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Identification of Nucleotide Patterns in Meconium Microbiota to Improve the Management of Meconium Aspiration Syndrome in Clinical Practice

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Abstract

For many years, airways, lungs and meconium, which is a thick green substance and accumulates in the baby's intestines during pregnancy, have been assumed to be sterile. However, this assumption has been revised by the development of microbiota analysis, which is used for the identification of microorganisms as a new promising technique; it has been found that various microorganisms exist in the lungs and in the meconium fluid. Meconium Aspiration Syndrome (MAS) as a common neonatal problem can give rise to the development of lung infections in newborns and even cases leading to death are encountered. Various strategies regarding antibiotic therapy have been developed against the risk of infection, but positive outcome could not be obtained for each case. In this study, 16S rRNA gene sequences of bacteria existing in meconium microbiota and infected lung microbiota were subjected to comparative sequence analysis. Our aim was to identify positions of nucleotide patterns between the hypervariable regions of the bacterial 16S rRNA gene sequences that could provide similar functions among bacterial groups. Furthermore, similarity analysis was conducted to identify molecular signatures via phylogenetic framework to understand the etiology of the infections after MAS. Interestingly, *Bifidobacteria* which are used as probiotics were found to be similar to *Actinomyces* which are known as opportunistic pathogens. Furthermore, *Clostridium leptum* was associated with pulmonary inflammation for the first time. This study proposes the usage of microbiota analysis to improve the MAS management in clinical practice.

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Meconium Aspiration Syndrome, Pneumonia, Newborn, Microbiota, 16S rRNA, Bioinformatics

Introduction

Newborn microbiota is interesting to many scientists since the colonization of many bacteria starts in this period of human life. Despite the studies of Hymanson and Hertz in 1917 and Burrage in 1927, which demonstrated the presence of bacteria in meconium –which has a thick consistency and accumulates during pregnancy in the baby’s intestine– of some newborns, it was commonly accepted that the airways, lungs and meconium are sterile (Burrage 1927; Chotirmall and Burke 2015; Hall 1934; Hymanson and Hertz 1917; Jiménez et al. 2008; Snyder 1936). However, recent studies have shown that this acceptance is not true and findings are promising to use microbiota as a biomarker (Dietert and Silbergeld 2015; Huang and Lynch 2011; Shukla et al. 2017; Zemanick et al. 2013). Besides, the first non-culture based microbiota analysis of meconium was –to the best of our knowledge– conducted by Mshvildadze et al. (Mshvildadze et al. 2010).

In a study, Macfarlane and Heaf have observed that newborns develop asthmatic symptoms due to the aspiration of meconium, suggesting that aspiration leads to abnormal respiratory functions through long-term effects on the respiratory system (Macfarlane and Heaf 1988). Following meconium aspiration, multiple life-threatening complications such as the development of hypoxia, respiratory and/or metabolic acidosis, pulmonary hypertension, hypoxic-ischemic encephalopathy and pneumothorax can be encountered (Espinheira et al. 2011; Shaikh et al. 2016). Other diseases potentially related to Meconium Aspiration Syndrome (MAS) include necrotizing enterocolitis, late-onset sepsis, autism and autism-like behavior disorders, fetal macrosomia, obesity, food sensitization and allergy, neonatal jaundice. Also autoimmune diseases such as type 1 diabetes, multiple sclerosis or Morbus Crohn were found to be related to MAS (Dobbler et al. 2017; Dong et al. 2018; Koleva et al. 2015; Stewart et al. 2012; Wilczyńska et al. 2019). In addition, the pro-inflammatory components of meconium lead to pneumonia and pulmonary inflammation, which in turn contribute to the development of bronchopulmonary dysplasia (Kopinova and Calkovska 2016; Speer 2003).

Thus, the aspiration of meconium by the baby due to maternal hypertension, maternal diabetes, hypoxia etc. often leads to the development of lung infections which make the prescription of partially non-specific prophylactic antibiotics mandatory (Yurdakök 2011). Antibiotics are used not only for prophylaxis, but also for treatment of MAS. It is difficult to distinguish between MAS and pneumonia, which leads to an increased prescription of antibiotics due to the high susceptibility to infection.

