Int J Clin Exp Med 2015;8(7):11075-11084
www.ijcem.com /ISSN:1940-5901/IJCEM0006296

Original Article

Molecular analysis of biofilms on the surface of neonatal endotracheal tubes based on 16S rRNA PCR-DGGE and species-specific PCR

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Received January 24, 2015; Accepted June 20, 2015; Epub July 15, 2015; Published July 30, 2015

Abstract: Ventilator-associated pneumonia (VAP) results in considerable morbidity and mortality in neonatal intensive care units. VAP is associated with polymicrobial biofilms that form on endotracheal tubes (ETTs). We aimed to evaluate the diversity and the bacterial community in biofilms on ETTs extubated from mechanically ventilated newborns. ETTs (N = 29) and aerobic sputum cultures were obtained from 20 mechanically ventilated newborns. Denaturing gradient gel electrophoresis (DGGE) was used to characterize the bacterial species in the biofilms on the ETTs. Species-specific PCR was used to detect common oropharyngeal *Streptococcus* species and known ETT-associated pathogens. DGGE profiling of ETT biofilms showed multiple banding patterns indicating a diverse bacterial community. The dominant bacterial species were *Klebsiella* spp. (29/29), *Streptococcus* spp. (27/29), and *Pseudomonas* spp. (24/29). The most frequently occurring *Streptococcus* species was *Streptococcus mitis* (N = 18). Oropharyngeal bacteria were present in 25 of 29 ETT specimens. *Streptococcus* spp. often co-existed with *K. pneumoniae* and/or *P. aeruginosa*. In contrast, only one bacterial species was isolated from each sputum culture, *K. pneumoniae* or *Acinetobacter baumannii*. Our results demonstrated that *Klebsiella* spp., *Streptococcus* spp., and *Pseudomonas* spp. were the most frequent microbes on the surface of neonatal ETTs. The co-existence of oral commensals and pathogenic bacteria on the same tubes may play a crucial role for biofilm formation.

Keywords: Mechanical ventilation, biofilm, denaturing gradient gel electrophoresis (DGGE), neonate, *Streptococcus* spp

Introduction

Ventilator-associated pneumonia (VAP) is one of the most common iatrogenic infections in neonatal intensive care units (NICU). VAP is associated with significantly prolonged hospitalization, increased medical costs, and a rising rate of morbidity. The VAP-related mortality rate is reported to be 32.2% [1]. Apart from factors such as birth weight, parenteral alimentation, and mechanical ventilation [2], endotracheal intubation is often considered an independent risk factor for VAP [3]. Scanning electron microscopy (SEM) studies have found that bacteria can colonize endotracheal tubes (ETTs) within 24 hours of implantation. The ETT provides an ideal biological niche for bacterial adhesion, and biofilms can form on both the inner luminal and outer surface [4]. In fact, it has been suggested that the term "endotracheal tube-associated pneumonia" is more accurate than "ventilator-associated pneumonia" [5].

Under most circumstances, biofilms contain multiple species of bacteria [6]. Multi-species biofilm formation is a dynamic process leading to a three-dimensional structure. As compared with planktonic bacteria, bacteria embedded in biofilms can adapt to the environment more easily by secreting polysaccharides, lipids, extracellular DNA, and proteins. Biofilm formation is also an effective immune evasion mechanism. The matrix of the biofilm prevents antimicrobial agents from penetrating into the interior of the biofilm, which contributes to the antibiotic-resistance of bacteria.

Current research focused on ETT-associated biofilms encompasses biofilm development, architecture, and interactions [6, 7]. Historically, VAP was diagnosed using conventional culture-based methods to assess the composition of the bacterial community. However, conventional methods are insufficient as up to 99% of bacteria in the natural environment cannot be cultured [8]. Indeed, over 90% of aspirates from ETTs do not yield positive culture results in the early stages. In contrast, culture-independent methods provide a more comprehensive profile of bacterial diversity and can be used to assess complex bacterial community dynamics. Denaturing gradient gel electrophoresis (DGGE) has been successfully used to investigate the composition of the bacterial community on ETTs that were removed from mechanically ventilated adults [9]. However, there are limited studies using the 16S rRNA PCR-DGGE method to characterize the bacterial community on neonatal ETTs. Understanding the microbiota of ETTs from mechanically ventilated neonates is crucial for effective antimicrobial therapy.

