and Gram negative bacteria tested at

the concentration 10 million-fold higher than target DNA of *A. cberii*. The PCR primers BAMQ#8 positively amplified the T1 sample and DNA specimens freshly retrieved from FFPE autopsy lung tissues of all four different lobes (RUL, RML, LUL, LLL) (**Figure 4A**). The C6 sample also tested positive by PCR. However, the size of the C6 amplicon appeared to be slightly larger than expected. DNA specimens prepared from the early-phase blood cultures involving SP4, BCF or BHI broth medium all tested negative by PCR using BAMQ#8 primers.

Specific primers were also developed for PCR detection of *M. hominis* of the *Phyllobacteriaceae* family in these samples. **Figure 4B** reveals that the *M. hominis*-specific primers MSMQ#5 amplified DNA sequences of *M. hominis* with high sensitivity using ~10 fg per reaction, but produced no positive products when using genomic DNA of *A. cberii* of the *Bradyrhizobiaceae* family at 10 million-fold higher concentration. The primer set MSMQ#5 also did not amplify genomic DNA from non-target *E. coli*, *P. aeruginosa*, *S. aureus*, *S. pyogenes* and human PBMC using 500 ng per reaction. As shown in part in **Figure 4B**, primer set BAMQ#8 would not amplify genomic DNAs from 20 different non-target species of commonly encountered Gram positive and Gram negative bacteria tested at the concentration 10 million-fold higher than target DNA of *M. hominis*. Consistent with the earlier results of the HTS-microbiome analyses, DNA previously prepared from the diseased lung tissue obtained at autopsy and DNA retrieved from the FFPE autopsy lung tissues tested negative by PCR using the *M. hominis*-specific primer set MSMQ#5. The early-phase blood cultures involving R5 and SP4 broth media also tested negative. On the other hand, the early-phase blood cultures involving BHI medium with or without FBS supplement (C9 and C8 samples) PCR-tested positive (**Figure 4B**).

Alignments of sequences amplified from the diseased lung tissues and the early-phase blood cultures using the PCR primer sets BAMQ#8 and MSMQ#5

All PCR amplicons obtained with the primer set BAMQ#8 in **Figure 4A** were retrieved from the agarose gel and initially subjected to Sanger sequencing. However, the amplicons obtained using DNAs extracted from different lobes of

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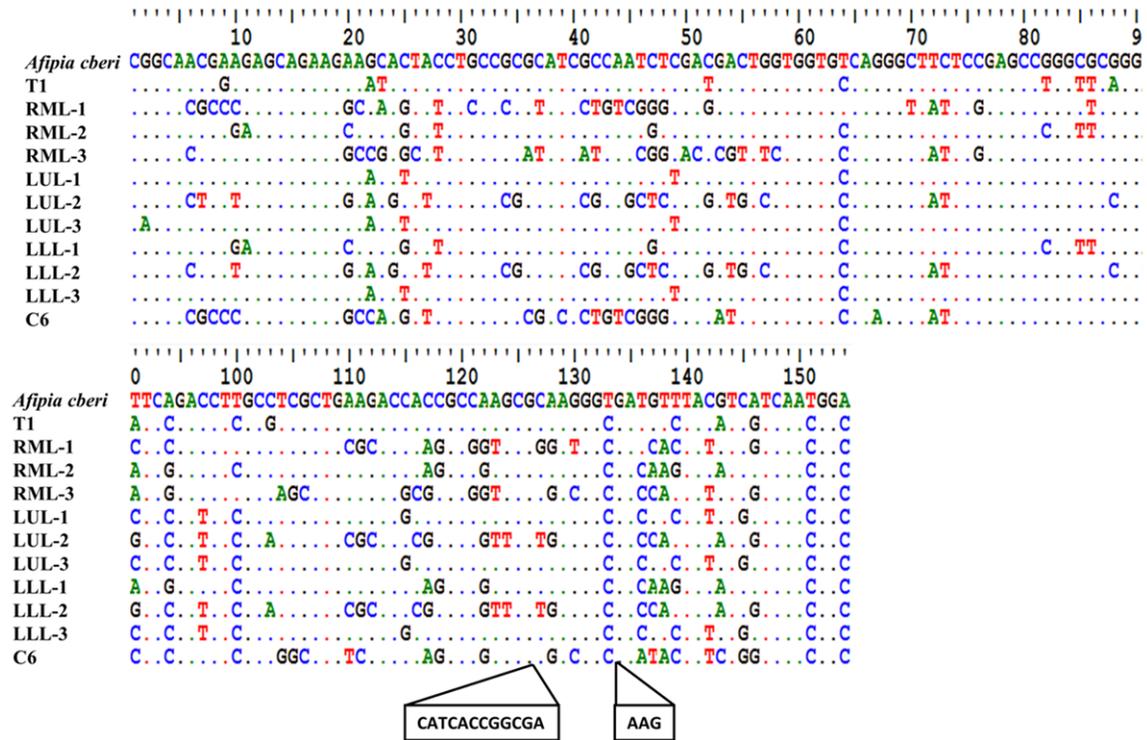