However, antibiotherapy in newborns has many concerns since there are many factors that influence the effects of antibiotics including gestational age, birth weight, intrauterine growth restriction, chronological age and, especially, kidney and liver function immaturity (Chirico et al. 2009). Another aspect is that some studies revealed that antibiotics impairs the immune system of the patient and thus leads to bacterial growth (Benoun et al. 2016; Ubeda and Pamer 2012). It should be added that antibiotherapy needs to be optimized concerning dosing, timing and route of administration if the bacterial composition of the microbiota is known due to the fact that optimization is strongly related to the appropriate selection of antibiotics. However, this is often not the case since traditional culturing techniques fail to identify the existence of many important bacterial clades. But principally, Gosalbes et al. detected extraordinarily high expression rates of antibiotic resistance genes in meconium-associated bacteria which helps us to understand the non-responsiveness to antibiotherapy in some cases and the associated mortality (Gosalbes et al. 2016). The present study is an attempt to reveal the relation of meconium microbiota and lung microbiota in the presence of pneumonia. It is aimed to shed light on the advancement of more specific antibiotherapies or to reduce the usage of antibiotics in some MAS cases, respectively.

Material and methods

Targeting Bacteria and Retrieving 16S rRNA Sequences from Databases

After extensive database and literature search, meconium- and pneumonia-associated bacteria were obtained from the studies of Nagpal et al. and

Chamberlain, respectively (Chamberlain 2016; Nagpal et al. 2016). The 16S rRNA gene sequences of these bacteria were retrieved from the National Center for Biotechnology Information (NCBI) Nucleotide Database and the European Bioinformatics Institute (EMBL-EBI) Database.

Subsequent analyses were performed with 16 bacteria found in meconium microbiota of vaginally-born infants and 33 bacteria found in lung microbiota of patients diagnosed with pneumonia. The sequences of some of these bacteria (*Streptococcus agalactiae*, *Nocardia sp.* and *Serratia sp.*) could not be retrieved from a single database entry, thus these sequences were merged from several database entries. BLASTn was performed for these sequences and the similarity rates were ascertained as >90%. All these bacteria and the corresponding accession numbers are given in Supplementary S1. All supplementary tables and data (S1-S10) are available online at <http://dx.doi.org/10.17632/w5653pr7yh.1>.

Extraction of the Hypervariable Regions

V-Xtractor version 2.1 was used on Linux (Ubuntu 18.04.1 LTS, GNOME 3.28.2, OS Type 64-bit) to extract the hypervariable regions V1-V9 of the bacterial 16S rRNA genes (Hartmann et al. 2010). Necessary to that end was a sensitive method; we preferred HMMER version 3.2.1 which allows biological sequence analysis using profile Hidden Markov Models (Eddy 2011).

Comparative Sequence Analysis

The progressive alignment tool T-Coffee version 11.00.8cbe486 was performed on Linux in order to align multiple sequences. The results of the multiple sequence alignments were then displayed and analysed by using Jalview version 2.10.4b1 (Waterhouse et al. 2009). In this way, global and local similarities of each of the extracted hypervariable regions V1-V9 of the bacterial 16S rRNA sequences were curated (Notredame et al. 2000). The results of each hypervariable region was further analysed and 9 bacteria associated with pneumonia (namely *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*,

Staphylococcus aureus, *Peptostreptococcus sp.*, *Bacillus anthracis*, *Nocardia sp.*, *Actinomyces sp.* and *Neisseria meningitidis*) were found to be similar to meconium-associated bacteria.

Phylogenetic Tree

Phylogenetic trees were generated separately for each hypervariable region at <http://www.phylogeny.fr/> (Anisimova and Gascuel 2006; Castresana 2000; Chevenet et al. 2006; Dereeper et al. 2008; Dereeper et al. 2010; Edgar 2004; Guindon and Gascuel 2003). For this purpose, the multiple sequence alignment results of 16 meconium-associated bacteria and 9 bacteria associated with pneumonia, which were previously found to be similar, were used.