The aim of this study was to characterize the diversity of the bacterial community in biofilms on ETTs extubated from mechanically ventilated newborns using the 16S rRNA PCR-DGGE method. In addition, species-specific PCR was performed to determine whether oropharyngeal *Streptococci* species and other known potentially pathogenic bacteria were colonized on the ETT.

Materials and methods

Sample collection

The protocol was approved by the Ethics Committee of the Chongqing Medical University, and written informed consent was obtained from all parents or guardians. Mechanically ventilated newborns were recruited from the NICU of the Children's Hospital of Chongqing Medical University in China between October 2011 and March 2012. Demographic and clinical characteristics of the patients were recorded. Sputum specimens were collected before extubation as previously described [10]. Specimens were subjected to routine aerobic culture immediately after suctioning according to standard microbiological methods recommended by the American Society for Microbiology (ASM). The resulting colonies were Gram stained and identified using a MicroScan WalkAway-40 automated bacterial identification and susceptibility test instrument.

The distal part of the ETT is generally the most common area for biofilm formation [4]. Therefore, a 2-cm long cross-sectional segment was taken from the distal part of each ETT and cut longitudinally using a sterile scalpel for SEM and molecular analysis. All extubated ETTs were deposited in sterile tubes and transported to the laboratory within 1 hour. All the samples were stored at -80°C until further processing.

Scanning electron microscopy (SEM)

SEM was used to investigate the formation of biofilms on the interior luminal surface of the distal part of the ETT. The SEM samples were processed using standard techniques. Briefly, the ETT specimens were fixed for at least three hours with 2.5% glutaraldehyde and washed twice in sterile phosphate buffered saline (PBS). Dehydration was performed in graded concentrations of ethanol-30, 50, 70, 90, and 100%. Each sample was gold coated using a gold sputter and viewed with an S-3000N Hitachi SEM (Hitachi High-Technologies, Japan).

DNA extraction

The planktonic microorganisms on both outer and inner surfaces of the ETT were removed by washing with sterile PBS. Microorganisms within the biofilms were detached from the surface of the ETT by sonication and vortex. The bacterial suspension was placed in a 2-mL sterile tube, and the DNA was extracted using the TIANamp Bacteria DNA Kit (Tiangen, China) according to the manufacturer's instructions. DNA extracts were stored at -20°C until further analysis.

16S rRNA PCR and DGGE

The variable V3 region of 16S rRNA was amplified using a universal bacterial primer set (357f, 5'-GC-clamp-CCTACGGGAGGCAGCAG-3' and 518r, 5'-ATTACCGCGGCTGCTGG-3'). A 40-bp GC-clamp (CGCCCGGGGCGCGCCCCGGGC-GGGGCGGGGGCAGGGG) was attached to the 5' end of the 357f primer for analysis of the PCR products by DGGE. Primers were produced by Sangon (Shanghai, China). Each 50 μl PCR reaction mixture contained: 25 μl Premix Taq (TaKaRa), 1 μl of primer, 18 μl ddH_2O, and 5 μl template DNA. PCR was performed using a

Table 1. PCR primers for species-specific PCR

Bacteria species	Primer sequence (5'-3')	Product size (bp)
P. aeruginosa	F-GGGGGATCTTCGGACCTCA	956
	R-TCCTTAGAGTGCCCACCCG	
K. pneumoniae	F-CGACCTGATTGCATTCGCCAC	521
	R-CTTCGCGCCGCGTGATACGCG	
S. pneumoniae	F-ACGCAACTGACGAGTGTGAC	353
	R-GATCGCGACACCGAACTAAT	
S.mutans	F-TCGCGAAAAAGATAAACAAACA	479
	R-GCCCCTTCACAGTTGGTTAG	
S. salivarius	F-GTGTTGCCACATCTTCACTCGCTTCGG	544
	R-CGTTGATGTGCTTGAAAGGGCACCATT	
S. oralis	F-TCCCGGTCAGCAAACTCCAGCC	374
	R-GCAACCTTTGGATTTGCAAC	
S. mitis	F-TGAAATCGAGGTTGGCCTAC	259
	R-CGTTTAGGAAAATCTC(G/T)CCCTT	

GeneAmp PCR system 9700 (ABI, Foster City, CA) under the following conditions: initial denaturation at 94°C for 2 min; 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; followed by extension at 72°C for 5 min. Prior to DGGE analysis, the PCR products were examined by electrophoresis in a 2% agarose gel and visualized under UV light (BenchTop 3UV, USA).