Figure 5. Alignment of amplicon sequences obtained using the BAMQ#8 primer set from different lobes of the patient's diseased lung tissues and from early-phase R5 culture of a blood sample with that of *A. cberii*. Sequences of amplicons from T1 and C6 samples were obtained using the Sanger Method. Sequencing of amplicons from FFPE autopsy lung tissues, right middle lobe, left upper lobe and left lower lobe (RML, LUL and LLL), that failed using the Sanger method were sequenced through MPS using MiSeq. Sequences of the three most-abundant reads from each amplicon sequenced by MPS were used for the alignment. The sequence variations seen between the high-abundance reads from the autopsy lung tissues and those of *A. cberii* are marked. The amplicon of the C6 sample was found to be distinct with 15 nts longer in size compared to the expected amplicons from *Bradyrhizobiaceae* microbes. Since phylogenetic analysis showed that more than 90% of microbial populations in the C6 sample were *Methylobacteriaceae* microbes (**Figure 1**), the C6 amplicon sequence was likely from microbes of *Methylobacteriaceae*, not *Bradyrhizobiaceae*.

FFPE autopsied lung tissues had mixed populations of sequences and yielded uninterpretable signals by Sanger sequencing. We then conducted MPS to study the sequence composition of amplicons derived from 3 different lobes of the FFPE autopsy lung tissues. The top three reads of the BAMQ#8 amplicon sequences obtained with FFPE autopsy lung samples from each lobe of were aligned against *A. cberii* and amplicon sequences amplified from the T1 sample using the BAMQ#8 primer set. The sequence amplified by the BAMQ#8 primer set using the T1 sample appeared to be closely related to that of *A. cberii*, although some sequence differences were noticed. Various degrees of sequence variations were also found among the most highly abundant reads amplified from different lobes of the patient's FFPE autopsy lung tissues and that of *A. cberii*

(**Figure 5**). The amplicon obtained using the blood culture C6 sample was found to be 15 nucleotides longer and had many more sequence differences in comparison with *A. cberii* (**Figure 5**). Since an earlier analysis showed that more than 90% of microbial population in the early-phase blood culture of the C6 sample consistent of *Methylobacteriaceae* microbes (**Figure 2**), the BAMQ#8 PCR amplicon sequence of the C6 sample was likely derived from those of the *Methylobacteriaceae* family and not the *Bradyrhizobiaceae* family.

