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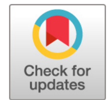

Dilution Factor of Quantitative Bacterial Cultures Obtained by Bronchoalveolar Lavage in Patients with Ventilator-Associated Bacterial Pneumonia

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ABSTRACT Ventilator-associated bacterial pneumonia (VABP) is a difficult therapeutic problem. Considerable controversy exists regarding the optimal chemotherapy for this entity. The recent guidelines of the Infectious Diseases Society of America and the American Thoracic Society recommend a 7-day therapeutic course for VABP based on the balance of no negative impact on all-cause mortality, less resistance emergence, and fewer antibiotic treatment days, counterbalanced with a higher relapse rate for patients whose pathogen is a nonfermenter. The bacterial burden causing an infection has a substantial impact on treatment outcome and resistance selection. We describe the baseline bronchoalveolar lavage (BAL) fluid burden of organisms in suspected VABP patients screened for inclusion in a clinical trial. We measured the urea concentrations in plasma and BAL fluid to provide an index of the dilution of the bacterial and drug concentrations in the lung epithelial lining fluid introduced by the BAL procedure. We were then able to calculate the true bacterial burden as the diluted colony count times the dilution factor. The median dilution factor was 28.7, with the interquartile range (IQR) being 11.9 to 53.2. Median dilution factor-corrected colony counts were 6.18 $\log_{10}$(CFU/ml) [IQR, 5.43 to 6.46 $\log_{10}$(CFU/ml)]. In a subset of patients, repeat BAL on day 5 showed a good stability of the dilution factor. We previously showed that large bacterial burdens reduce or stop bacterial killing by granulocytes. (This study has been registered at ClinicalTrials.gov under registration no. NCT01570192.)

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KEYWORDS bacterial burden, bronchoalveolar lavage, ventilator-associated bacterial pneumonia

The practice guidelines for hospital-associated bacterial pneumonia (HABP)/ventilator-associated bacterial pneumonia (VABP) recently released by the Infectious Diseases Society of America and the American Thoracic Society (1) recommend a 7-day duration of antibiotic therapy, irrespective of the causative pathogen(s). This recommendation is primarily based on the prior work of Chastre et al. (2), who found no significant difference in mortality between 8 versus 15 days of therapy (all-cause mortality, 18.8% versus 17.2%; $P =$ not significant) in a randomized, double-blind (until day 8) trial comparing 8 versus 15 days of therapy for bronchoalveolar lavage (BAL)-proven VABP. There was a statistically significant increase in the rate of relapse (40.6% versus 25.4%; 90% confidence interval for between-group risk difference, 3.9 to 26.6) in patients in the group infected with either *Pseudomonas aeruginosa* or *Acinetobacter* spp. (nonfermenter bacteria) receiving therapy for 8 days. However, there was also a significantly lower rate of resistance emergence (42.1% versus 62.3%; $P < 0.04$) and there was a higher number of antibiotic-free days (18.4 ± 8.0 versus 15.3 ± 8.4 days; $P = 0.01$) in the group receiving therapy for 8 days.

Given the high rate of relapse in both therapy duration groups, it is imperative to critically examine the indices of therapy intensity that may have a salutary impact on these relapse rates. The goal of antibiotic therapy is to drive the organism burden to eradication or reduce it to a point where the patient's immune system can clear the remaining organisms. We have shown previously in a murine pneumonia model that the capacity of granulocytes to clear microbes from an infection site can be saturated or overwhelmed when the bacterial burden is large (3). We have also demonstrated (4) with the aminoglycoside plazomicin that early aggressive therapy sufficient to substantially reduce the bacterial burden to below 5 $\log_{10}$(CFU/g) allows the granulocytes to regain bacterial killing function, resulting in a 1.0- to 1.5-$\log_{10}$(CFU/g) additional decline in the bacterial burden in the absence of further antimicrobial therapy. Having aggressive or immediately adequate antimicrobial therapy may then play a critical role in improving the outcome (5), decreasing rates of relapse, and shortening the time on mechanical ventilation.

One of the pieces of information, other than microbiology laboratory pathogen susceptibility data, that may help define adequate therapy is the concentration of the antibiotic(s) chosen for initial therapy at the primary infection site (here, at the primary infection site in patients with VABP). We have recently examined patients with suspected VABP who then had a BAL as an admission criterion for the study. In this set of patients, besides quantitating the number of pathogens in BAL fluid, we also determined the urea concentration in BAL fluid and plasma. This allowed us to calculate the dilution factor introduced by the BAL. This information allows the real baseline bacterial burden to be calculated. It also provides important information regarding the amount of bacterial cell kill required for granulocytes to regain their bacterial killing function.

