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Non-invasive diagnostics of pathogenic bacteria using a breath sampler in children with cystic fibrosis

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Abstract

Cystic fibrosis (CF) is a common autosomal recessive disease causing thick, viscous secretions leading to pulmonary infections with pathogenic bacteria. As part of routine patient care, colonization and infection with these bacteria is monitored with cough swab or sputum cultures and sometimes bronchoalveolar lavage. In this cross-sectional proof-of-concept study in a cohort of CF patients we collected swabs or sputa and exhaled breath samples with the modular breath sampler (MBS), a newly developed two-way non-rebreathing sampling device. Pathogen specific polymerase chain reactions (PCRs) were performed on the MBS samples and compared with the results obtained with conventional diagnostics (i.e. culturing of swabs and sputa). A control group of stable asthma patients was used as negative control for the MBS measurements. The pathogens detected using MBS and conventional culturing differed: *S. aureus* was found more often in swab or sputum samples whereas *Pseudomonas aeruginosa* and *S. pneumoniae* were found more often in MBS samples. We hypothesize that this is due to sampling of different compartments, MBS samples are derived from the lower respiratory tract while cultures from cough swabs and sputa are dominated by pathogens residing in the upper respiratory tract. Another important difference is the readout, i.e. culture versus PCR. The majority of CF patients in whom *P. aeruginosa* was found did not have recent positive cultures suggesting higher sensitivity of MBS-based than conventional diagnostics. The majority of parents/patients found the MBS easy to use and less of a burden than respiratory sampling.

1. Introduction

Cystic fibrosis (CF) is a relatively common autosomal recessive disease. There are around 1500 patients with CF in the Netherlands, of whom 900 are children [1]. CF is caused by mutations in the CF transmembrane conductance (CFTR) gene. The most common mutation is delF508, which is present in approximately 70% of CFTR alleles of patients with CF. The CFTR gene encodes a transporter or channel for the influx and efflux of chloride and is present in epithelial cells. A defect in this channel results in thick and viscous secretions, affecting multiple organ systems [2, 3]. In the respiratory tract it leads to problems with breathing and (bacterial) infections and eventually it can lead to lung damage, like bronchiectasis and loss of lung function.

The recurrent respiratory infections in patients with CF are often caused by (opportunistic) bacteria and can occur early in life. Some bacteria like