Results and Discussion

In recent years, many platforms were established, and pipelines were designed to analyse human microbiota. In 2007, the Human Microbiome Project (HMP) was launched which further contributes to our understanding of the human microbiota composition. However, there is no data concerning the lung, pneumonia or meconium available on the website of HMP (<https://hmpdacc.org/hmp/>); thus, much needs to be investigated in future. Because of the limited microbiome data regarding lung or pneumonia or meconium, respectively, we were forced to use the existing limited data and tried to indicate an approach for the management of MAS with our study with the aim to reveal that there is the pressing need for much more studies on this field.

One in every seven pregnancies ends with meconium-stained amniotic fluid and 5% of these infants develop MAS (van Ierland and Beaufort 2009). MAS has a wide range of severity and even in some cases, it is the leading cause of morbidity and mortality – depending on the conditions and complications which develop after parturition. Although many studies show that antibiotic use in infants cause many problems, antibiotic administration is one of the general applications to counteract this syndrome in newborn follow-up clinics (Miller et al. 2018; Rogawski et al. 2017).

In this study, the role of meconium microbiota in the development of lung infections was investigated by using the new data emerged from the microbiota analyses. Identifying molecular signatures via phylogenetic framework helps us to understand the etiology of the infection after MAS. Clinical usage of antibiotics in newborns could be redesigned by using these molecular signatures.

Staphylococcus and *Streptococcus* exist both in

meconium and in infected lungs according to the existing data. Abundance of these bacterial clades may indicate a target for diagnosis. Quantitative analyses on these bacteria of newborn meconium immediately after birth may help to enhance rational use of antibiotics in newborns.

The results of the comparative sequence analysis propose several significant similar nucleotide patterns which may indicate useful targets for

