16S rRNA gene profiling: Direct and indirect applications for clinical microbiology

Munckhof, E.H.A. van den

PART 1: DIRECT CLINICAL APPLICATION

CHAPTER 2

Evaluation of a stepwise approach using microbiota analysis, species-specific qPCRs and culture for the diagnosis of lower respiratory tract infections

Ellen H.A. van den Munckhof
Maurits N.C. de Koning
Wim G.V. Quint
Leen-Jan van Doorn
Maurine A. Leverstein-van Hall

European Journal of Clinical Microbiology & Infectious Diseases (2019) 38: 747-754

ABSTRACT

In clinical practice, the diagnosis of lower respiratory tract infections (LRTIs) is based on culture. The aim of this study was to evaluate whether a stepwise approach using microbiota analysis, species-specific quantitative real-time (q)PCRs and culture has the potential to be a more accurate and efficient diagnostic approach than culture alone. Sixty-two sputa obtained in a routine clinical setting from patients with a suspected LRTI were included. All sputa were analysed by culture, microbiota analysis based on the 16S ribosomal RNA gene and multiple species-specific qPCRs. Microbiota and culture data were compared to investigate whether cut-off values for microbiota analysis could be determined. For microbiota analysis, a relative abundance of 25% was identified as the cut-off value for the detection of both genera *Streptococcus* and *Haemophilus*. Microbiota analysis combined with species-specific qPCRs resulted in a significant increase in the number of positive sputa (73% vs 58%; $p = 0.003$) as well as in the number of identified pathogens (51 vs 37; $p = 0.049$) compared to culture. A stepwise approach using microbiota analysis, species-specific qPCRs and culture has the potential to be used in clinical settings for the diagnosis of LRTIs in the near future.

INTRODUCTION

Lower respiratory tract infections (LRTIs) are a leading cause of human morbidity and mortality worldwide (1). The standard microbiological method for identification of pathogens involved in LRTIs is culture of bacteria from sputum followed by species identification with matrix-assisted laser desorption ionization time of flight analyser (MALDI-TOF) (2) and antibiotic-susceptibility testing of the cultured putative causative microorganism. This approach is limited by poor diagnostic accuracy due to competition during selective culture and non- or poorly cultivable pathogens such as *Mycoplasma pneumonia*, *Chlamydia pneumoniae* and *Legionella pneumophila* (3). In research, accurate characterization of complex microbial communities is done by 16S ribosomal RNA (rRNA) gene sequencing (microbiota analysis). In recent years, microbial communities of different body sites have been subject of study in relation with disease. However, few studies have been performed in relation to the establishing the diagnosis of a disease.

We questioned whether a stepwise approach using microbiota analysis followed by species-specific (q)PCRs and/or culture has the potential to be a more accurate and efficient diagnostic approach than culture alone. Microbiota analysis can be used as a first screening step to divide sputa into those with and without DNA from potential pathogenic bacterial genera. Sputa without DNA from these genera can be reported as negative. The sputa with potential pathogenic genera require further analysis as a second step. The type of analysis depends on whether the potential pathogenic genus is (i) atypical (*Mycoplasma*, *Chlamydia*, *Legionella*), (ii) *Streptococcus*, *Haemophilus*, *Moraxella* or *Staphylococcus*, or (iii) others.

When an atypical genus is detected, a species-specific qPCR should be performed to discriminate between non-pathogenic species and *M. pneumoniae*, *C. pneumoniae* or *L. pneumophila*, because the 16S rRNA gene lacks sufficient resolution to allow bacterial identification lower than to the genus level (4, 5).

When the genus *Streptococcus*, *Haemophilus*, *Moraxella* or *Staphylococcus* is detected, a species-specific qPCR is required to discriminate between non-pathogenic species and *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* or *Staphylococcus aureus*. For these potential pathogenic species, quantification is required to discriminate between infection and colonization of the respiratory tract. For *S. pneumoniae*, a concentration of 1.00E+05 gene copies/mL has been described as a significant cut-off value to discriminate between infection and colonization (6). For *H. influenzae*, *M. catarrhalis* and *S. aureus*, no cut-off value has yet been determined.

When *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *S. aureus* or other potential pathogenic genera are detected by microbiota analysis such as Enterobacteriaceae, *Pseudomonas*, *Acinetobacter* and *Stenotrophomonas*, culture e.g. for antibiotic susceptibility testing, can be performed on indication as a third step. This step might be restricted to one selective agar plate per pathogen.

The aim of this study was to evaluate microbiota analysis combined with species-specific qPCRs for the identification of pathogens in sputum. First, data of microbiota analysis and species-specific qPCRs were compared with culture to investigate whether cut-off values for microbiota analysis as well as for the qPCRs targeting *H. influenzae*, *M. catarrhalis* or *S. aureus* could be determined.

