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A Pilot Study of the Noninvasive Assessment of the Lung Microbiota as a Potential Tool for the Early Diagnosis of Ventilator-Associated Pneumonia

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BACKGROUND: Ventilator-associated pneumonia (VAP) remains a common complication in critically ill surgical patients, and its diagnosis remains problematic. Exhaled breath contains aerosolized droplets that reflect the lung microbiota. We hypothesized that exhaled breath condensate fluid (EBCF) in hygroscopic condenser humidifier/heat and moisture exchanger (HCH/HME) filters would contain bacterial DNA that qualitatively and quantitatively correlate with pathogens isolated from quantitative BAL samples obtained for clinical suspicion of pneumonia.

METHODS: Forty-eight adult patients who were mechanically ventilated and undergoing quantitative BAL (n = 51) for suspected pneumonia in the surgical ICU were enrolled. Per protocol, patients fulfilling VAP clinical criteria undergo quantitative BAL bacterial culture. Immediately prior to BAL, time-matched HCH/HME filters were collected for study of EBCF by real-time polymerase chain reaction. Additionally, convenience samples of serially collected filters in patients with BAL-diagnosed VAP were analyzed.

RESULTS: Forty-nine of 51 time-matched EBCF/BAL fluid samples were fully concordant (concordance > 95% by κ statistic) relative to identified pathogens and strongly correlated with clinical cultures. Regression analysis of quantitative bacterial DNA in paired samples revealed a statistically significant positive correlation ($r = 0.85$). In a convenience sample, qualitative and quantitative polymerase chain reaction analysis of serial HCH/HME samples for bacterial DNA demonstrated an increase in load that preceded the suspicion of pneumonia.

CONCLUSIONS: Bacterial DNA within EBCF demonstrates a high correlation with BAL fluid and clinical cultures. Bacterial DNA within EBCF increases prior to the suspicion of pneumonia. Further study of this novel approach may allow development of a noninvasive tool for the early diagnosis of VAP. CHEST 2015; 147(6):1494-1502

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ABBREVIATIONS: BALF = BAL fluid; CFU = colony-forming unit; DPPC = dipalmitoylphosphatidylcholine; EBCF = exhaled breath condensate fluid; HCH/HME = hygroscopic condenser humidifier/heat and moisture exchanger; PCR = polymerase chain reaction; RT-PCR = real-time polymerase chain reaction; SICU = surgical ICU; SP-B = surfactant-associated protein B; VAP = ventilator-associated pneumonia; VUMC = Vanderbilt University Medical Center

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Ventilator-associated pneumonia (VAP) is among the most common health-care-associated infections in severely ill and injured patients, accounting for substantial morbidity, increased length of ICU and hospital stay, and excess cost and mortality.[1-3] Critically ill and injured patients are particularly prone to VAP, due to several factors that both increase the risk and confound the diagnosis of pneumonia, making accurate and timely diagnosis of pneumonia problematic.[1,4-9] Atelectasis, pulmonary contusions, acute lung injury, aspiration pneumonitis, and the systemic inflammatory response syndrome are all common following major operative interventions or severe trauma and can mimic pulmonary infections. The standard clinical criteria for the diagnosis of VAP overestimates the rate twofold when compared with quantitative culture using BAL or protected brush specimens.[10-13] Quantitative scoring systems of clinical and radiographic findings have failed to increase the diagnostic accuracy when compared to quantitative cultures.[14-18] Quantitative culture techniques increase the specificity of the diagnosis and may improve overall outcomes.[11] However, these techniques mandate the development of clinical symptoms to introduce clinical suspicion, requiring approximately three additional days for culture and sensitivity results, necessitating empirical antibiotic coverage while results are pending. Thus, existing diagnostic strategies delay therapy until the infection is well established and require significant empirical therapy, contributing to unnecessary antibiotic exposure.[19]

While the diagnosis of pneumonia is difficult to establish in a rapid or specific fashion, significant evidence supports reduced morbidity and mortality when appropriate antibiotic therapy is initiated early in patients with VAP.[20-23] Inadequate empirical antibiotic coverage for VAP is associated with a twofold increase in mortality, and retrospective studies suggest that delays as short as 30 min from the onset of fever in infected patients may increase mortality.[20-24] However, unnecessary antibiotic exposure is associated with increased risk for subsequent infectious complications, colonization, and infection with resistant pathogens, and increased hospital costs.[21,25-31] Thus, early identification of patients with pneumonia is necessary to improve outcomes in this population.

