Online data supplement

Persistent Infection with *Pseudomonas aeruginosa* in Ventilator-associated Pneumonia

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METHODS

Study population

The study protocol was reviewed and approved by the Institutional Review Board of the State University of New York at Buffalo. All mechanically ventilated patients for at least 72 hours and a clinical picture compatible with pneumonia were screened for enrollment. The diagnosis of pneumonia was suspected when a new or progressive infiltrate were present along with at least two of the following signs and symptoms: 1) purulent respiratory secretions; 2) fever, defined as body temperature ≥ 38^{o}C or hypothermia defined as body temperature ≤ 35^{o}C; and/or 3) leukocytosis defined as WBC≥ 10,000/mm^{3} or leukopenia with total white blood cell count < 4,500/mm^{3} or > 15% immature neutrophils (bands) regardless of total peripheral WBC. Written informed consent was obtained from all subjects or their legal representatives. Only patients with first episode of *Pseudomonas aeruginosa* VAP defined as bacterial growth of 10^{4} colony forming unit (CFU)/ml or more from bronchoalveolar lavage (BAL) were enrolled. Exclusion criteria included polymicrobial infection and discordant antimicrobial therapy. For the purpose of this study, discordant antimicrobial therapy was defined as the use of an antibiotic to which an isolate recovered from BAL fluid was not susceptible *in vitro*. In all patients, tidal volumes were maintained as 6–8 mL·kg^{-1} using a pressure-controlled or pressure-support mode. Positive end-expiratory pressure (PEEP) levels were set according to a strict protocol, in which optimal PEEP was defined as the lowest level of PEEP with maximum PaO_2.

Data Collection

Clinical data recorded on study enrollment included age, gender, reasons for mechanical ventilation, duration of mechanical ventilation before study onset, prior antibiotic therapy, temperature, leukocyte count, ratio of PaO2/FIO2, time to first antibiotic dose from VAP onset, the Acute Physiology and Chronic Health Evaluation II score (E1), and the Multiple Organ Dysfunction Score (MODS) (E2).

Collection and processing of respiratory specimens

A BAL was obtained at time of suspected VAP onset prior to antimicrobial therapy and was repeated on day 8 after completion of antibiotic therapy. One-half the collected samples was sent for microbiology processing, and the rest was filtered through two layers of sterile gauze and centrifuged at 500 x *g* for 10 min at 4°C to separate the supernatants from the cell pellet. The cell free supernatant was stored in small aliquots at –70°C for further analysis. Cells were resuspended in phosphate-buffered saline and counted by means of a Neubaeur chamber. The viability of the cells was assessed by trypan blue. Differential cell counts were performed on cytopsin preparations stained with a modified Giemsa-based Diff-Quick stain (Baxter Scientific Products; McGraw Park, IL). BAL fluid total proteins levels were measured by a modified Lowry assay (E3).

To assess the clonal distribution of *P. aeruginosa* isolates, DNA typing was conducted on all samples obtained at VAP onset using repetitive-element based PCR (rep-PCR) (E4). In brief, extraction of DNA from *Pseudomonas aeruginosa* grown overnight in Luria–Bertani broth was performed with QIAamp DNA Mini kits (QIAGEN, Inc., Valencia, Calif.) according to manufacturer's instructions. Genomic

DNA was measured at A_{260} using the Genequant Pro Calculator (Amersham Biosciences) and diluted to a final concentration of 30 ng μl^{-1}. DNA was amplified by rep-PCR methodology using the DiversiLab Pseudomonas Kit for DNA. 50ng of genomic DNA, rep-PCR primer mix, rep-PCR master mix, 2.5 units of AmpliTaq DNA polymerase, and 2.5 uL 10X PCR Buffer (Applied Biosystems, Foster City, CA) were added for a total of 25 uL per reaction. The thermal cycling parameters were set at: initial denaturation of 94°C for 2 min; followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 70°C for 90 sec; and a final extension at 70°C for 3 min. The DNA amplicons were analyzed using the DiversiLab System which includes fragment separation using microfluidics lab-on-a-chip technology and the 2100 Bioanalyzer. Analysis was performed with the web-based software (beta v. 3.0) using the Pearson correlation coefficient to determine distance matrices and Unweighted Pair Group Method with Arithmatic Mean (UPGMA) to create dendrograms. Sample relationships were designated as follows: indistinguishable – no band differences, similar – one band difference, and different – two and greater band differences.

After end of therapy, patients were considered to have a relapse of VAP when a second episode of VAP occurred by a bacterial strain of similar serotype to the first VAP episode, otherwise it was considered a superinfection (E5).

