Title: Investigating Transfusion-Related Sepsis using Culture-Independent Metagenomic Sequencing

Running Title: Metagenomics for Investigating Septic Transfusions

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Key Points

Question: Can the assessment and prevention of transfusion-transmitted infections be enhanced by incorporating clinical metagenomic next generation sequencing?

Findings: We describe three septic transfusion cases, two of which involved fatalities, where culture-independent metagenomic sequencing of blood products afforded rapid and actionable assessment of pathogen identity, abundance and genetic relatedness.

Meaning:

Transfusion-transmitted infections continue to result in significant morbidity and are challenging to prevent and diagnose. We found that culture-independent metagenomic sequencing enhanced traditional hospital infection control strategies used to investigate septic transfusion events.

Abstract

Importance: Transfusion-related sepsis remains a major hospital infection control issue due to challenges with diagnosis, prevention and management. Novel, unbiased, culture-independent diagnostic approaches are needed.

Objective: To present a case series of septic transfusion events in which culture-independent metagenomic next generation sequencing (mNGS) of blood products afforded rapid and actionable assessment of pathogen identity, abundance and genetic relatedness.

Design, Setting and Participants: In this series of three septic transfusion cases, mNGS of direct clinical blood specimens was performed in addition to standard culture-based approaches for clinical investigation. Cases involved Gram-negative sepsis and stemmed from hospital infection control investigations at two tertiary care hospitals in Northern California. Pathogen detection leveraged ID-Seq, a new open-access bioinformatics portal. Novel approaches to distinguish environmental microbiota from relevant pathogens were developed. Phylogenetic analysis was performed to assess microbial relatedness and understand transmission events.

Main Outcomes and Measures: Detection of pathogens from patient blood samples and transfused products using culture-independent mNGS. Assessment of microbial genomic relatedness to verify transmission events using a combination of mNGS and sequencing of cultured bacterial isolates.

Results: mNGS of direct clinical blood specimens afforded precision detection of pathogens responsible for each case of transfusion-related sepsis, and enabled discovery of a novel *Acinetobacter* species in a platelet product that had become contaminated despite

photochemical pathogen reduction. In each case, longitudinal assessment of pathogen burden elucidated the temporal sequence of events associated with each transfusion-transmitted infection. We found that informative data could be obtained from culture-independent mNGS of residual platelet products and leftover blood specimens that were either unsuitable or unavailable for culture, or that failed to grow due to prior antibiotic administration. In addition, we developed new methodology to enhance the accuracy of mNGS-based assessment of transfusion-related pathogens that share taxonomic similarity to background microbial contaminants commonly found in metagenomic sequencing assays.

Conclusions and Relevance:

In each case, culture-independent metagenomic sequencing of blood products afforded rapid and actionable assessment of pathogen identity, abundance and genetic relatedness. Together, these challenging cases demonstrated the potential for mNGS to advance existing methods for investigating transfusion-transmitted infections.

Introduction

While transfusion-associated infections have globally decreased over the past 50 years, bacterial contamination of the platelet supply remains a significant public health challenge, with approximately one in 1,500 – 5,000 units containing detectable organisms[1–5]. Room temperature storage facilitates more bacterial growth in platelets compared to other blood products, with skin flora, asymptomatic donor bacteremia, and direct introduction from environmental sources accounting for the majority of contaminants[3,6]. Even though current culture-based screening approaches may miss a significant fraction of contaminated units, clinically reported sepsis occurs in only one in 15,000-100,000 transfusions, presumably due to frequent concurrent antibiotic administration, low bacterial inoculum, or attribution of transfusion-related outcomes to a patient's pre-existing infection or illness[3,7].

Pathogen-reduction of platelet products using amotosalen, a nucleic acid cross-linking agent activated by ultraviolet (UV)-A irradiation, has been routinely performed in Europe for over a decade and recently received United States Food and Drug Administration approval[8]. Photoinactivation is broadly effective against diverse bacterial pathogens, and while failure at high bacterial loads has been described, few reports of sepsis have been reported following treatment[9,10].

Here we describe three transfusion-related Gram-negative sepsis investigations including one involving a pathogen-reduced platelet product. For each, we performed metagenomic next generation sequencing (mNGS) in addition to standard microbiologic diagnostics and found that mNGS enabled rapid and precise taxonomic identification and phylogenetic analysis of bacterial isolates, as well as culture-independent assessment of direct clinical samples.