DGGE analysis of the PCR amplicons was performed using the DCode universal mutation System (Bio-Rad, CA, USA) based on a protocol described by Muyzer et al. [11]. Twenty microliters of PCR product from the amplified DNA of each sample were added to an 8% (w/v) polyacrylamide gel. Electrophoresis was carried out using a denaturing gradient ranging from 35-65% to separate the amplicons. The denaturant (100%) contained 7 M urea and 40% formamide. Electrophoresis was performed for 30 min at 45 V followed by 16 h at 85 V in 1xTAE buffer at a constant temperature of 60°C. After electrophoresis, gels were stained using 20 μl SYBR Green I (Bio Teke) in 200 mL 1×TAE buffer for 30 min in the dark. Stained gels were imaged using the GelDoc system (Bio-Rad), and gel images were analyzed using Quantity One software, v 4.4 (Bio-Rad).

Cloning and sequencing

After imaging, 16 of the 16S rRNA bands were identified for excision and further characterization. The bands were excised with a sterile razor blade and placed in sterile tubes, and 50 μl of ddH_2O were added to each tube. DNA was released into the water from the gel at 4°C during an overnight incubation. The eluate, containing the template DNA, was amplified for the second time using the primer 357f and the primer 518r without the GC clamp under the conditions described above. The PCR product was purified with the TaKaRa Mini BEST Plasmid Purification kit (TaKaRa, China). Additional cloning was performed with the pMD 18-T Vector System (TaKaRa, China) according to the manufacturer's instructions. The PCR product was cloned into the pMD18-T vector and transformed into *Escherichia coli* competent DH5a cells (Tiangen, China). The transformed cells were loaded onto Luria-Bertani agar plates mixed with ampicillin (100 mg/ml) and incubated overnight at 37°C. A single colony was picked and sequenced with an ABI 3730xl sequencer (Applied Biosystems, Carlsbad, CA, USA) by Sangon (Shanghai, China) according to the manufacturer's instructions. The BLAST program (http://blast.ncbi.nlm.nih.gov/blast) was used to find the most similar sequences in the NCBI Genbank database. To be considered a match, sequences had to have at least 97% sequence identity with the typical genus phenotypes in the public database.

Species-specific PCR processing

Species-specific PCR was undertaken to detect the common oropharyngeal *Streptococcus* group microorganisms in biofilms on ETTs, and the ETT-associated pathogens *P. aeruginosa* and *K. pneumoniae*. The assays were performed using primers and conditions that have been described previously [12-17]. The seven sets of species-specific PCR primers used in this study are shown in **Table 1**.

Results

Neonatal demographic and clinical characteristics

A total of 29 extubated ETTs were obtained from 20 newborns during the study period. The clinical information of the neonates is present-

Table 2. Clinical data and the results of DGGE, species-specific PCR and sputum culture