MATERIALS AND METHODS

Study design

All sputa obtained between November 2014 and January 2015 from patients with a suspected LRTI who were hospitalized or visiting the outpatient clinic of the Haaglanden Medical Centre Bronovo hospital (The Hague), Alrijne hospital (Leiden) or Alrijne hospital (Leiderdorp, The Netherlands) were sent directly after collection to the medical microbiology laboratory for routine diagnostic analysis based on culture and MALDI-TOF. Patients were notified that remainders of their samples might be used for evaluation of diagnostic methods. If patients objected, samples were discarded. For all included sputa, microbiota analysis was performed. A qPCR assay targeting *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* or *S. aureus* was performed when the corresponding genus was identified by microbiota analysis. In case of a discrepant result between culture and microbiota analysis combined with species-specific qPCRs, microbiota analysis, species-specific qPCRs and/or whole genome sequencing was performed on the bacterial DNA harvested from the corresponding primary inoculated plates and/or subcultures.

Routine diagnostic analysis

The quality of each sputum sample was assessed by microscopic examination using Gram staining. If (polynuclear) leukocytes and alveolar cells were observed, the sputum sample was inoculated on blood, chocolate and MacConkey agar plates (Becton, Dickinson and Company, NJ, USA) and incubated for 18-24 h at 35 °C in a 5% CO_2 incubator or at 35 °C in ambient air. Subcultures were prepared for species identification by MALDI-TOF with software version 1.6.7.1000 (Bruker Corporation, Billerica, USA). Colonies morphologically suspected to be *S. pneumoniae* were tested for optochin susceptibility by Kirby-Baur disk diffusion, which was defined as an inhibition zone of ≥ 14 mm after 18 h incubation.

Pre-treatment and DNA extraction

The remaining sputum was homogenized with an equal volume of Sputasol (Oxoid Ltd, Basingstoke, UK). Bacteria of the primary inoculated agar plates as well as from the subculture plates were suspended in 2 and 1 mL cobas PCR medium (Roche Diagnostics, Meylan, France), respectively. The bacteria harvested from the culture plates were 10x

diluted in cobas PCR medium to avoid overloading of the DNA extraction system. For DNA extraction with the MagNA Pure 96 instrument, the MagNA Pure 96 DNA and Viral NA Small Volume kit (Roche Diagnostics) was used following the Viral NA Universal protocol. DNA of 200 µL sample was eluted in a final volume of 50 µL of elution buffer.

Microbiota analysis

A fragment of ~ 464bp of the V3-V4 region of the 16S rRNA gene was amplified using the primers: Bakt_341F (5'-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*CCTACGGGNGGCWGCAG-3') and Bakt_805R (5'-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*GACTACHVGGGTATCTAATCC-3'). These were described by Klindworth, et al. (7) with Illumina overhang adaptor sequences added (italicized sequences). Each 50 µL PCR reaction contained 5 µL (10x) Expand High Fidelity Buffer with 15 mM $MgCl_2$ (Roche), 2.6 U Expand High Fidelity Enzyme mix (Roche), 0.2 mM of each dNTP (Roche), 0.3 µM of each primer and 10 µL of extracted DNA. The PCR was run for 2 min at 94 °C followed by 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 1 min and a final extension step at 72 °C for 7 min. The PCR products with a visible band of ~ 531 bp on gel were subsequently purified and quantified using AMPure XP Beads (Agencourt Bioscience Corporation, Beverly, USA) and the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Paisley, UK), respectively. After library preparation using the Nextera XT kits (Illumina, San Diego, USA), sequencing was performed with the MiSeq desktop sequencer using the MiSeq Reagent Kits v2 500-cycles (Illumina). Sequencing data was processed following the QIIME pipeline. Sequences ≥ 100 bp in length with a quality score ≥ Q20 were clustered into operational taxonomic units using open reference-based approach that implements reference-based clustering following by *de novo* clustering. Clustering was conducted at a 97% similarity level using a pre-clustered version of the Augustus 2013 GreenGenes database. Unclassified reads were removed, and a sample was considered positive for a specific genus when more than 1% of the classified reads were assigned to that genus. Potential pathogenic genera included *Acinetobacter*, *Bacillus*, *Bordetella*, *Burkholderia*, *Coxiella*, *Chlamydia*, *Franciscella*, *Haemophilus*, *Legionella*, *Moraxella*, *Mycoplasma*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Staphylococcus*, *Stenotrophomonas*, *Streptococcus*, and Enterobacteriaceae (e.g. *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Proteus*, *Serratia*, *Yersinia*). The remaining bacteria were categorised as non-pathogenic bacteria.

Quantitative real-time PCRs

qPCRs using primers and probes described previously were performed for the detection and quantification of *M. pneumoniae* (8), *C. pneumoniae* (9), *L. pneumophila* (10), *S. pneumoniae* (11), *H. influenzae* (12), *M. catarrhalis* (13) and *S. aureus* (14). Each qPCR was carried out in a total volume of 10 µL, containing 5 µL 2x SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, CA, USA) and 2 µL of extracted DNA. Amplification reactions were performed under

the following conditions: 2 min at 95 °C followed by 45 cycles of 95 °C for 5 s and 60 °C for 30 s.