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The goal of our research is to develop tools that can improve the timeliness, specificity, and sensitivity of VAP diagnosis in critically ill patients. This would enable clinicians to diagnose pneumonia earlier in its course, select more-specific antimicrobial therapies, and more accurately monitor response to treatments. Exhaled breath contains aerosolized droplets of widely varying size (majority between 5 μm and 100 μm) that carry bacteria, as first described by Flugge in 1897.[32,33] These droplets reflect the pathogens in the lower respiratory tree, transmitting them to the environment.[32-35] Thus, we hypothesized that bacteria within these aerosolized breath droplets would collect within the hygroscopic condenser humidifier/heat and moisture exchanger (HCH/HME) filters between the endotracheal tubes and ventilator circuit and provide a quantitative assessment of pulmonary bacterial growth. The purpose of this current study is to examine if polymerase chain reaction (PCR) analysis of exhaled breath condensate fluid (EBCF) would correlate quantitatively and qualitatively with fluid samples from time-matched, semiquantitative BAL fluid (BALF) obtained for the clinical suspicion of pneumonia, thus providing qualitative and quantitative results in hours rather than the 3 days required by current techniques.

Materials and Methods

We previously reported that HCH/HME filters (Fig 1) serve as a reservoir for pathogens carried in exhaled breath condensate but do not allow bacterial proliferation, since they are bacteriocidal. As a result, bacterial cells remain intact and can be reliably quantified by real-time PCR (RT-PCR) (Fig 2).[36] Additionally, we have demonstrated that bacterial recovery is independent of species and that these isolated bacteria can be uniformly lysed and quantified without bias or loss of specific species. Finally, we have demonstrated that samples can be normalized by surfactant content via surfactant-associated protein B (SP-B) and dipalmitoylphosphatidylcholine (DPPC) measurements.

Patient Recruitment and Sample Collection

The study was approved by the Vanderbilt University Medical Center (VUMC) institutional review board (No. 101185) and funded by the Vanderbilt Institute for Clinical and Translational Research (CTSA 1 UL1 RR024975). The current standard of care for critically ill patients who were mechanically ventilated in the Vanderbilt surgical ICU (SICU) with suspected pneumonia is to undergo protocol directed, semiquantitative BAL for standard microbiologic culture and empirical antibiotic therapy until BAL results are available. Antibiotics are discontinued for BAL culture results $< 10^4$ colony-forming unit (CFU) of bacteria and deescalated to cover only pathogens $> 10^4$ CFU.

Between April and August of 2011, adult patients who were ventilated in the SICU, with a clinical suspicion of pneumonia, were enrolled. Two to 4 h prior to BAL, the existing HCH/HME filter unit was exchanged for a new “study” unit. Immediately prior to BAL, the study filter was removed and placed in a biohazard bag. Following the BAL, 2 mL excess BALF was placed in a sterile specimen vial for PCR analysis and residual BALF was sent to the clinical microbiology laboratory, per standard of care. The BALF sample and the time-matched EBCF collected from the study filter were processed separately in identical fashion, with positive and negative control samples. PCR findings from matched BALF