RESULTS

BAL fluid samples were available for 43 patients who had a BAL for study entry qualification on day 1, of whom 33 had an organism recovered at $\geq$10,000 colonies/ml. The recovered organisms are displayed in Table 1. As part of the protocol, enrolled and treated patients received a second BAL for evaluation of drug penetration into the epithelial lining fluid (ELF) whenever possible. BAL fluid samples were available for 20 patients to assess the dilution factor on day 5 versus that on day 1.

The dilution factor for the number of recovered organisms, along with the corrected (by the dilution factor) colony counts, is shown in Table 2. The day 5 mean dilution factor ($n = 20$) was 115, the median dilution factor was 47.6, the standard deviation was 185, and the 25th percentile and 75th percentile values were 17.2 and 123, respectively. From day 1, the same 20 patients had a mean dilution factor of 116, a median dilution

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TABLE 1 Organisms and bacterial burden from patients undergoing BAL

Organism	No. of patients	No. of patients from whom the organism was recovered at ≥10,000 colonies/ml (diluted)/total no. tested
Total	43	33/43
Gram-positive bacteria		
Staphylococcus aureus		6/7
Methicillin sensitive	4	
Methicillin resistant	3	
Streptococcus pneumoniae	1	1/1
Other streptococci	1	0/1
Gram-negative bacteria		
Pseudomonas aeruginosa	17	13/17
Enterobacter spp.		3/4
Enterobacter cloacae	3	
Enterobacter aerogenes	1	
Stenotrophomonas maltophilia	3	2/3
Klebsiella pneumoniae	2	2/2
Serratia marcescens	2	2/2
Escherichia coli	2	0/2
Hafnia alvei	2	2/2
Proteus vulgaris	1	1/1
Candida albicans	1	1/1

factor of 29.4, a standard deviation dilution factor of 189, and 25th and 75th percentile dilution factors of 18.3 and 103, respectively.

The correlation between the day 1 and day 5 dilution factors is shown in Fig. 1. The regression line was log(day 5 dilution factor) = 0.851 × log(day 1 dilution factor) + 0.299 (r^2 = 0.714; P = 0.000003). This and the data in Table 2 demonstrate that the dilution factor range is broad but relatively stable over the first 5 days of therapy.

DISCUSSION

The treatment of VABP remains a challenge. Ultimately, patient survival is the single most important variable, but VABP is associated with several other outcomes that are also important. As noted above (2), there is a very high rate of resistance emergence during therapy that is altered by the duration of antimicrobial therapy, with the rate of resistance emergence ranging from 42.1% with 8 days of antibiotic therapy to 62.3% with 15 days of treatment. The rate of relapse is also substantial, particularly for those infected with Gram-negative bacilli.

Understanding the bacterial burden at the baseline may be important in further limiting resistance emergence and in minimizing relapses. The likelihood of resistance emergence is altered by the presence of primary resistant mutants in the bacterial population. It is also determined by the drug(s) chosen for therapy; by the frequency of mutation of the pathogen to resistance to the antimicrobial agent(s), which is also

TABLE 2 Dilution factor introduced by BAL and the dilution factor-corrected baseline bacterial burden in patients with ventilator-associated pneumonia on day 1

Parameter	Dilution factor (n = 33)	Dilution factor-corrected no. of $\log_{10}$(CFU/ml) (n = 17)[a]
Mean	85.2	5.88
Median	28.7	6.18
SD	153	0.68
25th percentile	11.9	5.43
75th percentile	53.2	6.46

[a]Number of patients whose baseline bacterial burden (undiluted) of Gram-negative pathogens was ≥10^4 and for whom a dilution factor was also calculated on day 1.