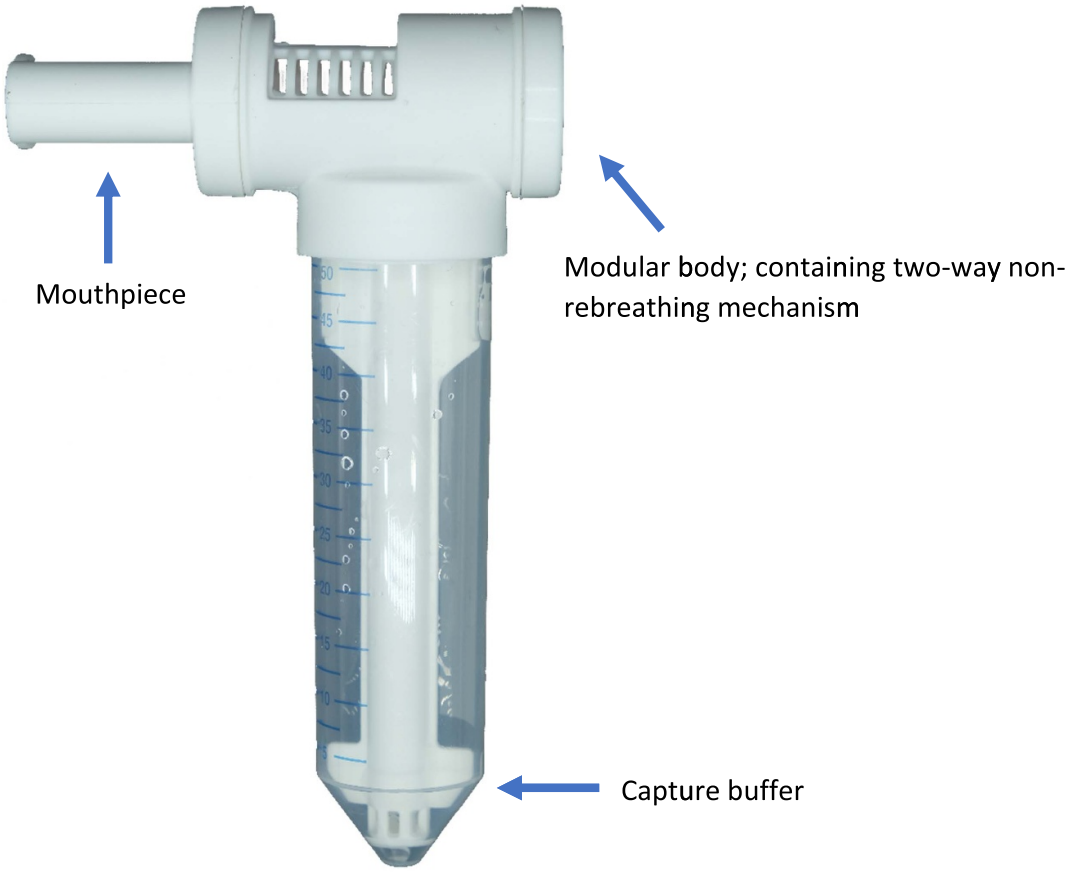

Figure 1. MBS.

Staphylococcus aureus and nontypeable *H. influenzae* are commonly detected in young children, whereas *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex are more prevalent in older children and adults [4, 5]. During a pulmonary infection, patients develop symptoms such as cough, dyspnea or exercise intolerance. When these symptoms are new or increased from baseline, this is called an exacerbation. Multiple factors contribute to an exacerbation, including chronic pulmonary inflammation and infection with pathogenic bacteria and viruses [6, 7].

Colonization and chronic infection of the respiratory tract with pathogenic bacteria is associated with a worser outcome. Especially chronic infection with *P. aeruginosa* is associated with accelerated decline of lung function, more frequent exacerbations and a worse prognosis [8]. Therefore, when found for the first time, current guidelines advise prompt eradication of *Pseudomonas aeruginosa* [9–11]. The Leeds criteria are used in clinical practice and research to define *Pseudomonas aeruginosa* infection status in CF patients [12].

Children with CF have a follow-up appointment at the CF outpatient clinic four times per year where sputum or cough swab cultures are taken to monitor colonization and presence of pathogenic bacteria in the airways [9–11, 13]. When the patient presents with signs of an exacerbation, additional cultures are often taken to tailor antibiotic therapy. Unfortunately, the results of a sputum or cough swab culture do not always give a reliable reflection of the presence of pathogenic bacteria in the lower airways and are only available days after the initiation of treatment. With bronchoalveolar lavage (BAL) the lower airways can be sampled, but routine use of this invasive sampling technique in young children has been discouraged [13].

The modular breath sampler (MBS) is a newly developed two-way non-rebreathing sampling device that collects aerosols from exhaled breath in a liquid (see figure 1). The unique low-tech approach allows for decentralized sample collection at ambient conditions without the need for supportive electric equipment, such as the case for obtaining the established exhaled breath condensate [14]. In short, patients breathe tidally through a nose-mouth mask or mouthpiece and the exhaled breath is guided through a liquid interface where the aerosols containing whole pathogens or fragments thereof are captured. This proprietary capture buffer can be directly analyzed without sample preparation using a panel of targeted polymerase chain reactions (PCRs) to detect

pathogenic bacteria. The MBS has been studied in the context of hospitalized patients with a SARS-CoV-2 infection with promising results [15].

Detecting bacteria from the lower airways by the MBS has advantages over sputum or cough swabs which are often contaminated with bacteria from the upper airways. Furthermore, for children it can be difficult to cough up enough sputum and they can experience the sampling as unpleasant or invasive. Exhaled breath analysis could be a more accurate, less invasive and more patient friendly method to investigate the presence of pathogenic bacteria in the lower airways [16].