We constructed a phylogenetic tree based on the heterogeneity among the sequences with the most highly abundant reads amplified from different lobes of the diseased lung (**Figure 6**). Some of the BAMQ#8 PCR amplicon sequences were clustered with those of microbes in

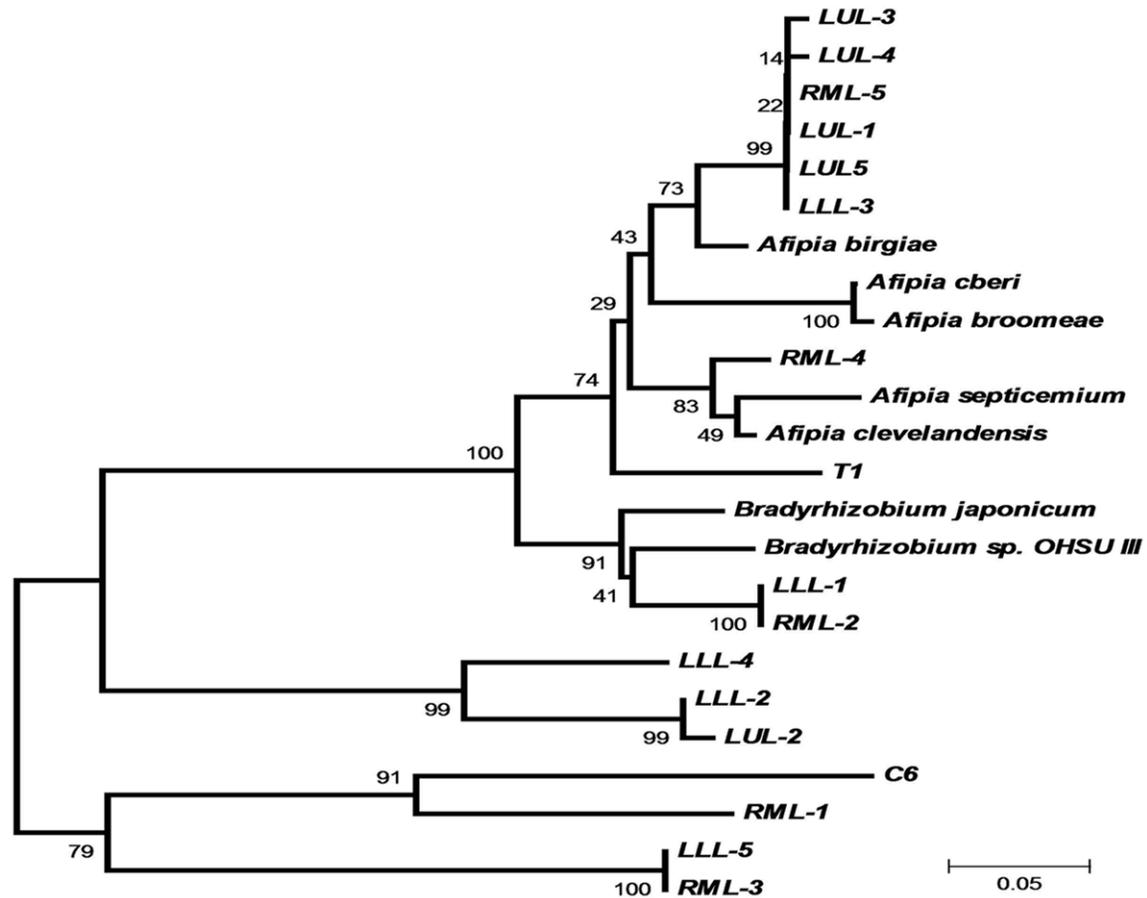


Figure 6. Phylogenetic analysis based on heterogeneity of sequences amplified from different lobes of the patient's diseased lung tissues using the BAMQ#8 PCR primer set. Sequences of PCR amplicons from FFPE autopsy lung tissues using the BAMQ#8 primer set revealed a high heterogeneity. There were evidently wide variations among different strains or species members of *Bradyrhizobiaceae* microbes in the patient's diseased lungs. None of the highly abundant or top-number sequence reads from different lobes of the patient's diseased lung were the same as the sequence of *A. cberi*.

Afipia genus, while others were clustered with those in the *Bradyrhizobium* genus. But, many sequences with apparently more variations were not clustered with those in either genus of the *Bradyrhizobiaceae* family. The PCR amplicons amplified by primers MSMQ#5 from the early-phase blood culture involving BHI medium (C8) and BHI medium with FBS (C9) (**Figure 4B**) were also retrieved and subjected to Sanger sequencing. The amplicon sequences were highly uniform, matching 100% to the sequence of *M. hominis*.