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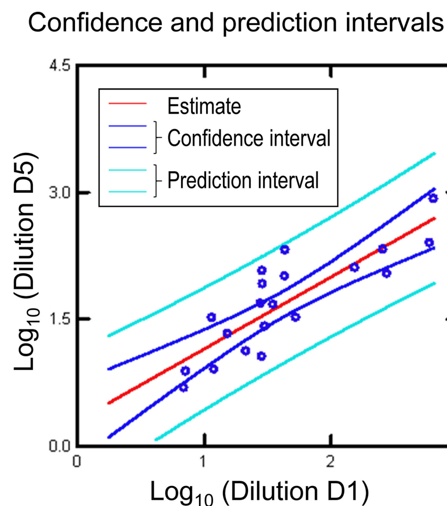

FIG 1 Regression between day 1 (D1) and day 5 (D5) dilution factors caused by BAL ($n = 20$). The lower and upper 95% confidence interval about the regression line and the 95% prediction interval (which refers to the observations) are indicated. The regression line was log(day 5 dilution factor) = 0.851 × log(day 1 dilution factor) + 0.299 ($r^2 = 0.714$; $P = 0.000003$).

influenced, for example, by whether the organism is a hypermutator; and finally, by the size of the total bacterial burden (6, 7).

Quantifying the size of the bacterial burden allows the physician to make rational decisions regarding the ultimate choice of therapy after the empirical phase. Table 2 demonstrates that when the bacterial burden in BAL fluid determined by quantitative culture was $\geq 10^4$ CFU/ml, accounting for the dilution factor disclosed that 50% of patients had an actual baseline bacterial burden in ELF of $\geq 10^{6.2}$ CFU/ml. When placed in the proper clinical perspective, our data demonstrate that a high percentage of patients with VABP have a baseline bacterial burden that exceeds the inverse of the frequency of mutation to resistance. The mutational frequency is often in the range of $1/10^7$ to $1/10^8$ (8). These patients both have a high concentration of organisms in the sampled area and, in addition, often have a substantial (multilobar) involvement of their pneumonic process. As the amount of lung involvement increases, the total bacterial burden increases and the number of less susceptible bacteria in the population at the baseline increases. These patients are at a particularly high risk for the emergence of resistance to an antibiotic administered as monotherapy and may benefit most from a combination chemotherapeutic approach.

In addition, in order to extubate patients quickly (minimize the number of ventilator days), have them leave the intensive care unit (ICU) (minimize the number of ICU days), and lower the rate of relapse, particularly in the treatment of VABP caused by Gram-negative nonfermenter bacteria, we need to maximize the rate and extent of killing of the infecting pathogen. To accomplish this goal, optimization of the host defense is also necessary. Clearly, granulocytes are a major line of defense in this circumstance. It has previously been demonstrated (3, 4) that high concentrations of bacteria can saturate granulocytes to overwhelm their antimicrobial functions and suppress their killing of infecting pathogens. Adequate antimicrobial chemotherapy has demonstrated (Fig. 2) an ability to reduce the saturation of granulocyte antimicrobial functions and allow the granulocytes to add >1 $\log_{10}$(CFU/g) kill over 24 h in the absence of further drug therapy (4). In the example shown in Fig. 2, the investigational aminoglycoside plazomicin was administered to mice with *P. aeruginosa* pneumonia using a dosing algorithm which humanized the pharmacokinetic profile of the drug in the plasma of the mice for the first 24 h of the experiment, at which time the first cohorts of mice were sacrificed for quantitative culture of lung tissue specimens. Other cohorts

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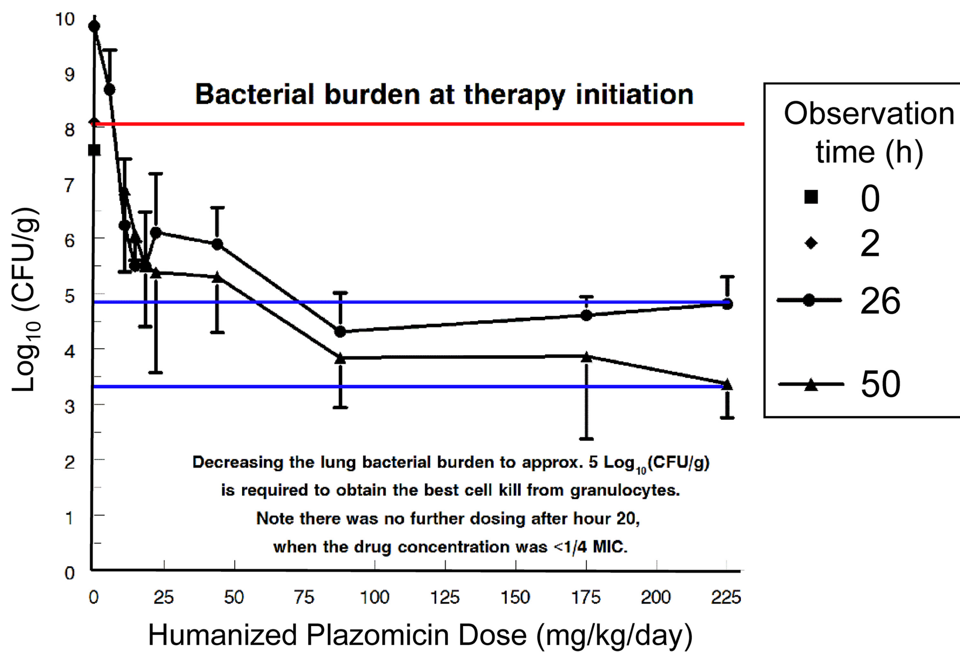