The total bacterial load (16S rRNA gene) was established using a primer set (Fw 5'-GAAAGCGTGGGGAGCAAA-3', Rv1 5'-GCCGTACTCCCCAGGCGG-3' and Rv2 5'-GTCGTACT-CCCCAGGCGG-3') based on Bogaert et al. (15) and 20x EVA green (Biotium, Inc., Fremont, CA, USA). Each reaction was carried out in a total volume of 10 µL, containing 5 µL (2x) LC480 Probes Master mix (Roche Diagnostics) and 2 µL of extracted DNA. Amplification reactions were performed under the following conditions: 5 min at 95 °C followed by 45 cycles of 95 °C for 10 s, 60 °C for 50 s and 72 °C for 1 s.

All amplification reactions were performed using a LightCycler 480 Instrument (Roche Diagnostics). For quantification, a 10-fold dilution series of a plasmid was included in each run and the second derivative analysis method was used for data analysis.

Whole genome sequencing

DNA was quantified with the Quant-iT PicoGreen dsDNA Assay Kit and a library was prepared using the Nextera XT kits. After library preparation, the PCR products were sequenced with the MiSeq desktop sequencer using the MiSeq Reagent Kits v3 600-cycles (Illumina). The bacterial species was determined by performing BLAST analysis on the with CLC bio software (QIAGEN, Aarhus, Denmark) generated consensus sequences.

Statistical analysis

McNemar and Wilcoxon signed rank tests of the statistical software package SPSS v.17.0 were used to evaluate the effect of the cut-off value on the microbiota data and to compare culture with microbiota analysis combined with species-specific qPCRs for identification of pathogens in sputum.

Data availability

The datasets generated and analysed during the current study are available in the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) repository with the accession number PRJNA518755.

RESULTS

Culture

In total 62 sputa were obtained from 57 patients, of which four were admitted to the intensive care unit (ICU) (**Supplementary Table S1**). Of the 62 sputa, 26 (42%) were collected after antibiotic treatment had been started. In total 37 pathogens were identified from 36 sputa by the routine diagnostic approach based on culture (**Table 1**). No bacterial pathogens were identified in the remaining 25 sputa.

Determination of cut-off values for microbiota analysis

First, microbiota and culture data were compared to investigate whether cut-off values for microbiota analysis could be determined. In total 110 potential bacterial pathogenic genera were identified from all 62 sputa by microbiota analysis (**Table 1, Supplementary Table S2**). The most frequently identified potential pathogenic genera by microbiota analysis were *Streptococcus* and *Haemophilus* in 50/62 (81%) and in 31/62 (50%) sputa, respectively. In contrast, a *Streptococcus* sp. was identified as pathogen by culture in only 3/62 (5%) sputa and a *Haemophilus* sp. in 14/62 (23%) sputa. Comparison of the relative abundance of these genera between culture-positive and culture-negative sputa showed that all culture-positive sputa had a relative abundance > 25% for *Streptococcus* or *Haemophilus*, except for three sputa (relative abundance of 7-13% for *Haemophilus*). However, these three sputa were culture-positive for *Haemophilus parainfluenzae*, a species which role in RTIs remains unestablished. By using this cut-off value, the total number of *Streptococcus* and *Haemophilus* positive sputa reduced significantly from 50 to 25 ($p < 0.001$, McNemar test) and from 31 to 13 ($p < 0.001$, McNemar test), respectively.

Table 1. Number of bacterial pathogens identified by culture and microbiota analysis in 62 sputa

Genus	Number of identified bacterial pathogens				
	Total culture positive	Microbiota analysis without a cut-off value		Microbiota analysis with a cut-off value of 25% for the genera *Streptococcus* and *Haemophilus*	
		Total positive	Microbiota analysis negative and culture positive	Total positive	Microbiota analysis negative and culture positive
Acinetobacter	0	1	0	1	0
Enterobacteriaceae	7	8	1	8	1
Haemophilus	14	31*	1[1]	13*	41
Moraxella	4	8	0	8	0
Mycoplasma	0	2	0	2	0
Proteus	1	0	1	0	1
Pseudomonas	2	4	0	4	0
Staphylococcus	4	3	2	3	2
Stenotrophomonas	2	3	0	3	0
Streptococcus	3	50**	0	25**	0
Total	37	110	5	66	8

[1] *Haemophilus parainfluenzae* only

*/**Total positive sputa reduced significantly ($p < 0.001$, McNemar test) by using a cut-off value of 25% for microbiota analysis