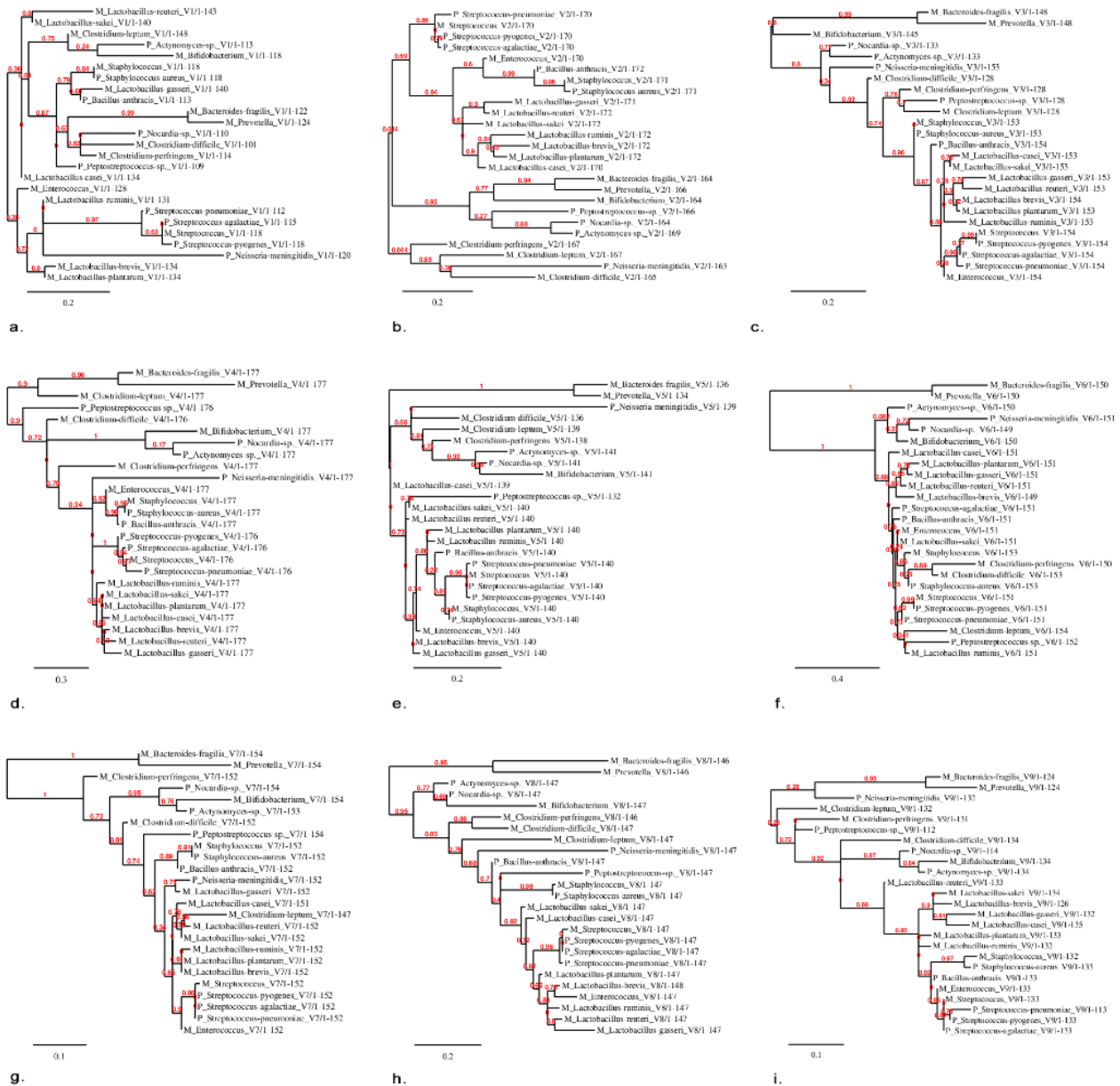


Figure 1. Phylogenetic Trees of Hypervariable Regions V1-V9. The phylogenetic trees generated separately for each hypervariable region V1-V9 (a-i.) of the 16S rRNA genes of the bacteria of meconium (M) and bacteria associated with pneumonia (P).

diagnostic assays. To analyze those similarities, phylogenetic trees were constructed with the sequences of each hypervariable region V1-V9 altogether of the corresponding bacteria (Figure 1). Positions of nucleotide patterns varying between the respective hypervariable regions of the 16S rRNA genes of bacteria found in meconium and bacteria

leading to lung infections have been analysed and are shown in Figure 2.

Peptostreptococcus that shows pathogenic activity for pneumonia under traumatic conditions has been found 95% similar to *Clostridium leptum* in meconium according to the phylogenetic tree generated by using the hypervariable regions V1-V9