Direct identification of infections by *Bradyrhizobiaceae* microbes in the autopsy lung tissues by FISH

We designed a *Bradyrhizobiaceae*-specific DNA probe (P1-2) from a region of the 16S rRNA

gene sequence that was highly conserved among microbes of the *Bradyrhizobiaceae* family and exhibited substantial differences from rRNA sequences of microbes of the *Phyllobacteriaceae* family for the FISH study. The selected *Bradyrhizobiaceae*-specific 16S rRNA gene sequence, as expected, also displayed major differences when compared with those of gamma-proteobacteria such as *E. coli* and *P. aeruginosa*. **Figure 7** shows that the Cy3-labeled P1-2 probe hybridized specifically with *A. cberi* in FFPE cell pellets of cultured A549 human lung cells infected with *A. cberi*, but not with *M. hominis* in similarly processed FFPE cell pellets of A549 human lung cells infected by *M. hominis*. Nuclei of human cells in the sections of the FISH study were counterstained by DAPI (blue). Interestingly, A549 lung cells in cultures infected by *A. cberi* or *M. homi-*

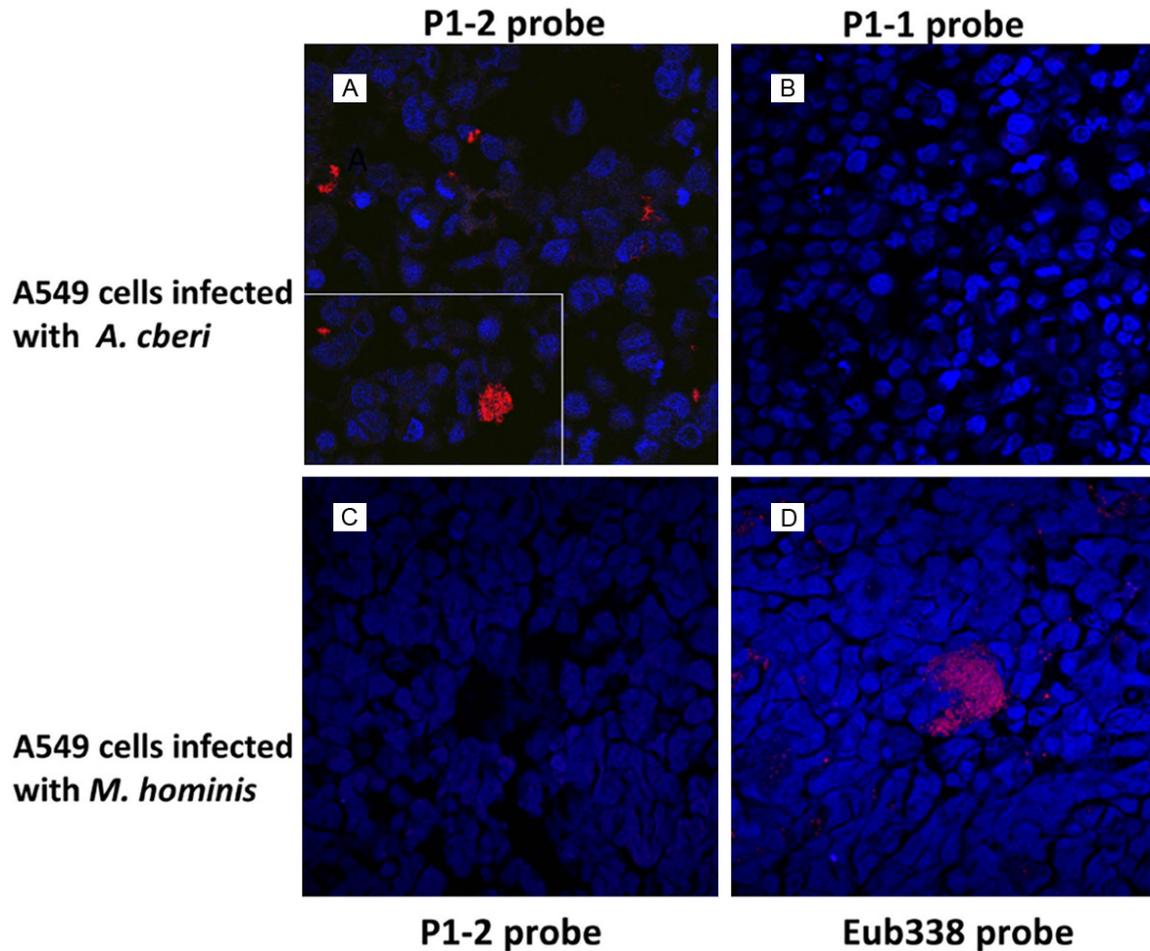


Figure 7. Detection and identification of *Bradyrhizobiaceae* microbes in infected cell cultures by FISH using the *Bradyrhizobiaceae*-specific P1-2 probe. The Cy-3 labelled P1-2 probe positively identified (red) *A. cberi* in sections of FFPE pellets of A549 lung cells experimentally infected by *A. cberi* (A). The inset shows positively hybridized clumping of *A. cberi* in the infected A549 cell culture. A FISH study using the Cy-3 labelled P1-1 probe with a sequence of reversed polarity compared to P1-2 as a negative