The hypothesis of our cross-sectional proof-of-concept study was that exhaled breath analysis using the MBS can detect pathogenic bacteria associated with pulmonary infection in children with CF. We anticipated to find different results when comparing MBS with culture results and therefore added a control group of stable asthma patients. These patients served as negative controls as previous research shows that, in general, they are not colonized or infected by pathogenic bacteria like patients with CF [17, 18]. The *Pseudomonas aeruginosa* status of patients according to the Leeds criteria was recorded at the time point of inclusion to put detection of this bacterium (either by culture or MBS) in perspective.

The primary objective of this study was to investigate the diagnostic yield of a PCR panel of pathogenic bacteria using exhaled breath samples collected with the MBS in children with CF during symptom-free periods and exacerbations. Secondary objectives were comparison of the PCR results with simultaneously taken respiratory cultures and evaluation of the ease of use of the MBS with a short questionnaire.

2. Methods

2.1. Sample size

We aimed to include 100 samples from CF patients and 30 samples from asthma control patients in this cross-sectional proof-of-concept study. Age matching of study subjects and controls was done based on the age at participation. From retrospective culture data we knew that around half of the cough swab or sputum cultures in our CF patients were positive for at least one pathogenic bacterium. Therefore, we expected a sample size of 100 study subjects and 30 control subjects to be sufficient to answer our research question.

2.2. Ethics

Ethical approval was waived by the institutional ethical review board as they considered the study procedures to be of negligible burden and not harmful for the patients. Furthermore, we acted according to the 'Code verzet' (code resistance) by the Dutch Pediatric Society, which implies that resistance to study activities by children is carefully monitored and evaluated and that the study is discontinued in cases of too much resistance.

2.3. Inclusion and exclusion criteria

All children with CF visiting the CF outpatient clinic of the Radboudumc could participate in the study, whether they visited the clinic for a routine check-up or for an exacerbation. Children could participate multiple times. The in- and exclusion criteria for our study were the following:

Inclusion criteria:

- Study subjects: children with a diagnosis of CF in whom a cough swab or sputum culture is taken;
- Control subjects: children with well-controlled 'doctor-diagnosed asthma';
- Age between 0 and 18 years;
- Informed consent by parents (and by patients when 12 years of age or older).

Exclusion criteria:

- Oxygen need;
- Severe dyspnea;
- Severe tachypnea;
- Having problems with breathing through a nose-mouth mask or mouthpiece;
- For asthma subjects: known presence of bronchiectasis.

2.4. Study procedures

Children who visited the CF outpatient clinic were asked to participate in this study. When consent was given, the child was asked to breathe through the MBS tidally during 2 min. If the patient experienced too much resistance despite the low resistance of the MBS, the measurement was stopped. After this procedure, the capture tube containing the breath sample (liquid) was disconnected from the MBS body and closed with the included cap. Subsequently, the tube was labeled and stored at −20 °C. A cough swab or sputum culture was also acquired as part of standard care during the appointment at the CF outpatient clinic.

After the samples were drawn, the physician filled in a short questionnaire about ease of use of the MBS together with the patient and parent.

2.5. Laboratory procedures

Cough swabs or sputum samples were analyzed with gram staining and cultured on various plates, including Columbia blood agar, chocolate agar plate, Mannitol salt agar, McConkey agar, Sabouraud agar and *Burkholderia cepacia* selective agar for at least 5 d. Matrix assisted laser desorption/ionization

time-of-flight analyzer was used to identify the colonies detected on plate.

The MBS-samples were defrosted at 4 °C overnight and 100 μl was aliquoted in a 96-well plate. The 96-well plate was sealed and incubated 45 min at 56 °C, before being transported to QM Diagnostics B.V. (Nijmegen, The Netherlands) where all samples were analyzed utilizing the OpenArray® technology by ThermoFisher (Waltham, Massachusetts, United States). Analysis was performed according to the manufacturer's protocol, 10 μl MBS-sample was used for 20 cycle preamplification and six targets were measured on a OpenArray® in duplicate. The following assays were utilized: Ba04646259_s1 (*S. aureus),* Ba06439619_s1 *(Streptococcus pneumoniae),* AIRS-BQC *(Pseudomonas aeruginosa),* Ba06439625_s1 *(Haemophilus influenza),* Ba06439622_s1 *(Moraxella catarrhalis),* Ba06439620_s1 *(M. pneumoniae),* and Ba04932083_s1 (*Klebsiella pneumoniae*).