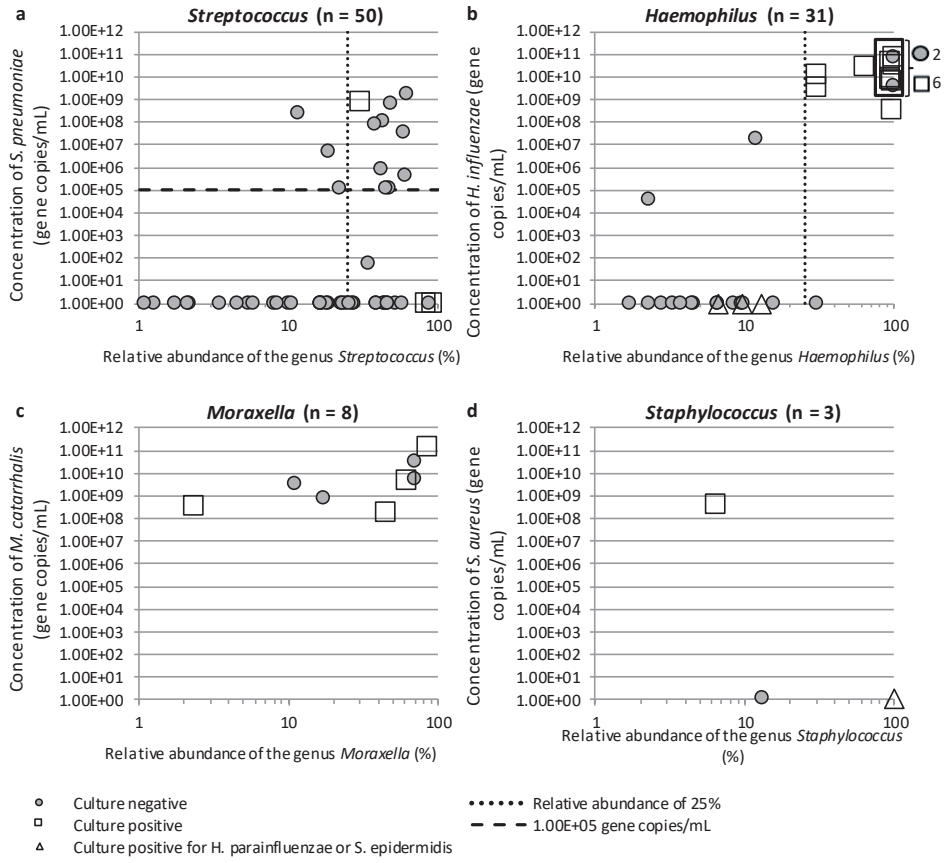

Figure 1. Culture and qPCR data of the sputa positive by microbiota analysis for the genera (**a**) *Streptococcus*, (**b**) *Haemophilus*, (**c**) *Moraxella* and/or (**d**) *Staphylococcus*. The sputa negative by qPCR are situated on the horizontal axis.

Determination of cut-off values for species-specific qPCRs

Before microbiota analysis combined with species-specific qPCRs could be evaluated for the identification of pathogens in sputum, the qPCRs targeting *H. influenzae*, *M. catarrhalis* and *S. aureus* were compared with microbiota analysis and culture to determine whether cut-off values were required to discriminate between infection and colonization. For *S. pneumoniae*, a concentration of 1.00E+05 gene copies/mL has been described as a significant cut-off value to discriminate between infection and colonization (6). Of the 25 sputa with a relative abundance ≥ 25%, 10 (40%) were positive for *S. pneumoniae* by qPCR. Nine (90%) of these 10 sputa contained a concentration of *S. pneumoniae* above this significant cut-off value of which one was also culture-positive (**Figure 1a**). Two other culture-positive sputa were negative for *S. pneumoniae* by qPCR.

For *Haemophilus*, 12/13 (92%) sputa with a relative abundance ≥ 25% were positive by qPCR (**Figure 1b**). All 12 sputa contained a relatively high concentration of *H. influenzae* ranging from 3.35E+08 to 7.45E+10 gene copies/mL, including all culture-positive sputa.

The genus *Moraxella* was identified in eight sputa by microbiota analysis and all eight sputa, including all culture-positive sputa, were positive for *M. catarrhalis* by qPCR (**Figure 1c**). All contained a relatively high concentration of *M. catarrhalis* ranging from 1.76E+08 to 1.23E+11 gene copies/mL.

The genus *Staphylococcus* was detected in three sputa by microbiota analysis and one of them was found positive for *S. aureus* by qPCR (**Figure 1d**). One of the remaining two sputa was obtained from a patient located on the ICU and the pathogen was identified by culture as *Staphylococcus epidermidis*, which is rarely pathogenic. These data indicated that species determination and quantification by qPCR for the genera *Haemophilus*, *Moraxella* and *Staphylococcus* was of no additional value in this study, which did not change by normalisation of the qPCR data using the total bacterial DNA load (**Supplementary Figure S3**).