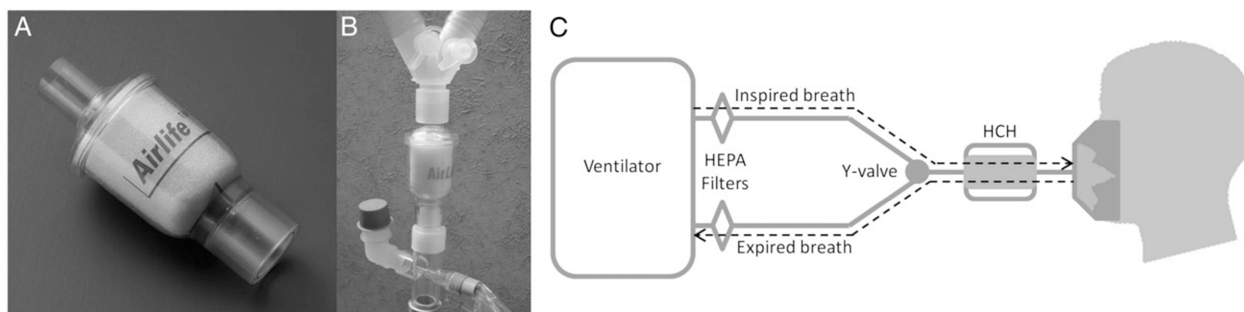

Figure 1 – *An HCH/heat and moisture exchanger filter unit in isolation and as part of the ventilator circuit. HCH = hygroscopic condenser humidifier; HEPA = high-efficiency particulate air.*

and EBCF samples were compared retrospectively with clinical microbiology results of the corresponding BALF sample.[37]

To assess quantitative changes in bacterial DNA over time within the EBCF in patients who were ventilated, serial HCH/HME filters were collected from a small number of patients who developed VAP during mechanical ventilation. Stored samples and clinical course were analyzed retrospectively after positive BAL cultures were identified.

Sample Processing

DNA Extraction: EBCF samples from HCH/HME filters and BALF samples were prepared as previously described.[36] Briefly, cells were concentrated (independently) on 13-mm-diameter, 0.45 micron Durapore membranes (Millipore Corp). Total DNA was purified from the membranes using the DNeasy Blood and Tissue Kit (Qiagen NV) with the following modifications. Lysis buffer consisted of 20 mM tris(hydroxymethyl)aminomethane, 136 mM sodium chloride, pH 7.4, plus 2 mg/mL lysozyme (Sigma-Aldrich Co LLC), 5 mM ethylenediaminetetraacetic acid, and 10 units/mL lysostaphin (Sigma-Aldrich Co LLC). Each concentrated cell sample was resuspended in 200 uL lysis buffer and incubated for 2 h at 37°C with 200 rpm shaking. Then, 20 uL proteinase K solution and 200 uL buffer AL from the DNeasy kit was added, and incubated at 56°C for 30 min.

Quantitative PCR: Sample analysis was performed as described previously.[36,38] Eight genomic targets, seven bacterial species-specific genes, and a species-independent bacterial 16S rDNA were used.[38-44] These seven species represent 70% of SICU pathogens causing VAP, as reported to the Centers for Disease Control and Prevention. Primer details are given in Table 1. All reactions were conducted in triplicate at 10 uL total volume, consisting of 5 uL SsoFast EvaGreen Supermix (BioRad Laboratories Inc), 1 uL DNA template, 1 uL primer mix, and 3 uL nuclease-free distilled water. Reactions were run on a CFX96 Real-Time PCR Detection System (BioRad Laboratories Inc). The cycling parameters were as follows: an initial incubation of 2 min at 98°C, then 40 cycles of 5 s at 98°C and 10 s at 60°C with real-time fluorescence measurement. Purified DNA samples were first analyzed by universal 16S quantitative PCR to conserve time and reagents by not further studying negative samples. Positive samples were then subjected to the full panel of species-specific probes. Absolute quantification was achieved using four concentrations (100, 10,000, 100,000, and 1 million copies) of each amplified sequence that had been ligated into TOPO-TA vectors, expressed in transformed *Escherichia coli*, extracted and purified, then quantified by fluorescent NanoDrop (Thermo Fisher Scientific Inc) using SYBR Green II (Life Technologies Corp) as a dye and a DNA standard.