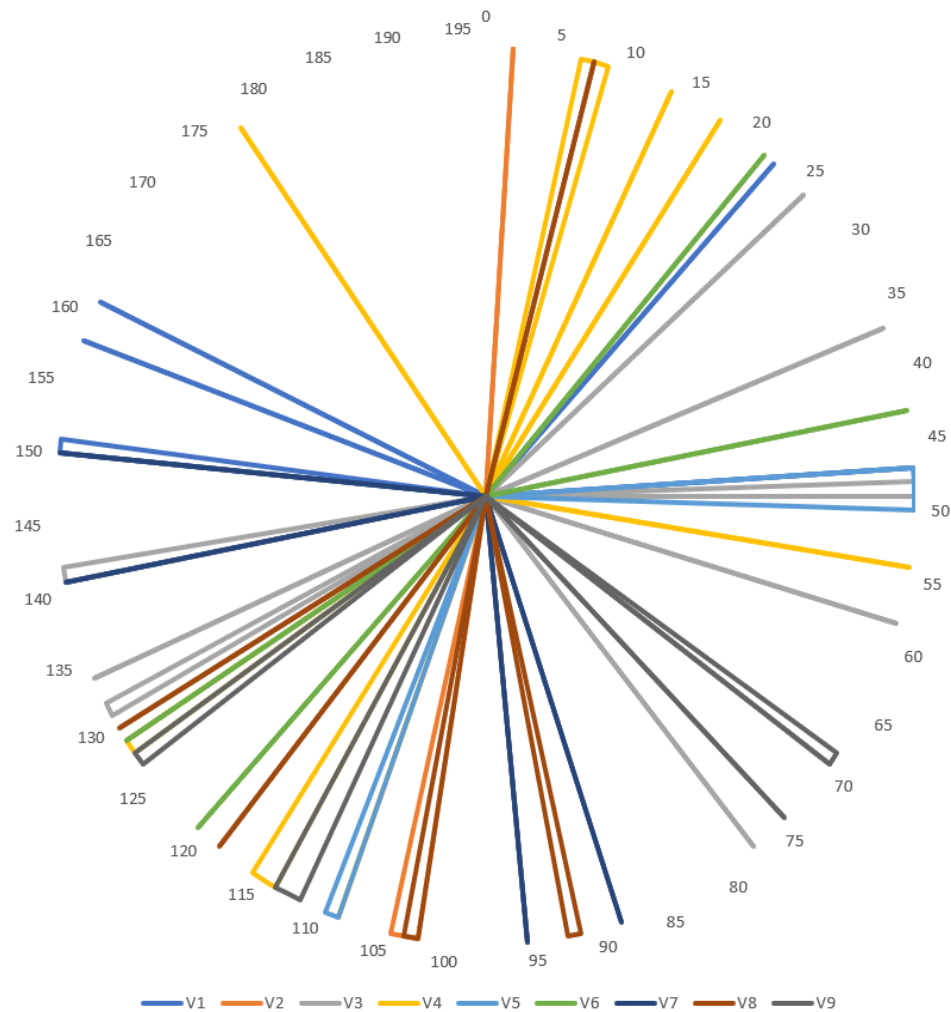


Figure 2. Nucleotide Pattern Positions. Positions of nucleotide patterns varying between the respective hypervariable regions V1-V9 of the 16S rRNA genes of the bacteria of meconium^a and bacteria leading to lung infections^b.

^a*Clostridium leptum*, *Bacteroides fragilis*, *Prevotella*, *Bifidobacterium*, *Clostridium perfringens*, *Clostridium difficile*, *Enterococcus*, *Staphylococcus*, *Streptococcus*, *Lactobacillus gasseri*, *Lactobacillus ruminis*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus sakei*, *Lactobacillus plantarum* and *Lactobacillus brevis*.

^b*Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Staphylococcus aureus*, *Peptostreptococcus sp.*, *Bacillus anthracis*, *Nocardia sp.*, *Actinomyces sp.* and *Neisseria meningitidis*.

– all merged together consecutively (Figure 3). Although *C. leptum*'s role in inflammatory bowel disease and in the development of asthma was shown in some studies, it was never associated with pulmonary inflammation (Huang et al. 2015;

multiple genes associated with virulence factors, harmful metabolites and antibiotic resistance, whereas one of these antibiotic resistance genes is possibly transferable (Wei et al. 2012). In order to confirm the similarity of *Actynomyces*