Methods

Ethics Statement

Investigations were carried out according to a no-subject-contact study protocol approved by the UCSF Institutional Review Board (IRB# 17-24056) which permitted analysis of de-identified leftover clinical microbiology samples from collaborating institutions and subsequent review of study subjects' electronic medical records.

Microbial culture

Bacterial cultures were inoculated into BacT/Alert (bioMérieux) blood culture bottles using standard clinical protocols. Species identification was performed using MALDI-TOF mass spectrometry (Bruker).

Sequencing and sample processing

Metagenomic sequencing was completed prior to bacterial isolate whole genome sequencing, to eliminate the possibility of contaminating metagenomic samples with isolate nucleic acid. All samples (culture-free plasma and blood product samples as well as cultured isolates) were inactivated by adding either DNA/RNA Shield (Zymo Research, Irvine, CA) or an extraction kit lysis buffer, and bashed with ceramic beads on a TissueLyser (Qiagen, Hilden, Germany) for either 2 minutes at $15s^{-1}$ or 1 minute at $30s^{-1}$. For case 1, metagenomic samples were extracted with the Zymo Quick-DNA/RNA Miniprep Kit and cultured isolate samples were extracted with the Zymo Quick DNA Fungal/Bacterial kit. Case 2 samples were extracted with the Zymo Quick-DNA/RNA Microprep Plus kit. For case 3, metagenomic samples were extracted with the Zymo Duet DNA/RNA kit and cultured isolate samples were extracted with the Qiagen AllPrep DNA/RNA kit on a Qiacube robot.

Ten to 100 ng of DNA from each sample was sheared with NEB fragmentase for 5 minutes (25 minutes for the case 3 isolates) and used to construct sequencing libraries with the NEBNext Ultra II Library Prep Kit. Adaptor ligated samples underwent between 12 and 18 cycles of amplification with dual unique indexing primers using the NEBNext Ultra II Q5 polymerase. Libraries were quantified with Qubit and quality checked with a Bioanalyzer High Sensitivity DNA chip. Samples were pooled and underwent paired end 150 base pair sequencing on an Illumina MiSeq or NextSeq 550. eTable 1 lists the number of reads obtained for each sample.

Bioinformatics and phylogenetic analyses

Detection, taxonomic identification, and abundance quantitation of microbes from raw sequencing reads was first performed using the IDseq pipeline according to described protocols[12]. To control for background environmental and reagent contaminants, no-template water control samples were incorporated alongside extracted nucleic acid and carried forward throughout library preparation and sequencing. Genome assembly was performed by first trimming the raw sequencing reads in fastq files using TrimGalore[17] and assembling using Unicycler[18] with default parameters. To identify the *Acinetobacter* species in case 1, BLAST+[19] was used to query the National Center for Biotechnology Information (NCBI) nt database with the assembled genomes, and related genomes were found using a Mash/Mini Hash search via PATRIC[20,21]. For case 3 samples, sequence type was determined using SRST2[22]; other *Klebsiella pneumoniae* sequences in NCBI databases belonging to the same sequence type were found using a Mash/Mini Hash search via PATRIC[20,21].

Phylogenetic analysis for case 1 and case 3 was used to determine the relationship between the samples in this study and the closely related NCBI samples. First, trimmed reads were aligned against a reference genome (case 1: *A. baumannii* CP017642.1, case 3: *K. pneumoniae* CP015392.1) using Snippy v4.3.6[23]. Then a SNP alignment was obtained by

variant calling using bcftools v1.9[24]. We filtered SNPs with QUAL<30, and filtered genotypes with major allele depth (FMT/AD) < 10 or major allele frequency (FMT/AF) < .9. Finally, the maximum likelihood phylogeny based on the SNP alignments was built in RAxML v8.2.12[25] using '-m ASC_GTRCAT --asc-corr=lewis' options.

Data Availability

Raw sequencing data is available via NCBI BioProject ID: PRJNA544865.

Results

Investigation #1: *Acinetobacter* septic platelet transfusion

Patient A, a man with relapsed acute lymphoblastic leukemia undergoing chemotherapy, received two doses of pathogen-reduced apheresis platelets on the day of planned home discharge. No side effects were experienced after transfusion of the first platelet unit, however after receiving the second unit two hours later, he developed chills and rigors followed by fever and critical hypotension. He received fluids and vancomycin before being transferred to the intensive care unit where meropenem, vasopressors, respiratory support and continuous renal replacement therapy were administered. He recovered fully and was discharged home 16 days later on parenteral antibiotics.