Patient number	*ETT number*	*Intubated Period (d)*	*Disease*	*Species-specific PCR*							*Sputum culture*
				S. mitis	*S. oralis*	*S. mutans*	*S. salivas*	*S. pneumoniae*	*K. pneumoniae*	*P. aeruginosa*	
1	1	4	Premature, Respiratory failure	+					+	+	Normal flora
2	2	5	NRDS, Respiratory failure	+						+	Normal flora
3	3	1	NRDS, Respiratory failure							+	Normal flora
4	4	2	Pneumonia, Respiratory failure							+	Normal flora
5	5	4	NRDS, Respiratory failure	+	+					+	Normal flora
6	6	5	NRDS, Respiratory failure							+	Normal flora
7	7	4	Premature, Respiratory failure	+				+	+	+	Normal flora
8	8	7	NRDS, Respiratory failure	+					+	+	*Klebsiella pneumoniae*
9	9	3	Pneumonia,	+						+	*Klebsiella pneumoniae*
10	10	3	NRDS, Respiratory failure		+				+	+	*Klebsiella pneumoniae*
11	11	2	Pneumonia, Respiratory failure	+					+	+	*Klebsiella pneumoniae*
12	12	4	Pneumonia	+					+	+	*Klebsiella pneumoniae*
13	13	2	NRDS, Respiratory failure								*Klebsiella pneumoniae*
14a	14	1	Pneumonia, Respiratory failure	+	+				+	+	*Klebsiella pneumoniae*
14b	15	6		+	+				+		*Klebsiella pneumoniae*
15a	16	6	NRDS						+	+	*Klebsiella pneumoniae*
15b	17	1							+	+	*Klebsiella pneumoniae*
15c	18	2		+						+	*Klebsiella pneumoniae*
16a	19	1	Pneumonia						+	+	No bacterial growth
16b	20	5		+							No bacterial growth
17a	21	3	NRDS	+						+	Normal flora
17b	22	4		+					+	+	Normal flora
18a	23	3	NRDS, Pneumonia				+		+	+	*Klebsiella pneumoniae*
18b	24	3						+	+	+	*Klebsiella pneumoniae*
18c	25	2		+						+	*Klebsiella pneumoniae*
19a	26	2	Pneumonia, Respiratory failure	+				+		+	*Acinetobacterbaumannii*
19b	27	1		+					+	+	*Acinetobacterbaumannii*
20a	28	3	Pneumonia	+							–
20b	29	2		+						+	–

Note: Normal Flora- defined as no detectable outgrowth of common VAP associated pathogens from the sputum culture. The sputum culture for patient 20 was excluded due to contamination so no data is reported. NRDS, Neonatal respiratory distress syndrome.

Figure 1. Representative SEM micrograph of an ETT associated biofilm. A dense amorphous material covers most of the surface. There is visible aggregation of bacteria adhered to the matrix.

ed in **Table 2**. The median gestational age was 29 weeks (29±7 weeks), and the postnatal age ranged from one to 10 days. The duration of intubation prior to ETT collection ranged from one to seven days (mean = 3.1 d). Patients 14-20 underwent continuous ventilation; therefore, they harbored more than one ETT. The underlying diseases included severe pneumonia, neonatal respiratory distress syndrome (NRDS), premature delivery, and respiratory failure.

Microbial morphology

A representative SEM micrograph (**Figure 1**) shows biofilm growth on the surface of the interior lumen of an ETT following extubation. Dense amorphous material covers most of the surface, and aggregates of bacteria adhere to the matrix. Cocci species account for the majority of the bacteria, and bacilli species are sparse.

Bacterial cultures of the sputum specimens

Sputum specimens were obtained from 19 of the 20 neonates. One specimen was discarded due to contamination. The microbiological profile of the sputum cultures is summarized in **Table 2**. *K. pneumoniae* was the most prevalent bacterium, which was found in 14 of 27 (52%) cultures. These results are consistent with previously published studies [18]. *Acinetobacter baumannii* was found in sputum cultures from two neonates (7%). Bacterial growth was observed in nine of the 27 (33%) specimens, but there was no indication of common pathogens associated with VAP. The sputum cultures from two of the neonates did not show any bacterial growth. No bacteria growth was detected from a short-term (less than 10 min intubation time) ETT used as a control (data not shown).