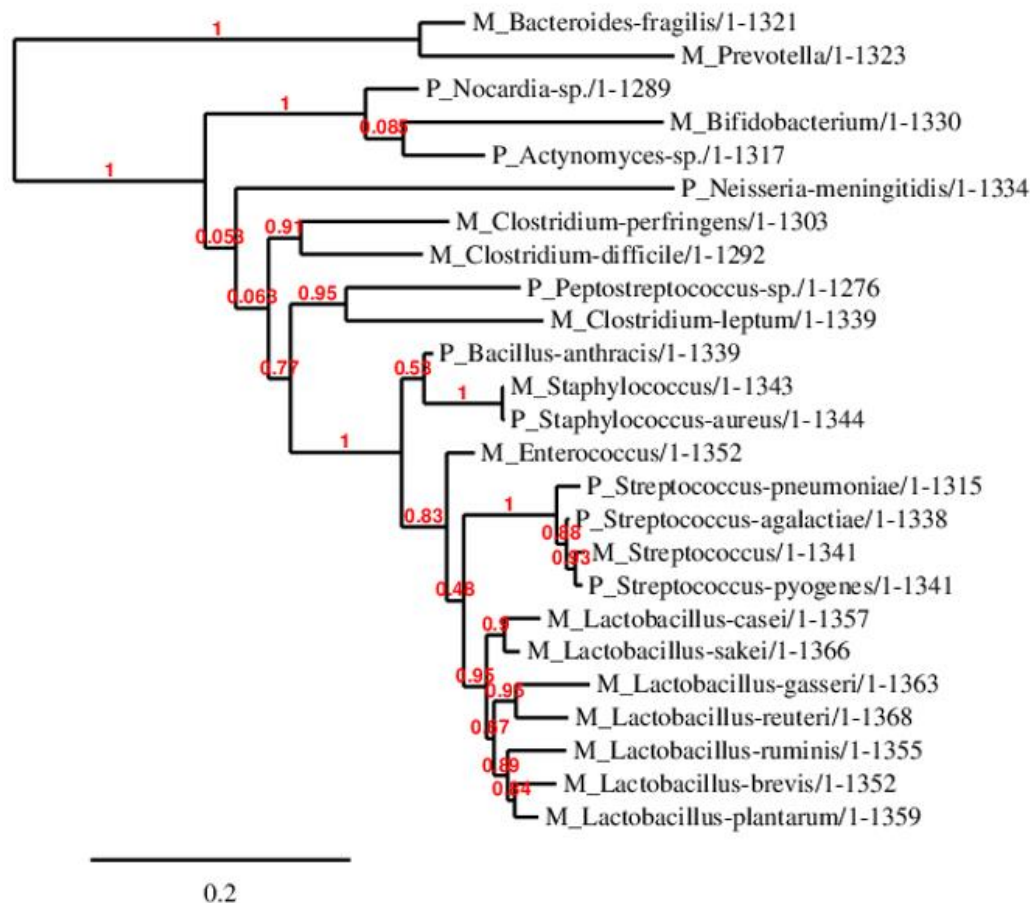


Figure 3. Phylogenetic Tree Comprising All Hypervariable Regions. The phylogenetic tree was generated by using the sequences of the hypervariable regions V1-V9 of the bacterial 16S rRNA genes which were all merged together consecutively. The abbreviation M indicates the association of the corresponding bacterium to meconium, while the abbreviation P shows the association to pneumonia. Care has to be taken to the similarity of *Peptostreptococcus* and *Clostridium leptum*.

Kabeerdoss et al. 2013). Another detected similarity was between *Actynomyces* and *Bifidobacterium* which is a bit surprising at first glance (Figure 1g and 1i). Besides being part of the human microbiota, *Actynomyces* are known for causing several diseases, whereas *Bifidobacterium* species are known to be beneficial and used as probiotics. However, some cases were reported in which *Bifidobacterium* species caused urinary, pleuropulmonary, obstetric and gynecologic infections, dental caries and even bacteremia (Bertelli et al. 2015; Weber et al. 2015). Moreover, *Bifidobacterium* species may possess

and *Bifidobacterium*, we performed additional comparative sequence analysis with the appropriate hypervariable regions of both bacteria and ascertained that there was a significant similarity: These bacteria's hypervariable regions V9 and V7 were the most similar sequences with similarity values 91% and 90%, respectively (Table 1). Thus, we propose that the pathogenicity of *Bifidobacterium* may be ascribed particularly to V9 and V7, respectively, since hypervariable regions may play an important role in the pathogenicity of bacteria. This pathogenicity could be in the form of

Table 1.

Similarity of *Actynomyces* and *Bifidobacterium*. The similarity percentages were calculated from the multiple sequence alignment results performed with the sequences of the appropriate hypervariable regions of both bacteria.