Blood cultures obtained 4.5 hours post-transfusion returned positive for *Acinetobacter calcoaceticus/baumanii (ACB) complex*. Residual material obtained from a saline rinse of the returned platelet bag also grew *ACB complex* as well as *Staphylococcus saprophyticus*. Culture of a non-transfused platelet co-component from the same donor, concurrently pathogen-inactivated with the transfused product five days earlier, was negative. At the time of the investigation, hypotheses considered included failure of the pathogen-reduction process, contamination of the pathogen-reduced platelet bag during transport, or pre-existing sub-clinical bacteremia in the patient.

To investigate this case further, we first sequenced the cultured *ACB* complex isolate from the patient's blood and the *ACB* complex isolate from a wash of the transfused platelet bag. In total, sequencing and analysis for this case was performed in 45 hours. *De novo* genome assembly revealed a novel *Acinetobacter* species most closely related (92% average nucleotide identity) to *A. nosocomialis* and *A. pitii,* members of the *ACB* complex **(Figure 1A)**. Phylogenetic analysis revealed zero single nucleotide polymorphisms (SNPs) over the 3.9 Mb genome between the novel *Acinetobacter* species cultured from the blood and from the platelet bag, indicating that the two isolates were identical.

Culture-independent mNGS detected a high abundance of *ACB complex* in the transfused product (230,000 reads per million, rpM) and in the patient's plasma following transfusion (190 rpM) **(Figure 1B).** By comparison, the untransfused co-components and patient's plasma before transfusion had very few alignments to *ACB complex*, in the range of expected background levels (0.25 and 2.2 rpM, respectively). Assessment of these low abundance alignments at the species level revealed that they most likely represented mis-assigned reads from other *Acinetobacter* species, notably *A. johnsonii*. More specifically, the co-components and pre-transfusion samples had only a small percentage of *Acinetobacter* genus reads that best aligned to an *ACB species*, while in the transfused product and post-transfusion samples, the vast majority of genus *Acinetobacter* reads aligned to *ACB species* (e**Figure 1A**). The orders-of-magnitude difference in percent reads mapping to *ACB complex*, along with the change in composition of *Acinetobacter* assignments between samples, indicated that only the transfused product and patient's post-transfusion plasma were contaminated by a species within the *ACB complex*.

Investigation #2: Fatal *Pseudomonas aeruginosa* septic transfusion

Patient B was admitted for management of acute on chronic heart failure and implantation of a right ventricular assist device. During surgery, the patient received three units of platelets, three units of fresh frozen plasma, and two units of cross-matched packed red blood cells (PRBCs). Intraoperatively, the patient became hemodynamically unstable. Cultures drawn three hours post-surgery ultimately returned positive for *Pseudomonas aeruginosa,* raising concern for a septic transfusion reaction (**Figure 2**).

Despite administration of antipseudomonal antibiotics, the patient's clinical stability continued to deteriorate, and he did not survive beyond post-operative day three. mNGS was performed on plasma samples collected pre- and post-transfusion from the patient, on aliquots from the blood culture bottles that eventually turned positive, and on residual packed red blood

cells (PRBCs) remaining following transfusion. No remaining material from the platelets or transfused plasma was available for mNGS or other diagnostic testing. Cultured bacterial isolates were unavailable for sequencing.

mNGS revealed no *P. aeruginosa* in the pre-transfusion plasma sample nor in the residual PRBCs, but identified a high abundance of *P. aeruginosa* in post-transfusion plasma (**Figure 2**) that decreased over time in the setting of antibiotic treatment. Serial measurement of procalcitonin demonstrated a normal pre-transfusion level (0.034 ng/mL, reference interval: <0.15 ng/mL) but significantly elevated concentrations following surgery (range: 42.73 to >100 ng/mL [above detection limit]). Phylogenetic analysis indicated strong relatedness to *P. aeruginosa* strain BWHPSA041.