2.6. Statistical analysis

Data from patient characteristic, medical history, Leeds criteria at time of inclusion, culture results, PCR results from the MBS samples and answers from the questionnaire were entered in an online Castor database and later imported to SPSS (IBM SPSS statistics version 25). Concerning Leeds criteria; four categories of *Pseudomonas aeruginosa* infection status are defined: (a). Never; a patient who never had a *Pseudomonas aeruginosa* positive culture, (b). Free; a patient who had at least one positive culture >12 months before assessment, (c). Intermittent; a patient with a positive culture in 50% or less of cultures in the preceding year or four. Chronic; a patient with positive cultures in more than 50% of cultures in the preceding year.

Descriptive statistics were used to present data from patient characteristics, culture and PCR results. McNemar and Chi square tests were used to compare differences in results between culture and MBS and MBS between study and control group respectively.

Table 1. Baseline characteristics of the participants in the CF- and asthma-group.

	CF-group ($n = 44$)	Asthma-group ($n = 32$)
Age at first MBS-measurement (mean and standard deviation)	8 ± 4,22	10 ± 4,30
Gender (male)	24 (55%)	17 (53%)
CF-mutation		N/A
F508del (homozygous)	37 (84%)	
F508del (heterozygous)	6 (14%)	
Other	1 (2%)	
Participants with multiple samples	26	0
Measurements per participant		
1	18	32
2	10	
3	10	
4	5	
5	1	

Table 2. Baseline characteristics of the MBS-measurements in the CF-group.

MBS-measurements	$n = 93$
CFTR modulator therapy[a]	79 (85%)
Microbiological sample taken	
Cough swab	66 (71%)
Sputum culture	27 (29%)
Exacerbation	10 (11%)
Antibiotics given	25 (27%)
Lung function, predictive FEV1 (%)	82.2 ± 15.9
Quartile 1	24.0–75.3
Quartile 2	75.3–83.0
Quartile 3	83.0–91.8
Quartile 4	91.8–117.0
Leeds criteria	
Never	40 (43%)
Free	39 (42%)
Intermittent	11 (12%)
Chronic	3 (3%)

[a] Medication developed to regulate/improve the CFTR gene function in patients with CF [25].

3. Results

Between April 2019 and May 2021, 131 MBS samples were obtained; 99 from patients with CF and 32 from patients with asthma. The baseline characteristics of both groups are shown in table 1. The average age at first participation in the study of both groups was respectively 8 and 10 years old. CF patients could participate more than once which explains the difference in age between these groups.

Six MBS samples in the CF-group did not have a simultaneous swab or sputum sample to compare the results with, therefore these samples were excluded. The remaining 93 MBS-samples were obtained from 44 CF-patients; several patients participated more than once (see table 2). The samples of the patients with multiple measurements were analyzed individually, as new infections could have developed over time.

Of the 93 samples in the CF-group; significantly more swab or sputum samples had a positive result compared to MBS sample: 62% versus 23% ($P < 0.05$). The exact results of both methods are shown in figures 2 and 3. In five cases (5,1%) the results of both methods were identical. *Staphylococcus aureus* was detected 55 times in a swab or sputum sample compared to only eight times in a MBS sample. On the other hand, *Pseudomonas aeruginosa* was measured once in a swab or sputum sample and