	Total Sequence Length	Count of Identical Nucleotides	Count of Different Nucleotides	Similarity (in %)
Hypervariable Region V1	118	85	33	72,03
Hypervariable Region V2	169	114	55	67,46
Hypervariable Region V3	145	105	40	72,41
Hypervariable Region V4	177	144	33	81,36
Hypervariable Region V5	141	120	21	85,11
Hypervariable Region V6	150	131	19	87,33
Hypervariable Region V7	154	139	15	90,26
Hypervariable Region V8	147	124	23	84,35
Hypervariable Region V9	134	122	12	91,04

antibiotic resistance, since antibiotic-binding sites are usually located within important structures of the bacterial 16S rRNA in order to achieve blockage of protein synthesis on the ribosome.

As it can be obtained from Table 1, V1-V3 are less similar when compared with the other hypervariable regions. We concluded that V1-V3 could be more suitable for the differentiation of bacterial species to the genus level. In line with our conclusion, Chakravorty et al. reported that V2 and V3 were the most suitable regions for bacterial differentiation (Chakravorty et al. 2007). Besides, the hypervariable regions V2 and V3 are also more appropriate for bacterial differentiation due to the high mutation rates in these regions (Bukin et al. 2019).

This study may be the first regarding the molecular evidence of the similarity between *Actynomyces* and *Bifidobacterium*. In fact, it would be appropriate if these analyses will be repeated with a larger data set and the results confirmed in future. Besides, further studies regarding the function of 16S rRNA hypervariable regions in the pathogenicity, but especially in the antibiotic resistance of bacteria should be performed in order to optimize or even to eliminate antibiotic treatment after MAS.

Conclusion

The successful treatment of MAS depends on the choice of the appropriate antibiotic, which may only be possible after a microbiota analysis. Even if it is not possible to carry out individual microbiota

analyses in the immediate future, at least one large-scale study can improve the prospects for successful treatment of MAS. In addition, identifying which hypervariable regions of the 16S rRNA gene are responsible for the pathogenicity, especially the resistance to antibiotics, of bacteria is also of great importance for the optimization of antibiotherapy.

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Conflict of interest

Authors declare no conflict of interest.

Supplementary Material

The following are the supplementary data related to this article (<http://dx.doi.org/10.17632/w5653pr7yh.1>).

Supplementary S1. Bacteria found in meconium (M) microbiota (Nagpal et al., 2016) and in lung (L) microbiota of patients diagnosed with pneumonia (Chamberlain, 2016) given together with the appropriate accession numbers of the 16S rRNA gene sequences.

Supplementary S2. Sequences of the hypervariable regions V1 of 16S rRNA genes of meconium-associated bacteria (M) and bacteria that cause pneumonia (P).

Supplementary S3. Sequences of the hypervariable regions V2 of 16S rRNA genes of meconium-

associated bacteria (M) and bacteria that cause pneumonia (P).

Supplementary S4. Sequences of the hypervariable regions V3 of 16S rRNA genes of meconium-associated bacteria (M) and bacteria that cause pneumonia (P).

Supplementary S5. Sequences of the hypervariable regions V4 of 16S rRNA genes of meconium-associated bacteria (M) and bacteria that cause pneumonia (P).

Supplementary S6. Sequences of the hypervariable regions V5 of 16S rRNA genes of meconium-associated bacteria (M) and bacteria that cause pneumonia (P).

Supplementary S7. Sequences of the hypervariable regions V6 of 16S rRNA genes of meconium-associated bacteria (M) and bacteria that cause pneumonia (P).

Supplementary S8. Sequences of the hypervariable regions V7 of 16S rRNA genes of meconium-associated bacteria (M) and bacteria that cause pneumonia (P).

Supplementary S9. Sequences of the hypervariable regions V8 of 16S rRNA genes of meconium-associated bacteria (M) and bacteria that cause pneumonia (P).

Supplementary S10. Sequences of the hypervariable regions V9 of 16S rRNA genes of meconium-associated bacteria (M) and bacteria that cause pneumonia (P).

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