control produced no positive signal in *A. cberi*-infected A549 lung cell cultures (B). The Cy-3 labelled P1-2 probe produced no positive signal in the A549 cell culture infected by *M. hominis* (C). In a parallel FISH study, single *M. hominis* cells and clumps of different sizes, in the A549 cell culture infected by *M. hominis* were positively identified (red) by the Cy-3 labelled universal eubacteria probe Eub338 [25] (D). Nuclei of A549 cells in all FISH study sections were counterstained (blue) with 4', 6-diamidino-2-phenylindole (DAPI).

nis for 36 hours exhibited significant morphological differences (**Figure 7A-D**). A Cy3-labelled probe of P1-1 with the reversed polarity in the same 16S rRNA gene region of P1-2 was used as a negative control that produced no positive hybridization signal with *A. cberi* in infected A549 cells (**Figure 7B**). **Figure 8** revealed that definite positive hybridization signals could be identified in all four different lobes of FFPE autopsy lung tissues studied by FISH using the Cy3-labelled P1-2 probe. The positive FISH signals found in areas of inflammation with or without apparent necrosis the autopsy lung tis-

sues had characteristics highly similar to those elicited by *A. cberi* in *A. cberi*-infected A549 cells (**Figure 8A-C**). Occasional positive signals were found to be associated with inflammatory cells and degenerating pneumocytes in the intra-alveolar space (**Figure 8D**).

Discussion

The standard method used to identify microbial agent(s) in an infection or sepsis in a patient involves recovering microbe(s) from infected tissue(s) and/or a blood culture from the