Evaluation of microbiota analysis combined with species-specific qPCRs

For evaluation of microbiota analysis combined with species-specific qPCRs for identification of pathogens in sputum, the stepwise approach as depicted in **Figure 2** was

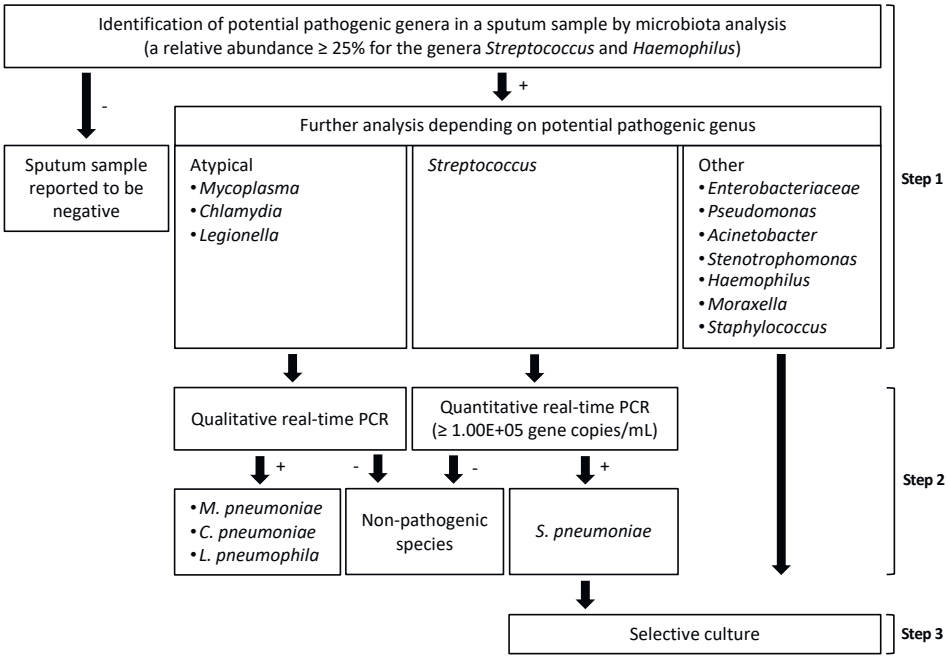

Figure 2. Final stepwise approach using microbiota analysis, species-specific qPCRs and culture for the diagnosis of lower respiratory tract infections

used. This approach included microbiota analysis with a cut-off value of 25% for the genera *Streptococcus* and *Haemophilus*, and species-specific qPCRs targeting atypical pathogens and *S. pneumoniae*. This resulted in identification of 51 pathogen in 45/62 (73%) sputa, which was a significant higher number of pathogens (p = 0.003, Wilcoxon signed rank test) and positive sputa (p = 0.049, McNemar test) compared to the 37 pathogens identified by culture in 36/62 (58%) sputa (**Figure 3**). Culture and microbiota analysis combined with species-specific qPCRs were in agreement in 36/62 (58%) sputa. In the remaining 26 sputa, 10 additional pathogens were identified by culture in 10 sputa, and 24 additional pathogens were identified by microbiota analysis combined with species-specific qPCRs in 22 sputa.

As discrepancy analysis for those bacteria, DNA harvested from the primary inoculated agar plates and subcultures were analysed by microbiota analysis, qPCR and/or whole genome sequencing. Of the 10 additional pathogens detected by culture, eight were detected by microbiota analysis (and qPCR) in the harvested bacterial DNA (**Supplementary Table S4**). These data indicated that these bacteria were present in the sputum but were detected by microbiota analysis only after selective culture had increased their relative abundance. The remaining two pathogens were misidentified in the clinical laboratory as *S. pneumoniae* instead of *S. pseudopneumoniae*.

Of the 24 additional bacteria identified as pathogen by microbiota analysis combined with qPCR, one was non-cultivable in the clinical laboratory, two were probably rarely pathogenic species and 13 were present on the agar plates but not recognized or reported as positive by the technician in the clinical laboratory (**Supplementary Table S5**). The remaining eight pathogens were only detected in the sputum sample and not in the harvested DNA by microbiota analysis and/or species-specific qPCRs.

DISCUSSION

To the best of our knowledge, this is the first study showing that a stepwise approach using microbiota analysis, species-specific qPCRs and culture has the potential to be a more accurate and efficient diagnostic approach for the diagnosis of LRTIs than culture alone. A significant higher number of pathogens and positive sputa were identified using microbiota analysis combined with species-specific qPCRs.

This study provides also a cut-off value for microbiota analysis for both the genera *Streptococcus* and *Haemophilus*. This cut-off value reduced the number of unnecessary species-specific qPCRs to be performed significantly, without missing sputa positive for *S. pneumoniae* or *H. influenzae* by culture.