Surfactant Assay: To aid in quality control and standardization, SP-B concentrations from EBCF samples, BALF samples, or both were determined as reported by Krämer et al[45] with the following modifications: Infasurf (ONY, Inc) was diluted in phosphate-buffered saline to create standards. Polyclonal rabbit anti-SP-B (Millipore Inc) was used at a 1:5,000 dilution in phosphate-buffered saline, 0.1% bovine serum albumin. Plates were developed with the Amplex ELISA Development Kit for Rabbit IgG (Invitrogen Corp) according to manufacturer's directions and read at 590 nm with 530 nm excitation on a Spectramax M5 (Molecular Devices LLC) in the VUMC Center Molecular Cell Biology Resource Core. DPPC concentrations in all samples were determined as previously described by Ivanova et al[46] using 200 ng/mL D62-DPPC (Avanti Polar Lipids Inc) as an internal standard and analyzed on a TSQ Quantum quadrupole mass spectrometer (Thermo Scientific Fisher Inc) in the VUMC Mass Spectrometry Core.

Statistical Methods: The binary outcome data (target amplified or not relative to controls) were categorized using a contingency table with EBCF results in the rows. The CIs for proportions, such as percent agreement, sensitivity, and specificity, were constructed using the Wilson score method with continuity correction.[47] CIs for the correlation coefficients were constructed using the Fisher Z transform method. The concordance coefficient κ, and an accompanying CI, was calculated from the matched sample data according to the description by Hanley[48] by considering the sample space to consist of all $2^7 = 128$ possible outcomes from performing seven distinct tests with binary response.

Results

Forty-eight subjects with 51 episodes of clinically suspected VAP who underwent BAL prior to the initiation of empirical antibiotic therapy were enrolled. The matched EBCF/BALF pairs were analyzed by RT-PCR for both qualitative and quantitative agreement of pathogens and compared with quantitative clinical cultures reported by the clinical microbiology laboratory.

Clinical Culture Results

Of 51 episodes of suspected VAP, 20 patients had confirmed VAP by quantitative BAL (cultures with $> 10^4$ CFU/mL) and continued deescalated antibiotic therapy for a full therapeutic course. RT-PCR of matched EBCF detected the pathogens in 100% of patients. In 31 episodes of suspected VAP, quantitative BAL did not confirm pneumonia ($< 10^4$ CFU/mL) and empirical antibiotics were discontinued. In 24 of these 31 episodes,