Analysis of type III secretory protein phenotype

P. aeruginosa isolates were cultured under TTSS-inducing conditions in MIN-S medium (E6). Cultures were incubated with shaking overnight at 37 °C prior to dilution to OD_{600} of 0.1 in fresh MIN-S medium and cultured for further 5 h. Bacterial cells were

harvested by centrifugation and the supernatant removed. Cell-free supernatant from each sample was concentrated using Centricon tubes (MWCO 10 KDa; Millipore, MA). Concentration of protein in all preparations was determined by the Biorad Dc protein quantification kit (Biorad; Hercules, CA). Standardized protein concentrations (20 μg) were loaded onto 12.5% Tris polyacrylamide gels (Biorad; Hercules, CA) and run under denaturing conditions. Polyacrylamide gels were transferred to PVDF membrane and immunoblotted with anti-PcrV, anti-ExoS or anti-ExoU as previously described (E7).

Cytotoxicity assay

PMNs were collected from whole blood obtained by venipuncture from healthy volunteers and purified by density gradient centrifugation. PMNs were washed twice and resuspended to 10^7/ml in modified HEPES-buffered saline. The viability of PMNs, which was determined by trypan blue staining, was more than 95%.

Each of the clinical isolates was cultured overnight in LB medium. Bacterial cells were pelleted and washed three times in sterile PBS, diluted to an OD_{600} of 0.1 and regrown in LB medium for a further 1.5 h. Following this, cultures were harvested, washed with Lactated Ringers and finally resuspended in 100 μl Ringers:PBS solution (2:1 ratio by volume). The viability of PMNs in the presence of bacterial isolates was assessed by conincubation of samples containing 5 x 10^6 CFU/ml of *Pseudomonas aeruginosa* and 5 x 10^6 PMNs/ml. Cytotoxicity was assayed 2 h post infection by lactate dehydrogenase release using the Cyto Tox96® kit (Promega; San Luis Obispo, CA) according to the manufacturer's instructions.

Neutrophil apoptosis

Neutrophil apoptosis was assessed by light microscopy (×200) analysis of cytospin cells stained with Wright's Giemsa method and identification of nuclear changes (condensation of chromatin and simplification of nuclear structure) characteristic of apoptosis (E8, E9). Two blinded investigators assessed the percentage of neutrophil apoptosis on cytospin preparations by analyzing 500 cells per slide each. The analysis was performed on two different slides from the same patient. Data were reported as the percentage of apoptotic cells. The percentage was obtained by using the mean value obtained by the two investigators. To validate the light microscopic method of assessment of neutrophil apoptosis, we used a second independent method based on Annexin V binding with quantification by flow cytometry. In brief, cell pellets were washed twice in PBS at pH=7.2 and incubated on ice with FITC-anti-CD15 and PE-anti-CD45 for 15 min. Allophycocyanin-annexin V (R&D Systems, Inc., Minneapolis, MN, USA) was then added for 15 min. After dilution in PBS (500 µl), samples were incubated with 5µl of 7-Amino Actinomycin D (7-AAD) at room temperature for 15 min and analyzed immediately by flow cytometry (FACStar; Becton Dickinson, Mountain View, CA, USA). PMN were identified on the CD15/side scatter (SSC) dot plot. Apoptotic PMNs were identified by the distribution of allophycocyanin-annexin V and 7-AAD pattern (annexin V^+, 7-AAD^-) (figure E1). The extent of neutrophil apoptosis was compared with the percentage of neutrophil apoptosis determined by nuclear morphology and light microscopy (linear regression slope 0.79, p=0.03; n =6). These results confirm the validity of Wright's Giemsa staining to assess apoptosis.

Neutrophil elastase

Elastase in bronchoalveolar lavage fluid was measured in duplicate by a commercial immunoenzymatic assay kit (PMN Elastase EIA™; Alpco Diagnostics, Windham, NH). The minimum detection level was 3.0 ng/mL. The maximum intra- and interassay coefficients of variation were 5.2% and 6.4%; respectively. A single technician who was blinded to the objectives of the protocol conducted all measurements.

Statistical analysis

Continuous variables were compared using unpaired Student's *t* test or the Mann-Whitney's *U*-test if the variables were not normally distributed. Categorical variables were compared using chi-square test with Yates correction or Fisher's exact test when necessary. Correlations were analyzed with Spearman's rank correlation. Parametric data are presented as mean ±SD and non-parametric data as median with 95% confidence interval or range. A p value of <0.05 was determined as significant. Calculations were carried out using SPPS 12.0.