Since high abundances of non-pathogenic *Streptococcus* spp. can be present in the lower respiratory tract, a next step with a species-specific qPCR was required to identify *S. pneumoniae* positive sputa (16-18). In addition, quantification of *S. pneumoniae*

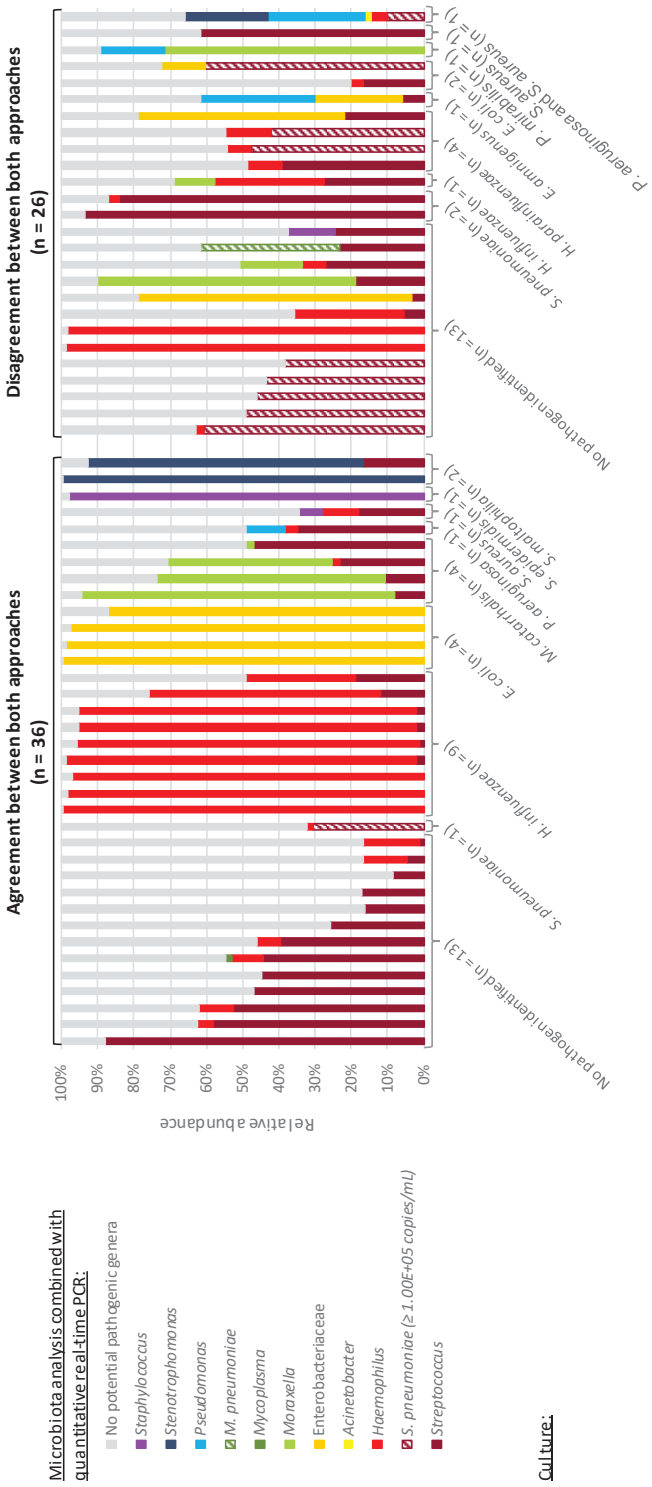

Figure 3. Comparison of culture with microbiota analysis combined with species-specific qPCR for identification of pathogens in 62 sputa. For microbiota analysis, a relative abundance of 25% for the genera *Streptococcus* and *Haemophilus* was used as cut-off value. For the *S. pneumoniae* qPCR, a concentration of 1.00E+05 gene copies/mL was used as cut-off value.

was required to discriminate between infection and colonization. Application of the widely accepted concentration of 1.00E+05 gene copies/mL as cut-off value resulted in categorization of one *S. pneumoniae* positive sputum sample as colonization (6).

For the genus *Haemophilus*, the data showed that a relative abundance of the genus *Haemophilus* above the cut-off value was only present in a sputum sample when *H. influenzae* was involved in a LRTI. Therefore, species determination and quantification by qPCR was of no additional value.

Species determination was also of no additional value for the genus *Moraxella*. All sputa positive for the genus *Moraxella* by microbiota analysis were positive and contained relatively high concentrations of *M. catarrhalis* according to qPCR. These data confirmed that potential pathogenic species colonizing the nasopharynx are only present in the lower respiratory tract when involved in a LRTI (16-18). However, this could not be confirmed for the genus *Staphylococcus* as there were only three positive sputa by microbiota analysis.

Comparison of culture and microbiota analysis combined with species-specific qPCRs for identification of pathogens revealed that clinically relevant bacteria are frequently missed by culture. This may be caused by the following: (i) the presence of uncultivable pathogens, (ii) the presence of pathogens with similar morphologic characteristics as non-pathogenic colonizers making their recognition by technicians difficult, (iii) the presence of slow-growing or fastidious pathogens overgrown by faster growing bacteria or (iv) the use of antibiotics prior to sample collection, which can have a substantial effect on the growth and subsequent identification of pathogens by culture (19-21). Only in four cases, the pathogen identified in sputum by microbiota analysis combined with species-specific qPCRs was not present on the corresponding primary inoculated agar plates and no antibiotics were used prior to sample collection. For these cases, the inhomogeneity of the sputum might explain the absence of these pathogens on the primary inoculated agar plates.