The diversity and sequencing results of DGGE

The DGGE profiles of the16S rRNA from the ETT associated biofilms are shown in **Figure 2**. The number of distinct DGGE bands from each ETT ranged from three to eight (mean = 5.6) (**Table 2**). There was a high diversity of DGGE profiles in each patient. To identify the bacterial species in the ETT associated biofilms, the 16 most prevalent bands were excised for further characterization (**Table 3**). All ETT samples showed polymicrobial colonization (**Table 4**). The three most common bacteria were *Klebsiella* spp. (identified in 100% of ETT specimens; 29/29), *Streptococcus* spp. (93%; 27/29), and *Pseudomonas* spp. (83%; 24/29). The high prevalence of *Klebsiella* reflects either the higher sensitivity of DGGE as compared to sputum culture or contamination from the extubation process, as *Klebsiella* spp. were also detected using DGGE on the short-term (less than 10 min intubation time) ETT used as a negative control (data not shown).The remaining sequences belonged to the genera *Bacillus* (19/29) and genera *Enterobacter* (6/29). Microorganisms that are unculturable were found in 16 of 29 (55%) specimens.

Interestingly, there was longitudinal consistency over time in individuals with more than one ETT analysis (patient 14-20). In these cases, an organism was present not only in the first speci-

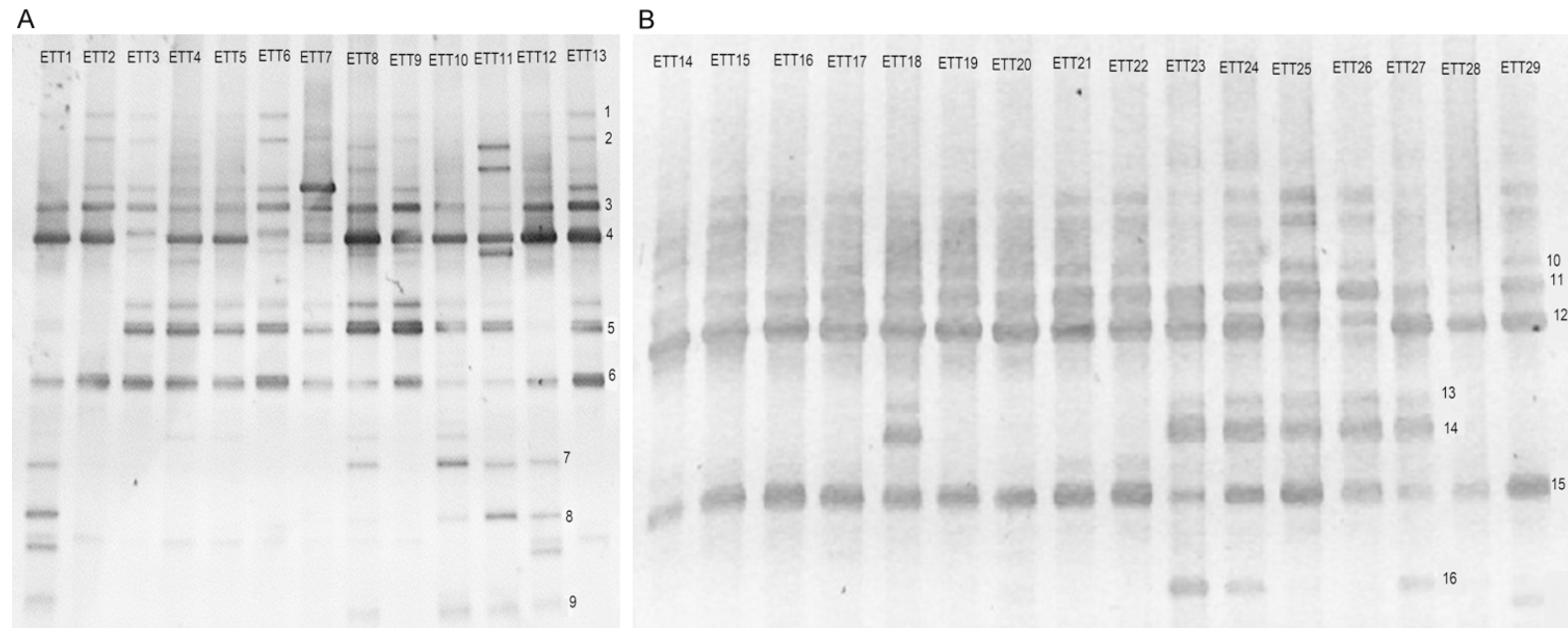

Figure 2. DGGE profiles of amplified 16S rRNA from ETT associated biofilms from 29 ventilated neonates. A. ETT specimens 1-13. B. ETT specimens 14-29. The Arabic numerals 1-16 indicate the DGGE bands that were excised for sequencing.