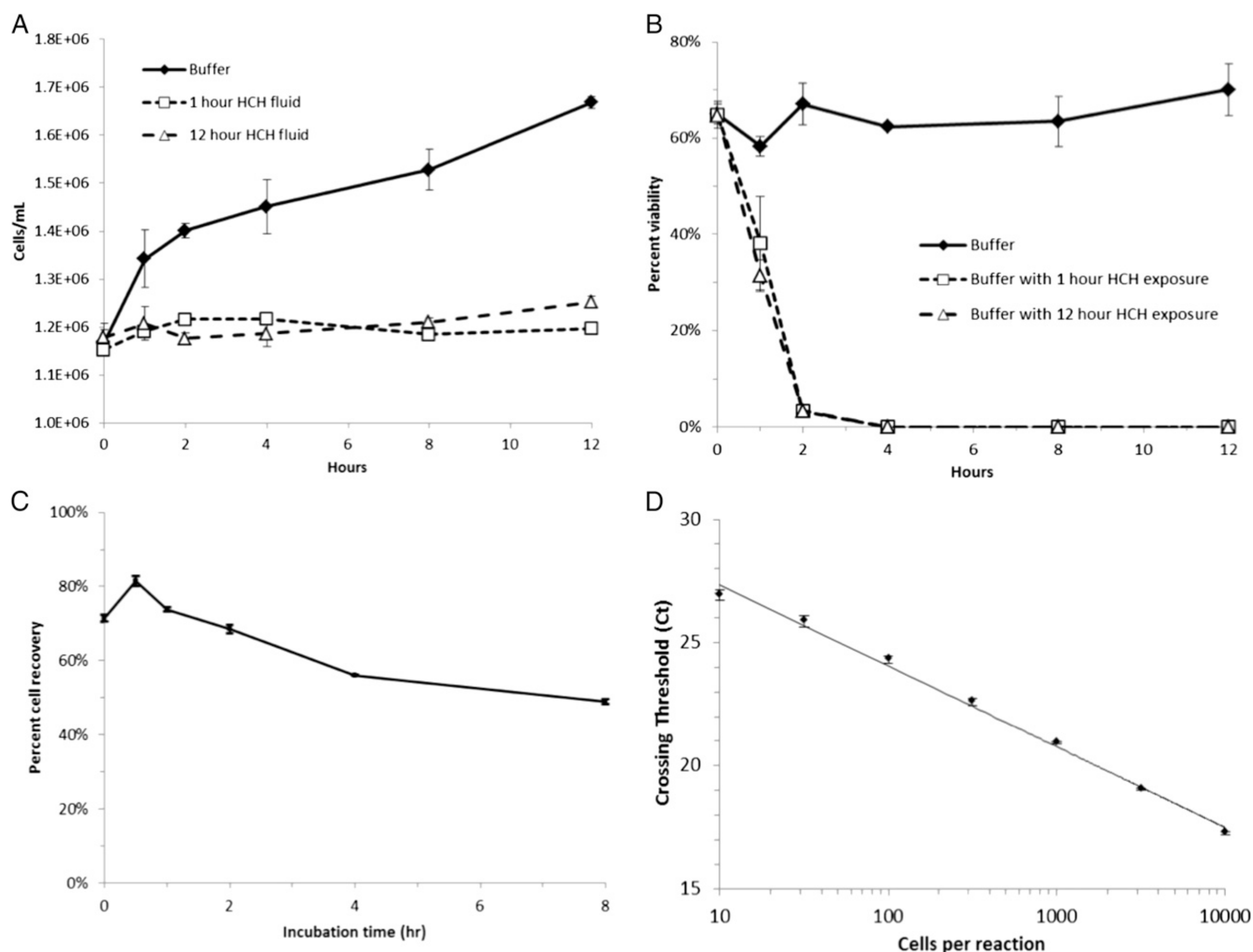

Figure 2 – *Extraction of information from HCH/heat and moisture exchanger (HCH/HME) filters is reliable. A, B, Filter environment does not allow proliferation of* Escherichia coli *and is bacteriocidal. C, A large, representative, and reproducible fraction of bacteria inoculated can be recovered from filters through quantitative assay of nonviable* E coli *by Coulter counter. D, Linear regression line* ($r^2 = 0.995$) *of* E coli *inoculum vs quantitation by real-time polymerase chain reaction, demonstrating the sensitivity and accuracy of polymerase chain reaction analysis of HCH/HME fluid. See Figure 1 legend for expansion of other abbreviation.*

BAL revealed no growth. RT-PCR detected low copies of bacterial DNA from both EBCF and BALF in eight cases when BAL clinical cultures did not.

Qualitative Agreement of PCR Detection of Bacterial Pathogens in EBCF and BALF

Fifty-one time-matched pairs of EBCF and BALF samples were collected from 48 patients. Three patients had repeat BAL procedures performed on different days. The qualitative results from the 51 time-matched pairs are summarized in Table 2, grouped by pathogen results in the 24 patterns observed. The qualitative PCR results for the matched (EBCF/BALF) pairs are in near-perfect agreement, with two minor exceptions (Table 2). In the first exception, $<$ 100 copies/mL of *Entercoccus faecalis* DNA was found in the BALF sample but not the EBCF sample, and no growth occurred in the standard microbiologic culture sample. In the second exception, $<$ 200 copies/mL of *Staphylococcus aureus* DNA was detected in the BALF sample but not in the EBCF sample, with no growth in microbiologic culture sample. In two matched pairs (Table 2), the clinical microbiologic cultures detected $< 10^3$ CFU/mL of *Klebsiella pneumoniae* not detected by PCR in either EBCF or BALF.