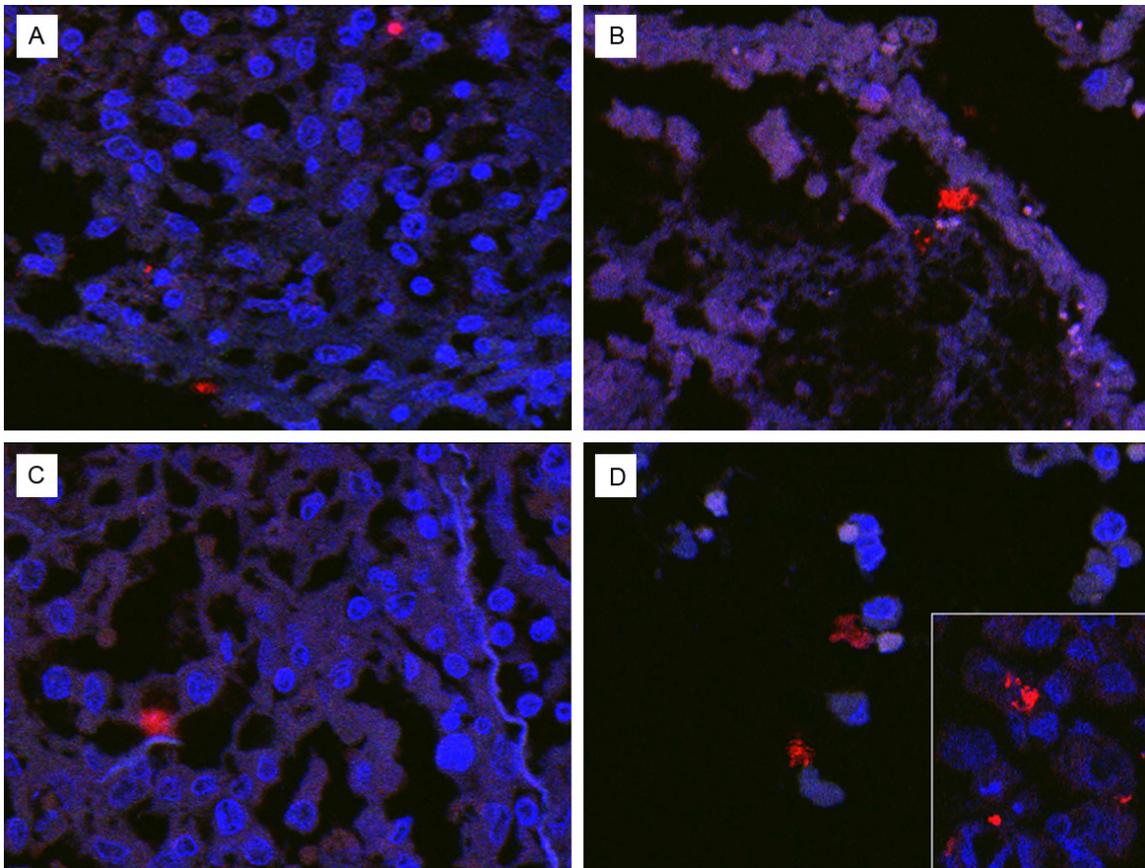


Figure 8. Detection and identification of *Bradyrhizobiaceae* microbes in FFPE autopsy lung tissues by FISH using the Cy-3 labelled *Bradyrhizobiaceae*-specific P1-2 probe. The Cy-3 positive signals (red) were identified in areas with many chronic inflammatory cells, their nuclei stained (blue) by DAPI (A & C). Positive signals could be found in areas with fulminant necrosis and alveolar degradation. Few viable cells with their nuclei positively stained by DAPI were present in this area (B). Positive signals were also seen associated with degenerating inflammatory cells and pneumocytes in the intra-alveolar space (D).

patient. However, this method can miss important insights into the possible diverse nature of microbes that exist in infected tissues and blood. The ability to grow in a laboratory culture system varies greatly among different microbes. MPS or HTS using various next generation sequencing (NGS) platforms has provided a critical means to assess *in situ* full microbial communities and to observe growing patterns of different microbial populations over time in the habitats, bypassing a cultivation-based bias [1-4]. A recent report summarized the tremendous growth in finding and understanding the presence of uncultivable microbial communities in the lungs of patients with various diseases and in healthy individuals using state-of-the-art culture-independent techniques [18]. In the present HTS-microbiome study using DNAs prepared from diseased lung tissue obtained at autopsy and from three early-phase cultures of

blood samples involving different media, we did a comprehensive analysis of microbial populations present in a patient who had a rapidly-progressing pulmonary disease.