Table 3. Sequencing results of the DGGE bands

Band number	NCBI BLAST result	Accession number	Identity (%)
Band 1	Unculturable bacterium	JQ462576.1	100%
Band 2	*Pseudomonas* spp.	JX949982.1	100%
Band 3	*Streptococcus* spp.	JX548436.1	100%
Band 4	*Streptococcus* spp.	JX548381.1	98%
Band 5	*Bacillus* spp.	JN316004.1	100%
Band 6	*Klebsiella* spp.	JX457349.1	100%
Band 7	*Klebsiella* spp.	JX310744.1	99%
Band 8	Unculturable bacterium	HQ776847.1	100%
Band 9	*Klebsiella* spp.	KC632210.1	100%
Band 10	*Pseudomonas* spp.	GQ375136.1	100%
Band 11	*Streptococcus* spp.	JX861486.1	100%
Band 12	*Bacillus* spp.	JX490096.1	100%
Band 13	*Klebsiella* spp.	JX457349.1	100%
Band 14	*Enterobacter* spp.	HM074378.1	100%
Band 15	*Klebsiella* spp.	JX282908.1	100%
Band 16	*Klebsiella* spp.	JX489160.1	100%

men but also the second or third. This was especially apparent for *Klebsiella* spp., *Streptococcus* spp., and *Pseudomonas* spp (**Table 4**).

Detection of microorganisms by species-specific PCR

The results of species-specific PCR from 20 neonates (29 ETT specimens) are shown in **Table 2**. The three most common species identified were: *P. aeruginosa* (found in 86% of samples tested), *S. mitis* (66%), and *K. pneumoniae* (51%). Two bacterial species, *S. oralis* and *S. pneumoniae* were found only in four neonates and three neonates, respectively. The least common bacteria identified were *S. salivas*, found in one neonate (patient 18), and *S. mutans*, which was not detected. Oropharyngeal bacteria were found in 25 of 29 samples. In 10 of 20 neonates, the polymicrobial communities on the ETT specimens included at least one *Streptococcus* species, *P. aeruginosa* and *K. pneumoniae*.

Discussion

The profiles of ETT-associated microbial communities from mechanically ventilated neonates are not well understood and have been based primarily on culture techniques, which fail to detect the majority of bacteria. Molecular methods such as the 16S rRNA PCR-DGGE method can be used to characterize polymicrobial communities. Using these molecular methods, we found a high microbial diversity in the airway sample of neonates. A change in the prevalence of detectable organisms may be an indicator of VAP [10]. In the current study, we use molecular methods to demonstrate the high microbial diversity and population characteristics of polymicrobial communities on the surface of ETTs extubated from neonates. Bacterial species detected in this study included *Klebsiella* spp., *Streptococcus* spp., *Pseudomonas* spp., and *Bacillus* spp.; these bacteria, isolated from biofilms, tended to aggregate together on the same tubes.

Although molecular detection techniques can provide valuable information, it is important to utilize both conventional culture techniques and molecular techniques to fully characterize the neonatal microbiota. In the current study, we found a high degree of convergence between sputum cultures and DGGE, for example, *K. pneumoniae* was the most prevalent isolate by both methods. *K. pneumoniae* has been considered a major pathogen in nosocomial infections. In particular, extended spectrum β lactamases (ESBL) producing strains are causes for concern as they are associated with high rates of morbidity and mortality among infants in the NICU. The bacteria detected in sputum cultures and DGGE were not always consistent. Sequences corresponding to *A. baumannii* were not found using the DGGE method, but it is one of the most prevalent isolates in sputum samples of VAP neonates in our hospital [2].