The binary outcome data (target amplified or not EBCF vs BALF) are shown in Table 3. The point estimate of the overall proportion of agreement between EBCF and BALF is 49 of 51, in excess of 96%. A 99% CI of the proportion of agreement is 0.81 to 1.0. Given the nominal nature of the data, the modest sample size, and the possibility that the seven distinct PCR measurements are correlated, an appropriately conservative omnibus measure of the degree of concordance between the EBCF and BALF results is provided by the statistic κ of 0.95 (99% CI, 0.87-1.0) (Table 4). If each organism and

TABLE 1] Pathogen-Specific Targets for Polymerase Chain Reaction Analysis

Organism[a]	Gene Target	Amplicon Size
Acinetobacter baumannii	Citrate synthase	722
Escherichia coli	β-Glucuronidase	486
Enterococcus faecalis	16s rDNA	138
Enterococcus faecium	D-ala:D-ala ligase	550
Klebsiella pneumoniae	16s-23s rDNA IS	260
Pseudomonas aeruginosa	OM lipoprotein	504
Staphylococcus aureus	Thermonuclease	106
Universal	16s rDNA	470

[a]Selected pathogens account for > 70% of surgical ICU ventilator-associated pneumonia pathogens.

sample is treated as independent and the data pooled, then 7 × 51 = 357 observations are available. Under these assumptions, an overall correlation (Cohen's W or φ) in excess of 0.98 (99% CI, 0.97-0.98) is calculated. The 99% CI for overall combined sensitivity is 0.92 to 0.98, and the overall combined specificity is 0.98 to 1.0 (Table 4).

Quantitative Agreement of RT-PCR Between EBCF and BALF

The quantitative correlation between each of the 51 matched EBCF and BALF samples was compared. Linear regression of the matched EEBCF/BALF pairs for each of the seven species-specific DNA targets and a universal 16S rDNA target is shown in Figure 3. A point estimate of the Pearson product moment correlation coefficient is 0.85 (99% CI, 0.81-0.88). While overall correlation is good, some samples demonstrated poor agreement, with low number of copies in one sample and high number in the other.

Analyses of EBCF and BALF for SP-B and DPPC were positive in all cases. Six consecutive EBCF samples from one patient provided an average of 310 ng/mL of SP-B and 261 ng/mL DPPC, with SDs of 428 ng/mL and 326 ng/mL, respectively. A matched BALF sample from the same patient contained comparable amounts (SP-B, 256 ng/mL; DPPC, 180 ng/mL).

Discussion

Tools to diagnosis VAP in a rapid, specific fashion would improve patient care quality. Studies in the literature support reduced morbidity and mortality when appropriate antibiotic therapy is initiated early in the course of VAP,[20-23] yet existing diagnostic approaches rely on symptoms that correspond with an advanced infectious process. In addition, traditional culture-based techniques require at least 48 h to establish or rule out infection, identify specific pathogens, and determine antibiotic sensitivity. These limitations contribute to poor antibiotic stewardship and substantially increased hospital costs.

Exhaled breath contains fluid particles carrying pathogens from the lower respiratory system[32-35] and prior study demonstrates that HCH/HME filters are reservoirs for pathogenic bacterial DNA.[36] Our current study assesses the qualitative and quantitative correlation of DNA in EBCF with BALF and microbiologic cultures for suspected pneumonia. Results indicate that RT-PCR of EBCF correlates highly, both qualitatively and quantitatively, with RT-PCR and standard microbiologic cultures of BALF, with the point estimate of their concordance (κ) > 95%. However, Figure 3 demonstrates that some samples demonstrated a low number of DNA copies in one specimen type but high number in the other. The origin of this disagreement is unclear at present and further study and refinement is required to advance this application.