A stepwise approach using microbiota analysis, species-specific qPCRs and culture may be more efficient since less cultures are needed, because a part of the sputa can directly be reported to be negative after microbiota analysis and species-specific qPCRs have been performed. For the remaining sputa, selective culture can be prepared on indication, since the pathogen has already been identified by microbiota analysis combined with species-specific qPCRs. In this study, performing culture based on the outcome of microbiota analysis combined with species-specific qPCRs would have resulted in inoculation of 50 agar plates instead of at least 173.

The major disadvantage of this stepwise approach is that microbiota analysis is still expensive and has a turnaround time of 48 h. However, in contrast to culture, faster, better and cheaper technology for microbiota analysis can be expected in the near future. For example, the relatively new MinION sequencing platform is faster than the most widely used MiSeq sequencing platform but still has a relatively high error rate (22).

In literature, multiple molecular approaches have been described for the diagnosis of LRTIs. Approaches showing promising results were based on multiplex qPCRs (23), a DNA microarray (24) or metagenomics (25). The disadvantage of using only multiplex qPCRs or a DNA microarray is the limited number of pathogens that can be targeted at once. Rarely pathogenic bacteria (e.g. *S. epidermidis*) are not targeted by these assays and will be missed. This is not the case for metagenomics, which enables identification of all bacteria down to (sub)species level in a sputum sample. The challenge of metagenomics would be to discriminate between colonization and infection since it does not give quantitative information. Another topic of discussion is the ethical diagnosis of the human DNA sequences that will be obtained with metagenomics (26).

A limitation of the current study is that it is a heterogeneous group of patients with different LRTIs, different comorbidities, and frequently chronic lung diseases. The study was not restricted to patients with pneumonia confirmed by X-ray. Another limitation of the study is the limited number of sputa, resulting in too low numbers of some pathogens e.g. *S. aureus*, to evaluate whether a cut-off value for microbiota analysis or species-specific qPCR could be defined. In addition, a larger sample set is required to determine the clinical relevance of the defined cut-off values for microbiota analysis. The advantage of this study is that it was performed on unselected clinical samples representative for a routine clinical setting.

In conclusion, the stepwise approach using microbiota analysis, species-specific qPCRs and culture has the potential to be a more accurate and efficient diagnostic approach than culture alone for the diagnosis of LRTIs. It has the potential to be used in clinical settings for the diagnosis of LRTIs in the near future when challenges of the cost of microbiota analysis are overcome.

ACKNOWLEDGEMENT

The authors are grateful to Ingrid Poot (DDL Diagnostic Laboratory) for her technical assistance.

REFERENCES

1. Collaborators GL. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory tract infections in 195 countries: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Infect Dis*. 2017;17(11):1133-1161.
2. van Veen SQ, Claas EC, and Kuijper EJ. High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization-time of flight mass spectrometry in conventional medical microbiology laboratories. *J Clin Microbiol*. 2010;48(3):900-7.
3. Edin A, Granholm S, Koskiniemi S, Allard A, Sjostedt A, and Johansson A. Development and laboratory evaluation of a real-time PCR assay for detecting viruses and bacteria of relevance for community-acquired pneumonia. *J Mol Diagn*. 2015;17(3):315-24.
4. Janda JM and Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol*. 2007;45(9):2761-4.
5. Pei AY, Oberdorf WE, Nossa CW, Agarwal A, Chokshi P, Gerz EA, Jin Z, Lee P, Yang L, Poles M, Brown SM, Sotero S, Desantis T, Brodie E, Nelson K, and Pei Z. Diversity of 16S rRNA genes within individual prokaryotic genomes. *Appl Environ Microbiol*. 2010;76(12):3886-97.
6. Stralin K, Herrmann B, Abdeldaim G, Olcen P, Holmberg H, and Molling P. Comparison of sputum and nasopharyngeal aspirate samples and of the PCR gene targets lytA and Spn9802 for quantitative PCR for rapid detection of pneumococcal pneumonia. *J Clin Microbiol*. 2014;52(1):83-9.
7. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, and Glockner FO. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res*. 2013;41(1):e1.
8. Winchell JM, Thurman KA, Mitchell SL, Thacker WL, and Fields BS. Evaluation of three real-time PCR assays for detection of Mycoplasma pneumoniae in an outbreak investigation. *J Clin Microbiol*. 2008;46(9):3116-8.
9. Kuoppa Y, Boman J, Scott L, Kumlin U, Eriksson I, and Allard A. Quantitative detection of respiratory Chlamydia pneumoniae infection by real-time PCR. *J Clin Microbiol*. 2002;40(6):2273-4.
10. Benitez AJ and Winchell JM. Clinical application of a multiplex real-time PCR assay for simultaneous detection of Legionella species, Legionella pneumophila, and Legionella pneumophila serogroup 1. *J Clin Microbiol*. 2013;51(1):348-51.
11. Park HK, Lee SJ, Yoon JW, Shin JW, Shin HS, Kook JK, Myung SC, and Kim W. Identification of the cpsA gene as a specific marker for the discrimination of Streptococcus pneumoniae from viridans group streptococci. *J Med Microbiol*. 2010;59(Pt 10):1146-52.
12. Abdeldaim GM, Stralin K, Olcen P, Blomberg J, Molling P, and Herrmann B. Quantitative fucK gene polymerase chain reaction on sputum and nasopharyngeal secretions to detect Haemophilus influenzae pneumonia. *Diagn Microbiol Infect Dis*. 2013;76(2):141-6.
13. Greiner O, Day PJ, Altwegg M, and Nadal D. Quantitative detection of Moraxella catarrhalis in nasopharyngeal secretions by real-time PCR. *J Clin Microbiol*. 2003;41(4):1386-90.
14. Pichon B, Hill R, Laurent F, Larsen AR, Skov RL, Holmes M, Edwards GF, Teale C, and Kearns AM. Development of a real-time quadruplex PCR assay for simultaneous detection of nuc, Panton-Valentine leucocidin (PVL), mecA and homologue mecALGA251. *J Antimicrob Chemother*. 2012;67(10):2338-41.