Although microbiome studies are highly effective for analyzing a complex microbial community, analysts need to be cautious. Reagent, water or other unknown system contamination in PCR can critically impact a sequence-based microbiome analysis. This contamination could be particularly challenging when studying low biomass samples without culture confirmation [19]. In this study, the concurrent 16S rRNA PCR products amplified in parallel from control culture broths of R5, SP4_F and BCF, as well as water samples using primers p91F/p13B, were minimal. Parallel sequencing of the PCR amplified products by MiSeq yielded only very low numbers of paired-ends reads (**Table 1**). The

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low numbers of sequence reads obtained with paired ends as well as the unpaired raw reads obtained from the culture broth and water controls best matched to some gamma-proteobacteria and *Bacteroides*. There were no sequence reads matching those of *Rhizobiales* microbes. In this particular case, the sequence-based finding has been confirmed by successful isolation of two new species of *Rhizobiales* microbes of the two specific families by culture from the blood sample [6]. Moreover, highly specific PCR primers and FISH probes developed from the genomic and 16S rRNA sequences of the *Rhizobiales* isolates clearly detected the target *Rhizobiales* microbes in former blood cultures and the diseased lung tissues (**Figures 4A, 4B and 8**).

Analysis of DNA specimens from both the autopsy lung tissue and the early-phase blood cultures involving BCF broth medium (C9) revealed that *Pseudomonadaceae*-related 16S rRNA gene sequences were the most highly abundant reads. Since the patient developed *Pseudomonas* infections at the terminal stage of illness after extracorporeal membrane oxygenation (ECMO) was initiated in the hospital, finding *Pseudomonadaceae*-related gene sequences by MPS was not unexpected. However, antibiotic treatments against infections of *Pseudomonas*-related microbes appeared to be effective. No *Pseudomonas*-related microbes were recovered in any of our previous microbial cultures, nor were they recovered in subsequent re-initiated culture using cryopreserved SP4 culture broth.

Importantly, the top sequence reads of *Bradyrhizobiaceae* found in the diseased lung (T1-2, 4.86% of total sequence reads) matched 100% to that of *A. cberi*, previously isolated from the re-initiated blood cultures using SP4 medium. However, the majority of sequences taxonomically classified as *Bradyrhizobiaceae* (~20% of total sequence reads) were those that had a >95% match with that of *A. cberi* (**Table 1** and **Figure 2**). Microbes in the family of *Bradyrhizobiaceae* are known to have very high levels of homology in their 16S rRNA gene sequences, and thus are poor determinants of species or genera diversity [20, 21]. Minor differences of 16S rRNA gene sequences in the *Afipia* genus or in the *Bradyrhizobiaceae* family could be significant. The differences of 16S rRNA gene sequences found in the diseased

lung indicated that there were likely a mixed group of different *Bradyrhizobiaceae* microbes in addition to *A. cberi*. The marked sequence variations found in the PCR amplicons obtained with the diseased lung tissue (T1) and FFPE autopsied lung tissues (RML, LUL and LLL) using the *Bradyrhizobiaceae*-specific primer set BAMQ#8 (**Figure 5**) further provide strong evidence for a highly mixed group of different species of *Bradyrhizobiaceae* microbes to be present in the diseased lung.

One of the most abundant sequences (~30% of total reads) found in the early-phase blood culture set up with BCF medium (C9) belonged to microbes of *Phyllobacteriaceae*, another *Rhizobiales* family (**Figure 3**). These sequence reads matched closely (>95% of 440 bp, **Table 1**) with that of *M. hominis* isolated subsequently from the re-initiated SP4 broth culture of the patient's blood sample. The sequence of the most abundant number reads identified in the C9 sample corresponding to those of *Mesorhizobium* in the family of *Phyllobacteriaceae* (C9_2 with 12.1% of total reads, **Table 2**) had only one nucleotide mismatch with that of *M. hominis*. However, the 16S rRNA sequence reads with a 100% match with that of *M. hominis* were identified as only 0.45% of total reads (**Table 1**). Therefore, there were also a mixed group of different species of *Phyllobacteriaceae* microbes, in addition to *M. hominis*, present in the patient's blood. The *M. hominis* strain that successfully grew from the previously cryopreserved SP4 broth of blood culture [6] was one among several different strains/species of *Phyllobacteriaceae* microbes present in the patient's blood. Differing from BAMQ#8 amplicons of *Bradyrhizobiaceae* microbes found in the diseased lung, MSMQ#5 amplicons of different strains/species of *Phyllobacteriaceae* microbes found in the patient's blood samples appeared to be genetically more homogeneous. All of the amplicons obtained using the *M. hominis*-specific primer set MSMQ#5 have exactly the same sequence of *M. hominis*.