The qualitative data demonstrate substantial agreement of RT-PCR between EBCF and BALF and that PCR is more sensitive than standard clinical quantitative culture of BALF. In two cases, *K pneumoniae* was identified on clinical culture at levels lower than the threshold for VAP. Whether these are contaminates or represent a failure of the PCR analysis cannot be determined. However, in all cases, RT-PCR detected the pathogens involved in VAP established by quantitative BAL and could have been used to eliminate empirical coverage for either gram-positive or gram-negative pathogens in 28 patients, and eliminate empirical coverage in 16 patients without detectable pathogens. The quantitative agreement of RT-PCR of EBCF and BALF is also strong. However, translating the number of copies of DNA to CFU detected by clinical culture requires further research. In addition, a better understanding of those mismatches is paramount to advance this line of research.

Another potential application of this technique could be to demonstrate changes in the lung microbial community that occur over the period of mechanical ventilation and precede the development of VAP. Results from serial sample analyses of HMEs from a small number of patients who develop VAP suggest that derangements in lung microbiota may precede the clinical suspicion

TABLE 2] Summary Results of 51 Matched Pairs Grouped by Pattern of Occurrence

No.	EBCF Bacteria by PCR	BALF Bacteria by PCR	BALF Bacteria by Culture	No.	EBCF Bacteria by PCR	BALF Bacteria by PCR	BALF Bacteria by Culture
1	None[a]	Sa	None	1	Pa	Pa	None
2	Ec	Ec	None	1	Sa	Sa	None
1	Ef	Ef	None	1	None	None	Kp[b]
2	Kp	Kp	None	15	None	None	None
1	Efs	Efs	Efs	1	Ab	Ab	Ab
	Pa	Pa	Pa				
1	Pa	Pa	Pa	1	Ef	Ef	Ef
	Sa	Sa	Sa				
1	Kp	Kp	Kp	3	Kp	Kp	Kp
	Pa	Pa	Pa				
1	Ec	Ec	Ec	2	Pa	Pa	Pa
	Sa	Sa	Sa				
1	Ef	Ef	Ef	9	Sa	Sa	Sa
	Sa	Sa	Sa				
1	Kp	Kp	Kp	1	Ec	Ec	Ec
	Sa	Sa			[a]	Efs	
1	Ab	Ab	Ab	1	Sa	Sa	Sa
	Pa	Pa					
	Sa	Sa	Sa				Kp[b]
1	Ab	Ab	Ab	1	Efs	Efs	None
	Efs	Efs					
	Pa	Pa			Sa	Sa	
	Sa	Sa	Sa				

Ab = *Acinetobacter baumannii*; BALF = BAL fluid; CFU = colony-forming unit; Ec = *Escherichia coli*; EBCF = exhaled breath condensate fluid; Ef = *Enterococcus faecium*; Efs = *Enterococcus faecalis*; Kp = *Klebsiella pneumoniae*; Pa = *Pseudomonas aeruginosa*; PCR = polymerase chain reaction; Sa = *Staphylococcus aureus*.
[a]Present at < 200 copies by PCR in BALF but not EBCF.
[b]Present in clinical culture at $\leq 10^3$ CFU/mL, not detected in EBCF or BALF by PCR.

of pneumonia, and these changes are detectable by EBCF analysis (data not shown). However, this study was not designed to examine this application and further research is being conducted to examine this concept.

Although significant literature examines exhaled breath in a variety of settings,[49-52] no studies have examined serial EBCF collected from reservoirs such as HCH/HME filters, or used quantitative bacterial DNA analysis. While the knowledge that pathogenic microorganisms

TABLE 3] Individual Organism Detection by PCR in EBCF and BALF

Target	Sensitivity	99% CI	Specificity	99% CI
Acinetobacter baumannii	1	0.87-1.0	1	0.87-1.0
Escherichia coli	1	0.87-1.0	1	0.87-1.0
Enterococcus faecalis	0.75	0.56-0.88	1	0.87-1.0
Enterococcus faecium	1	0.87-1.0	1	0.87-1.0
Klebsiella pneumoniae	1	0.87-1.0	1	0.87-1.0
Pseudomonas aeruginosa	1	0.87-1.0	1	0.87-1.0
Staphylococcus aureus	0.95	0.79-0.99	1	0.87-1.0

See Table 2 legend for expansion of abbreviations.