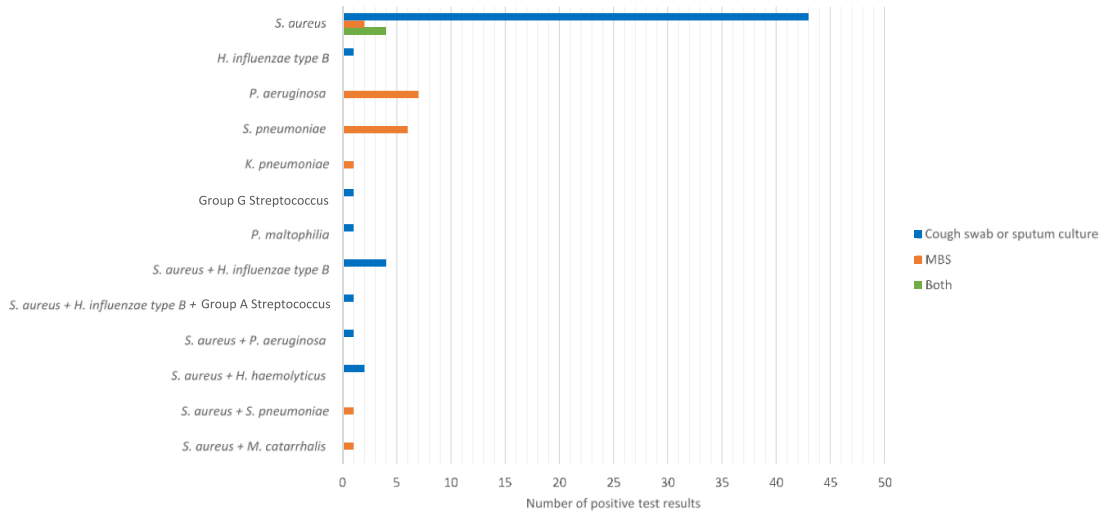

Figure 2. Positive results of swab or sputum and MBS samples in the CF-group.

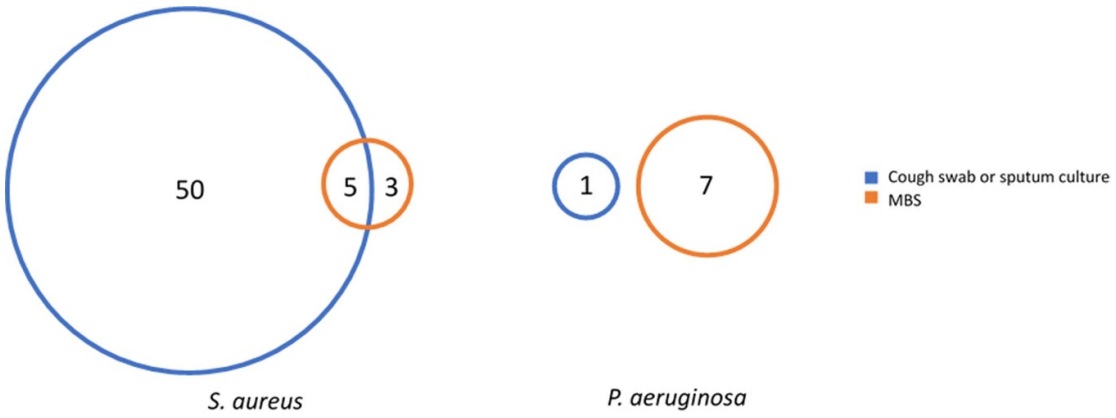

Figure 3. Venn-diagram of positive results for *Staphylococcus aureus* and *Pseudomonas aeruginosa* in swab or sputum and MBS-samples.

seven times in a MBS sample. *S. pneumoniae* was detected seven times using the MBS and was not detected in swab or sputum samples.

MBS samples were significantly more often positive in the CF group compared to the asthma control group: 23 versus 6% ($P < 0.05$). Only two samples in the asthma group had a positive result, with *S. aureus* detected in both. In addition, in one of these two samples *P. aeruginosa* was also found.

3.1. Pseudomonas aeruginosa

In total, eight separate patients with CF had a positive result for *P. aeruginosa* from either a swab or sputum sample or a MBS sample. The Leeds criterium of these patients at the time of sampling is shown in table 3: five had a criterium free, two intermittent and one never. In the follow up 9 months after sampling, only the two intermittent patients had positive *Pseudomonas aeruginosa* cultures.