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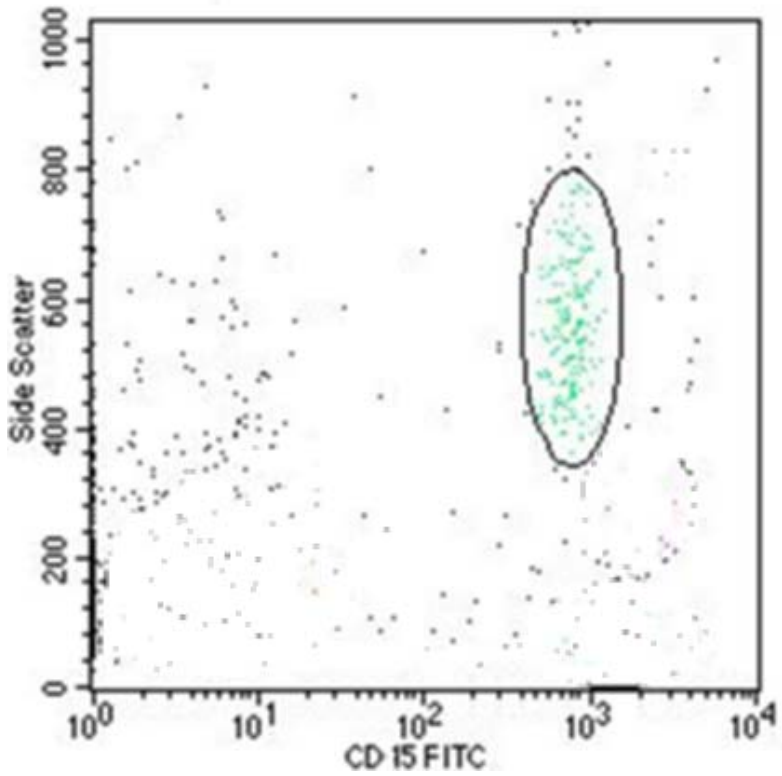
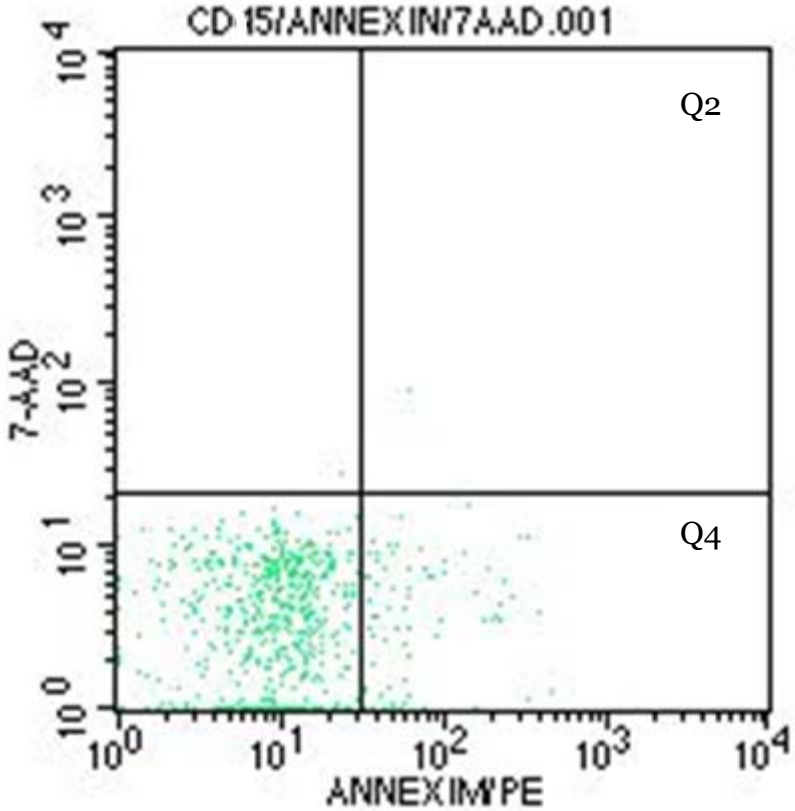
LEGENDS

Figure E1. Study of PMN apoptosis by flow cytometry. The fluorescence of anti-CD15 Ab was used to identify PMN as $CD15^+$ cells and to gate out other cells. A gate was drawn around the PMN population (*A*). Fluorescence analysis was performed on this gate. The combination of allophycocyanin-annexin V and 7-AAD distinguished between early apoptotic cells (Q4) and late apoptotic cells (Q2) (*B*).

(A)


Side Scatter
0
200
400
600
800
1000
10^0
10^1
10^2
10^3
10^4
CD 15 FITC

(B)


CD 15/ANNEXIN/7AAD.001
Q2
Q4
7-AAD
10^0
10^1
10^2
10^3
10^4
ANNEXIM PE