TABLE 4] Omnibus Statistical Measures of Agreement

Statistic	Point Estimate	99% CI
Targets, No.	357	N/A
Sensitivity	0.96	0.92-0.98
Specificity	1.0	0.98-1.0
Correlation, ϕ	0.98	0.97-0.98
Agreement	0.96	0.81-1.0
Concordance, κ	0.95	0.87-1.0

N/A = not applicable.

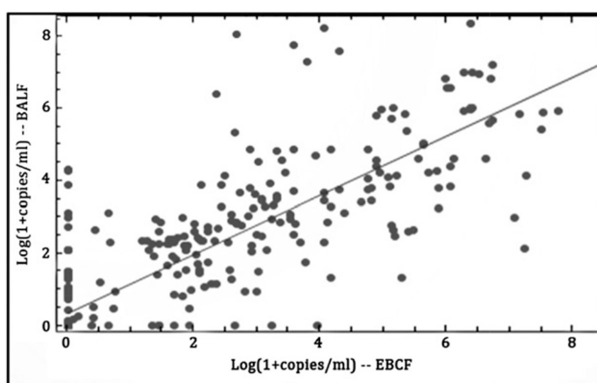

Figure 3 – *Linear regression of the bacterial target DNA/mL of patient sample for the 51 time-matched sample pairs EBCF vs BALF. The real-time polymerase chain reaction data from the seven specific targets and a universal 16s rDNA target, listed in Table 1, are shown. The Pearson product moment correlation is 0.85 (99% CI, 0.81-0.88), indicating a significant positive correlation. BALF = BAL fluid; EBCF = exhaled breath condensate fluid.*

are carried in the exhaled breath of humans and animals is not novel, quantification by genetic analysis over time is.[32,33,53,54] Most studies of exhaled breath examine volatile and nonvolatile compounds expressed over brief periods (seconds to minutes). Studies interrogating bacterial genetic material with PCR assays have either failed to test their system against the rigors of the ICU environment or continue to rely on invasive BAL techniques to acquire samples.[55,56] Other exhaled breath studies use short, minute-scale durations, increasing variability, and false-negative results.[57,58]

Our study has several limitations. This study only examines HMEs from patients with clinically suspected VAP and thus does not provide any information regarding EBCF in patients without suspicion. Additionally, in two cases, clinical laboratory detected *K pneumoniae* at low levels (< 10^3 CFU/mL) not present in either the EBCF or BALF by PCR. Whether this indicates a lack of PCR sensitivity or contamination of the clinical specimen cannot be determined. Whether all pathogens are carried equally in exhaled breath condensate under all patient conditions cannot be determined by this study design. RT-PCR of EBCF and BALF detected low number of copies of pathogenic DNA in several instances of negative cultures, suggesting greater sensitivity. While RT-PCR of EBCF and BALF correlate well, translating this to quantitative microbiologic results requires additional studies.

In conclusion, RT-PCR of EBCF appears adequately sensitive and specific to study the lung microbiota in critically ill patients who are ventilated. These results introduce the potential opportunity to diagnose VAP noninvasively in hours, rather than days, and may allow for early, targeted antibiotic therapy and the elimination of empirical therapy when PCR is negative. While these findings are preliminary and require additional investigation, they do provide substantial rationale for continued research in this area. Future application of this novel noninvasive approach to define normal and aberrant lung microbes in patients who are ventilated may ultimately improve the expediency and specificity of VAP diagnosis.

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Author contributions: A. K. M. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the analysis. A. K. M., P. R. N., J. M. J., R. J. I., and E. M. B. contributed substantially to study design; E. M. B. contributed to sample acquisition and analysis; A. K. M., P. R. N., J. M. J., R. J. I., and E. M. B. contributed to data analysis and interpretation; J. S. B., J. R.-K., and W. P. D. contributed to data interpretation; and A. K. M., J. S. B., J. R.-K., W. P. D., P. R. N., J. M. J., R. J. I., and E. M. B contributed to writing the manuscript.

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