3.2. Experiences

All patients and parents in the CF-group and 31 in the asthma-group were able to fill in the questionnaire about their experiences. The researcher, who was present during the MBS sample collection, filled in the questionnaire in all cases. The answers of the questionnaire are shown in table 4. In both groups combined, 113 patients (90%) experienced the MBS easy to use, while one patient (0,8%) found it difficult. According to the researchers, the procedure was easy 118 times (94%) and difficult twice (1,6%). In 118 cases (94%) sampling succeeded in one attempt and the patient was able to sample for a minimum of 2 min in 122 cases (98%). The patients in the CF-group were asked to compare the MBS sampling with the cough swab or sputum culture. 73 patients (79%) experienced the use of MBS to be less of a burden, two patients (2%) found the use of MBS more burdensome, and 18 patients (19%) did not prefer one method over the other.

Table 3. Information of the patients with a positive results for *P. aeruginosa*.

Sample type	Age (years)	Gender	Leeds-criterium at time of the study	*P. aeruginosa* cultured in the next 9 months
MBS	16	Female	Free	No
MBS	13	Male	Free	No
MBS	15	Male	Free	No
MBS	18	Female	Intermittent	Yes
MBS	13	Male	Never	No
MBS	4	Female	Free	No
MBS	10	Female	Free	No
Swab or sputum	18	Female	Intermittent	Yes

Table 4. Results of the questionnaire for patients and researcher concerning MBS use.

Questions for the patients	CF ($n = 93$)	Asthma ($n = 32$)[a]
How did the patient find the procedure?		
Easy	89 (96%)	24 (75%)
Neutral	3 (3%)	7 (22%)
Difficult	1 (1%)	0 (0%)
How did the patient find the procedure compared to culture?		
More of a burden	73 (79%)	
Neutral	18 (19%)	
Less of a burden	2 (2%)	
Could the patient do the procedure at home?		
Yes	91 (98%)	31 (97%)
No	2 (2%)	0 (0%)
Questions for the researcher		
Did the procedure succeed in one attempt without any interruptions?		
Yes	87 (94%)	31 (97%)
No	4 (4%)	0 (0%)
Was the patient able to sample for 2 min?		
Yes	91 (98%)	31 (97%)
No	2 (2%)	1 (3%)
How did the procedure go?		
Easy	89 (96%)	29 (91%)
Neutral	3 (3%)	2 (6%)
Difficult	1 (1%)	1 (3%)

[a] One patient did not fill in the questionnaire.

4. Discussion

In our study population of CF patients, MBS was significantly more often positive than in asthma patients. This confirms our hypothesis that MBS can detect pathogenic bacteria from exhaled breath in patients with CF which are not found in a control group of patients with asthma.

We observed several striking differences comparing MBS results with those from cough swab or sputum culture: (a) Much more positive cultures were found than MBS samples: 62% (58/93) versus 24% (22/93) $P < 0.05$; (b) The bacteria found by both techniques do not seem to overlap at all. Cultures showed *Staphylococcus aureus* as main organism whereas MBS results were positive mainly for *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*.

It is known that *Pseudomonas aeruginosa* is ubiquitously present in many different environments, we have therefore taken precautions to avoid contamination. All production batches of the MBS and the breath samples were processed in lateral flow cabinets in a biosafety level two laboratory. All clinical samples were taken in clean outpatient rooms using air filter ePM1-60% and in which cross-contamination between patients is monitored and prevented. In addition, the capture buffer was used as 'no template control' in the PCR analysis. Finally, looking at the frequency of detection of *Pseudomonas aeruginosa*, which is generally very low, further underlines the fact that contamination cannot explain the outcome of our analysis.