In the current study, both DGGE and species-specific PCR methods demonstrated a remarkable diversity of the bacterial community in the biofilms on the ETTs. Oropharyngeal bacteria, alone or in combination with other bacteria, were recovered from most of the specimens. Interestingly, *Klebsiella* spp., *Streptococcus* spp. and *Pseudomonas* spp. seemed to persist

Table 4. Distribution of Sequencing results of DGGE bands from neonatal ETTs

Patient ID	*ETT number*	*DGGE bands*	*Results of sequencing*					
			Klebsiella spp.	*Streptococcus spp.*	*Pseudomonas spp.*	*Bacillus spp.*	*Enterobacter spp.*	*Unculturable bacteria*
1	1	9	●	●		●		●
2	2	6	●	●	●			●
3	3	7	●	●	●	●		●
4	4	5	●	●		●		
5	5	5	●	●		●		
6	6	7	●	●	●			●
7	7	6	●	●	●	●		
8	8	8	●	●	●	●		●
9	9	7	●	●	●	●		●
10	10	6	●	●		●		●
11	11	7	●	●	●	●		●
12	12	7	●	●				●
13	13	7	●	●	●	●		●
14a	14	4	●	●	●		●	
14b	15	6	●	●	●		●	
15a	16	7	●	●	●	●	●	●
15b	17	4	●		●	●		
15c	18	3	●		●	●		
16a	19	7	●	●	●	●	●	●
16b	20	7	●	●	●	●	●	●
17a	21	3	●	●	●			
17b	22	3	●	●	●			
18a	23	6	●	●	●		●	●
18b	24	4	●	●	●	●		
18c	25	4	●	●	●	●		
19a	26	5	●	●	●	●		●
19b	27	5	●	●	●	●		●
20a	28	4	●	●	●			
20b	29	3	●	●	●			

on the tubes during the intubation period, which may be an important factor for pathogenesis. While conventional culture techniques can only detect few or even none of the bacterial species in biofilms [9], molecular methods may promote an improved understanding of the microbiota and allow us to better diagnose and treat ETT-associated infections. As automated systems become available, high throughput screening will facilitate the process.

Biofilm formation is a dynamic process; microorganisms in biofilms on extubated ETTs can reflect disease status. Cairns et al. suggested that normal oral microflora may act as the initial colonizers on ETTs and could promote subsequent biofilm development [9]. Co-aggregation of different bacterial species in biofilms was first described by Gibbons [19] in dental plaque, and is now known to occur in a variety of environments [20-22]. The mechanisms underlying co-aggregation of oral commensals and other bacteria are still unclear. One possibility is that quorum sensing (QS), a method of autoinducers (AIs)-mediated cross-talk between individual bacteria embedded in biofilms, could lead to polymicrobial biofilm formation. Autoinducer-2 (AI-2) is a well-studied QS signaling molecule produced by many Gram-positive and Gram-negative bacteria species [23, 24]. AI-2 can facilitate interspecies communication in microbial environments [25]. It has also been shown to promote co-aggregation by increasing the expression of adhesion molecules of patho-

genic bacteria [26]. In this context, we speculate that the AI-2 producers *K. pneumoniae* and *Streptococcus* spp. may facilitate biofilm formation incorporating *P. aeruginosa*.

Our study is associated with several limitations. First, DGGE data are descriptive rather than quantitative. Second, we had a limited sample size both in the number of patients and ETT specimens. Further study using a larger sample of ETT biofilms is necessary to elucidate whether there is a correlation between the microbial community on the ETT and different diseases.

In conclusion, we demonstrated that *Klebsiella* spp., *Streptococcus* spp. and *Pseudomonas* spp. were the most frequent microbes on the surface of neonatal ETTs, and they prefer to coexist in biofilms. These data provide a more in-depth knowledge of the ETT associated microbiota, which may have clinical significance for the management of mechanically ventilated neonates. We speculate that the AI-2 mediated signaling pathway may play a role in biofilm formation; however, additional studies are required to support this speculation.

Acknowledgements

We are grateful to Yu He, Zhengli Wang and Ying Dong for their valuable advice to the linguistic revision of the manuscript. This study was supported by the National Natural Science Foundation of China (No. 81370744), the fund from Ministry of Education (No. X3387), National Science and Technology Support Project (2012BAI04B05), the Scientific Research Foundation of Chongqing Municipal Health Bureau (NO. 2013-2-051) and the Scientific Research Foundation of The science and Technology Commission of Yuzhong District of Chongqing (No. 20140103).

Disclosure of conflict of interest

None.

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