The present study shows that *Rhizobiales* microbes of different families clearly favor different culture media, even though they may still not be able to grow to the extent that can be recovered or isolated by culture. The *Mesorhizobium* microbes of the *Phyllobacteriaceae* family were found mainly in cultures

involving BHI-based media (C8 and C9) (**Figure 4B**). In comparison, the large majority (more than 70%) of 16S rRNA gene sequence reads found in the blood culture using R5 medium (C6) were those of *Methylobacteriaceae*, another *Rhizobiales* family (**Figure 1**). The sequences with the two highest abundant reads corresponded to those of two different *Methylobacterium* genera of the *Methylobacteriaceae* family (C6_1 and C6_2, **Table 2**). On the other hand, the sequence with the most abundant reads in the early-phase blood culture using SP4 medium (C7_1) corresponded to those of the *Sphingomonas* genus of the *Sphingomonadaceae* family (**Table 2**). However, no species members of the *Methylobacteriaceae* or *Sphingomonadaceae* families were isolated from the re-initiated culture of cryopreserved SP4 culture broth. Instead, *A. cberi* of the *Bradyrhizobiaceae* family, and *M. hominis* of the *Phyllobacteriaceae* family, with their sequences found to be present only in very low numbers in the early-phase blood culture of SP4 medium, (15 or none after more than a million reads analyzed of the C7 sample), evidently continued to grow and were eventually isolated from the re-initiated culture after a long course of 2-3 months of incubation [6].

Highly sensitive *Bradyrhizobiaceae*-specific (BAMQ#8) and *M. hominis*-specific (MSMQ#5) PCR primers were developed to detect the target *Rhizobiales* microbes (at ≤ 10 fg/PCR reaction) in the patient's diseased lung tissues and the early-stage blood cultures. In the study, as in part shown in **Figure 4**, the specificity of the primer sets designed from comparative bacteria genomic sequence alignments have been specifically examined experimentally against genomic DNAs from more than 20 different non-target species of commonly encountered bacteria at the concentration more than 10 million-fold higher than target DNA of *A. cberi* or *M. hominis*. In the future, these PCR primers may prove to be useful for molecular diagnostic studies and identification of *Rhizobiales* microbes in humans. Although the sequences obtained using the BAMQ#8 primer set are highly conserved among *Bradyrhizobiaceae* microbes, the intervening sequences of its amplicons were evidently heterogeneous among different species or strains (**Figure 5**). The heterogeneity of the amplicon sequences obtained using the BAMQ#8 primer set shows that they can be used to analyze the related-

ness of different strains/species compositions of *Bradyrhizobiaceae* microbes (**Figure 6**). A *Bradyrhizobiaceae*-specific 16S rDNA probe was also developed (**Figure 7**) for direct identification of microbial infections in the diseased lung tissues by FISH (**Figure 8**). Definite positive hybridization signals could be identified in all four different lobes of FFPE autopsy lung tissues with or without apparent necrosis using the Cy3-labelled P1-2 probe. The RNA preservation in the FFPE autopsy tissue of more than 15 years could clearly result in the lower number of cells with positive hybridization signals identified.

The study revealed the presence of a mixed group of *Rhizobiales* microbes of different taxonomic families in the patient's diseased lung and blood. Viral infections, particularly viremias involving RNA viruses, are often composed of swarms of viral variants [22, 23]. The degrees of intra-host variability could be associated with the onset and/or severity of the viral disease. However, the presence of a mixed group of different families of *Rhizobiales* microbes has not been previously reported in humans. More HTS-microbiome studies in various diseased and/or non-diseased states in the future could provide insightful understanding of the nature of the mixed group of microbes [24]. Any role these *Rhizobiales* microbes might have played individually or together in any disease process will require further investigation. In this aspect, highly sensitive molecular assays and specific probes developed in the study are expected to be highly valuable in rapid detection and identification of these microbes in humans.

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Disclosure of conflict of interest

None.

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