We hypothesize that this discrepancy in results between both techniques is explained by the fact that different compartments are sampled: upper versus lower airways. Another explanation could be the sensitivity of culture versus PCR in picking up different bacteria. Multiple studies have been performed

comparing PCR with culture results in swab or sputum samples for different bacteria. Deschaght *et al* investigated *Pseudomonas aeruginosa* detection with culture and qPCR in 852 swab or sputum samples of CF patients. They found concordance between PCR and culture in 89 samples, PCR positive and culture negative results in 26 and PCR negative and culture positive results in ten samples. All 26 PCR positive, culture negative samples turned culture positive during follow up. They concluded that qPCR may have a predictive value for impending *Pseudomonas aeruginosa* infection [19]. Héry-Arnaud *et al* followed a cohort of 96 CF patients without chronic *Pseudomonas aeruginosa* colonization for 3 years and performed culture and qPCR on swab or sputum samples. 36 of the 96 patients became culture positive during the follow up period. In 20 of 36 cases qPCR was positive earlier than culture resulting in a median detection gain time of 8 months [20]. Johnson *et al* investigated the diagnostic yield of different PCR techniques in 59 *Staphylococcus aureus* culture positive swab or sputum samples of CF patients using culture as the gold standard. Sensitivities of various techniques ranged from 34% to 85% [21]. Although not performed on exhaled air but on swab or sputum samples, these studies show that PCR can be a promising technique mainly for the early detection of *Pseudomonas aeruginosa* and may perform poorer when it comes to *Staphylococcus aureus*.

Looking at the Leeds *Pseudomonas aeruginosa* status, one of the seven patients where this bacterium was found with MBS had a status of never being colonized with Pseudomonas in the past and five of seven were free of colonization (at least one positive culture >12 months before assessment assessed by cough swab or sputum culture). In the 9 months after sampling, cultures in these patients remained negative for *Pseudomonas aeruginosa*. These findings suggest that the MBS-based method allows for early detection of (re)colonization with *Pseudomonas aeruginosa*, although data should be interpreted with caution in this proof-of-concept study. A follow up study is currently being set up, focusing on *Pseudomonas aeruginosa* to validate these findings.

Using cough as a sample, Ku *et al* investigated a sampler comparable to the MBS in a cohort of adult CF patients: the PneumoniaCheck™ [22]. With this device the patients are asked to cough into the device and pathogens are analyzed using PCR. In 20 CF patients they found more CF related bacteria with sputum versus cough samples: 100 versus 65%. However, contamination with bacteria from the upper airways (*Streptococcus mitis*) was found in 93% of sputum samples versus 0% in the cough samples suggesting sampling of the lower airways with their device. Patrucco *et al* investigated the PneumoniaCheck™ in a small cohort of pneumonia patients where they found a good correlation of the results with BAL samples for non-herpes viruses [23].

In the two subjects in the asthma control group, one subject was positive for *Staphylococcus aureus* and one for *Staphylococcus aureus* and *Pseudomonas aeruginosa*. A low colonization frequency with pathogenic bacteria has been described in asthma patients: Zhang *et al* describe results from induced sputum cultures in a group of adult patients with severe asthma showing positive cultures for *Pseudomonas aeruginosa* among other bacteria and links colonization with duration of asthma and having exacerbations in the past year [24]. It might be that Pseudomonas is an incidental finding in asthma patients.

The majority of CF and asthma patients experienced sampling with the MBS as easy and less of a burden than cough swab or sputum culture sampling.

5. Conclusion

In this cross-sectional proof-of-concept study we studied the diagnostic yield of exhaled breath combined with PCR-based detection in pediatric patients with CF using the MBS, an innovative two-way non-rebreathing breath sampler. Asthma patients served as negative controls of whom only 6.2% had a positive MBS sample compared to 23% in the CF group, confirming the reliability of the MBS-based measurements. There was a striking difference in the bacteria found with the conventional culture method versus the MBS-based detection, possibly caused by the fact that different compartments were sampled (i.e. upper versus lower respiratory tract, respectively) and due to a different readout. *Pseudomonas aeruginosa* was detected more often in MBS than in swab or sputum samples, also in subjects with a Leeds criterium of never or free of colonization with this bacterium. This indicates the added value of the MBS in the early detection of *Pseudomonas aeruginosa*. In general, patients, parents and researchers involved in this study experienced sampling with the MBS easy and less of a burden than cough swab or sputum culture sampling.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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