FIG 2 Demonstration of bacterial clearance attributable to granulocytes after adequate chemotherapy results in the decline of the bacterial burden to a point that returns the antimicrobial capability of the granulocytes. Blue lines, the bacterial burdens at hours 26 and 50. The difference between the lines is due to bacterial cell kill by granulocytes alone. Error bars represent standard deviations. Modified from the *Journal of Infectious Diseases* (4) with permission of the publisher.

of mice were observed for an additional 24 h without further antibiotic therapy before quantitative cultures of their lung tissue specimens were assessed. With this dosing algorithm, the residual drug concentration was approximately 1/4× MIC (MIC = 2 mg/liter for plazomicin) for the infecting pathogen (*Pseudomonas aeruginosa*) at the time with no further antimicrobial therapy. The two blue horizontal lines in Fig. 2 show the maximal kill obtained at 24 h of therapy (hour 26 of the experiment) and 24 h later (hour 50 of the experiment). This difference is due to the return of granulocyte function and is maximal when the bacterial burden is reduced to less than 5 $\log_{10}$(CFU/g).

Given that 25% of our patients had dilution factor-corrected bacterial burdens that exceeded 6.46 $\log_{10}$(CFU/ml) prior to initiation of antimicrobial therapy, the initial therapeutic choice should generate a minimum kill of 1.5 to 2.0 $\log_{10}$(CFU/ml). Maximization of antimicrobial therapy is most critical in patients with the largest actual bacterial burden to maximize the probability of attaining this magnitude of initial pathogen kill. Obtaining this goal has the possibility of helping suppress the emergence of resistance, lead to earlier extubation, and (it is hoped) diminish the possibility of a relapse. Determination of the true bacterial burden may be a helpful tool to understand the results of disappointing clinical trials in patients with VABP and design better interventions. Measurement of BAL fluid and serum urea concentration should be considered for future studies of VABP. The clinical characteristics of the patients or the technical aspects of BAL fluid samples with altered dilution factors also need further exploration.

MATERIALS AND METHODS

A brief description of the study is found in the supplemental material. The trial identifiers are ClinicalTrials.gov identifier NCT01570192, DMID protocol number 10-0060, and EudraCT registration number 2012-003483-46. For this study, we analyzed the BAL fluid sample that qualified patients for study inclusion and a repeat BAL fluid sample that was obtained on approximately day 5 for drug concentration measurement. (Each participating institution had the protocol evaluated and approved by their local institutional review board.)

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Bronchoalveolar lavage. A fiberoptic bronchoscope (FOB) was positioned so that the tip of the scope could be wedged in a distal airway. The sampling area was selected on the basis of the location of the radiographic infiltrate. In patients with diffuse pulmonary infiltrates or minimal new changes in a previously abnormal chest radiograph, sampling was directed to the area where endobronchial abnormalities were maximal. An initial aliquot of 20 to 30 ml was instilled, aspirated, and discarded. Serial 50-ml aliquots of sterile saline were administered through the suction channel of the FOB using a sterile syringe. The same syringe was used to aspirate back each BAL fluid aliquot. The pooled second and third aliquot samples constituted the BAL fluid sample (9).

Quantitative culture methodology. Bacterial culture of the pooled BAL fluid sample was performed according to the routine clinical protocol. A cytocentrifuged sample of BAL fluid was Gram stained. Aerobic cultures were performed by serial dilution or calibrated loop techniques. Bacteria present at a concentration of 1×10^3 CFU/ml BAL fluid specimen or higher were identified.