15. Bogaert D, Keijser B, Huse S, Rossen J, Veenhoven R, van Gils E, Bruin J, Montijn R, Bonten M, and Sanders E. Variability and diversity of nasopharyngeal microbiota in children: a metagenomic analysis. *PLoS One*. 2011;6(2):e17035.
16. Human Microbiome Project C. Structure, function and diversity of the healthy human microbiome. *Nature*. 2012;486(7402):207-14.
17. Dickson RP and Huffnagle GB. The Lung Microbiome: New Principles for Respiratory Bacteriology in Health and Disease. *PLoS Pathog*. 2015;11(7):e1004923.
18. Hang J, Zavaljevski N, Yang Y, Desai V, Ruck RC, Macareo LR, Jarman RG, Reifman J, Kuschner RA, and Keiser PB. Composition and variation of respiratory microbiota in healthy military personnel. *PLoS One*. 2017;12(12):e0188461.
19. Murphy TF and Parameswaran GI. Moraxella catarrhalis, a human respiratory tract pathogen. *Clin Infect Dis*. 2009;49(1):124-31.
20. Byrnes MC, Irwin E, Reicks P, and Brodsky I. Prospective, protocolized study evaluating effects of antibiotics on sputum culture results in injured patients. *Surg Infect (Larchmt)*. 2013;14(1):24-9.
21. Driscoll AJ, Deloria Knoll M, Hammitt LL, Baggett HC, Brooks WA, Feikin DR, Kotloff KL, Levine OS, Madhi SA, O'Brien KL, Scott JAG, Thea DM, Howie SRC, Adrian PV, Ahmed D, DeLuca AN, Ebruke BE, Gitahi C, Higdon MM, Kaewpan A, Karani A, Karron RA, Mazumder R, McLellan J, Moore DP, Mwananyanda L, Park DE, Prosperi C, Rhodes J, Saifullah M, Seidenberg P, Sow SO, Tamboura B, Zeger SL, Murdoch DR, and Group PS. The Effect of Antibiotic Exposure and Specimen Volume on the Detection of Bacterial Pathogens in Children With Pneumonia. *Clin Infect Dis*. 2017;64(suppl_3):S368-S377.
22. Kerkhof LJ, Dillon KP, Haggblom MM, and McGuinness LR. Profiling bacterial communities by MinION sequencing of ribosomal operons. *Microbiome*. 2017;5(1):116.
23. Gadsby NJ, Russell CD, McHugh MP, Mark H, Conway Morris A, Laurenson IF, Hill AT, and Templeton KE. Comprehensive Molecular Testing for Respiratory Pathogens in Community-Acquired Pneumonia. *Clin Infect Dis*. 2016;62(7):817-823.
24. Ozongwu C, Personne Y, Platt G, Jeanes C, Aydin S, Kozato N, Gant V, O'Grady J, and Enne VI. The Unyvero P55 'sample-in, answer-out' pneumonia assay: A performance evaluation. *Biomol Detect Quantif*. 2017;13:1-6.
25. Yan Q, Cui S, Chen C, Li S, Sha S, Wan X, Yang R, Xin Y, and Ma Y. Metagenomic Analysis of Sputum Microbiome as a Tool toward Culture-Independent Pathogen Detection of Patients with Ventilator-associated Pneumonia. *Am J Respir Crit Care Med*. 2016;194(5):636-9.
26. Feigelman R, Kahlert CR, Baty F, Rassouli F, Kleiner RL, Kohler P, Brutsche MH, and von Mering C. Sputum DNA sequencing in cystic fibrosis: non-invasive access to the lung microbiome and to pathogen details. *Microbiome.* 2017;5(1):20.

SUPPLEMENTAL APPENDIX

Supplementary information accompanies this paper at
https://link.springer.com/article/10.1007%2Fs10096-019-03511-4.