Urea instrumentation summary. Determination of the urea concentration was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a Prominence high-performance liquid chromatography (HPLC) column (Shimadzu) and an API5000 triple-quadrupole mass spectrometer (AB Sciex). Separation was achieved using a Gemini NX C_{18} 150- by 4.6-mm (particle size, 4-μm) HPLC column (Phenomenex) at 40°C with a run time of 5 min. The mobile phases consisted of water (mobile phase A) and methanol (mobile phase B), which were injected at a flow rate of 0.750 ml/min in the isocratic mode at 20% mobile phase B with an injection volume of 10 μl.

The mass spectrometer was operated in the positive-ion mode using a turbo ion spray (TIS) probe interface. A multiple-reaction-monitoring (MRM) *m/z* 60.1/44.0 was used for urea, and an MRM *m/z* 62.124/45.0 was used for the labeled internal standard, [$^{15}N_2$]urea. The parameters for the API5000 mass spectrometer (arbitrary units) were as follows: CAD (collision cell gas setting), 6; CUR (curtain plate gas setting), 30; GS1 (gas 1; nebulizer gas setting), 60; GS2 (gas 2; auxiliary gas setting), 60; IS (IonSpray voltage), 5,500; TEM (temperature of heater gas), 650°C; MRM, *m/z* 60.1/44.0; DP (declustering potential), 60; CE (collision cell energy), 28; CXP (collision cell exit potential), 15; dwell time, 200 ms; MRM, *m/z* 62.124/45.0; DP, 101; CE, 27; CXP, 10; dwell time, 200 ms. Calculated concentrations were performed using Analyst software v1.6.2 (ABSciex, Redwood City, CA).

Analysis of plasma urea concentrations in patient samples. A plasma sample for measurement of the urea concentration was collected from each patient at about the same time that the BAL was conducted. This sample was centrifuged and stored at −80°C until it was assayed for urea concentrations. Prior to analysis, all samples were thawed at room temperature. Once they were thawed, the samples were vortexed for 1 min, followed by centrifugation for 10 min at $0.835 \times g$. Twenty-five microliters of each sample was added to a 1.7-ml microcentrifuge tube, followed by the addition of 10 μl of the [$^{15}N_2$]urea internal standard (25 μg/ml in water) and 100 μl of LC-MS-grade water. The tubes were then capped, vortexed well for 1 min, and centrifuged at $16{,}168 \times g$ for 10 min. A 25-μl aliquot of the sample supernatant was then transferred to a 96-well plate, followed by the addition of 500 μl of LC-MS-grade water. The plate was then covered and vortexed gently for 1 min, prior to loading of a sample onto the LC-MS/MS system for analysis using a 10-μl injection volume.

Linearity of the urea concentration in plasma. The linearity of the urea concentration in plasma over a range of 5.00 to 500 μg/ml was demonstrated for each calibration curve over 3 separate runs, with a correlation coefficient (R) of ≥0.9994 and a linear regression coefficient (R^2) of ≥0.9987. The within-run accuracy as well as the between-run accuracy for each calibration curve was within ±10% of the nominal concentrations, and the respective coefficients of variation of the mean values were <0.1%. The calibration curve precision range was 0.7% to 4.3% within runs and 0.1% to 5.7% between runs. The within-run and between-run accuracies of quality control (QC) samples were within ±10% of the nominal concentrations, and the coefficients of variation were <0.7% of the mean values. For the QC samples, the within-run precision ranged from 0.4% to 8.2% and the between-run precision ranged from 0.2% to 9.2%.

BAL fluid urea concentration analysis. The method used to analyze the urea concentration in BAL fluid was the same as that described above for plasma. The calibration curves for the urea concentration in BAL fluid were linear over a range of 0.500 to 100 μg/ml, with a correlation coefficient (R) of ≥0.9989 and a linear regression coefficient (R^2) of ≥0.9978. The accuracy for each calibration curve was within ±15% of the nominal concentrations, and the respective coefficients of variation of the mean values were <15%. Calibration curve precision ranged from 0.2% to 9.7%. For the QC samples, accuracy was within ±10% of the nominal concentrations, and the respective coefficients of variation of the mean values were <5%. The precision for the QC samples ranged from 1.2% to 5.3%.

The dilution factor was calculated as described by Rennard et al. (10).

Statistics. The dilution factor was calculated as the estimate of the urea concentration in plasma divided by the estimate urea concentration measured in BAL fluid. All statistical evaluations were performed with the SYSTAT program for Windows (v13.0).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01323-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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