Several environmentally ubiquitous *Pseudomonas* species are common contaminants of mNGS library preparation reagents and thus we used IDSeq[12] to determine the percent of *Pseudomonas* genus reads that mapped specifically to *P. aeruginosa.* In all six post-transfusion plasma samples, as well as the blood culture bottle samples, over 99% of genus *Pseudomonas* reads mapped best to *P. aeruginosa*, while in water controls an average of 7.2% (range 0-12.1%) mapped best to *P. aeruginosa* (e**Figure 1B).** This result indicated that the *P. aeruginosa* observed in the post-transfusion plasma samples was not the result of contamination from mNGS library preparation reagents.

Investigation #3: Fatal *Klebsiella* septic platelet transfusion

Two immunocompromised pediatric patients developed septic shock following transfusion of platelets derived from a single donor, as recently reported[6]. Patient C, who had undergone successful autologous hematopoietic stem cell transplantation, developed hypotension, tachycardia and vomiting 15 minutes into the transfusion. Despite initiation of vancomycin and cefepime, fluid resuscitation, vasopressor support and intubation, Patient C died within five hours. Blood cultures ultimately returned positive for *Klebsiella pneumoniae*, as did a culture from residual material in the platelet bag.

Five hours earlier, Patient D, who was receiving empiric cefepime for neutropenic fever, had undergone transfusion with the second platelet unit derived from the same donor. He decompensated into septic shock nine hours following transfusion but ultimately survived following fluid resuscitation and vasopressor support. Blood cultures remained negative in the setting of concurrent antibiotic treatment, initially precluding determination of whether sepsis was due to a *Klebsiella*-contaminated platelet transfusion or to another etiology.

Culture-independent mNGS of pre- and post-transfusion plasma or serum samples revealed a marked increase in *Klebsiella pneumoniae* following transfusion in both patients, although patient D, who had negative blood cultures, had 12.9-fold fewer *Klebsiella* rpM detected compared to patient C (**Figure 3**). Genome assembly and phylogenetic analysis revealed only one SNP across the 4.2 Mb core genome of *K. pneumoniae* among Patient C's plasma sample, Patient C's blood culture isolate, and the residual platelet bag culture isolate. Across approximately 2700 bases of the *K. pneumoniae* genome detected in Patient D's plasma with a read depth greater than 10, zero SNPs relative to the other three samples were identified. The sequence type of *K. pneumoniae* in all four samples was determined to be ST491, and the closest ST491 sequence on Genbank differed from these sequences by 212 SNPs.

Together these data confirmed that the platelet unit represented a single source of infection and that despite Patient D having negative blood cultures, both patients became bacteremic with the same strain of *K. pneumoniae* transfused from the contaminated platelet components. As previously reported, routine culture-based screening of the donor's platelets at 24 hours remained negative at five days, although an additional platelet unit from the same donor shipped to a different hospital and quarantined before transfusion grew *K. pneumonaie* that was highly related based on whole genome sequencing analysis[6].

Discussion

Bacterial contamination of platelet products remains an important and underrecognized hospital infection control challenge despite existing screening methods and psoralen-based pathogen-reduction strategies[1–5]. Rapid recognition of potential transfusion-associated sepsis can permit quarantine of untransfused co-components from a potentially contaminated supply chain and assist with root cause analysis. Traditionally, culture-based methods including pulsed-field gel electrophoresis and more recently, whole genome sequencing, have been the central diagnostic tools for septic transfusion investigations. Here, we found that culture-independent mNGS extended the utility of these methods by directly detecting pathogens from clinical samples to assess genetic relatedness, obtain precise strain information, and interrogate levels of pathogen in a patient's bloodstream throughout the course of a septic transfusion event.

In each case examined, mNGS provided actionable information that clarified the sequence of events resulting in transfusion-related sepsis. In case 1, for example, potential explanations considered included: 1) failure of the pathogen inactivation process; 2) contamination of the platelet bag from an environmental source after pathogen reduction; and 3) pre-existing occult bacteremia resulting in retrograde introduction of bacteria into the platelet bag during transfusion. Temporal mNGS assessment of patient plasma demonstrated abundant *Acinetobacter* DNA in post-transfusion samples and in washes of the transfused platelet bag, but not in any pre-transfusion samples, consistent with a septic transfusion event and not pre-existing bacteremia. The findings of high abundance *Acinetobacter sp.* in the transfused unit but not untransfused co-components suggested that contamination occurred following pathogen reduction treatment, potentially from an environmental source during product handling, transport or storage. Transfusion-related sepsis from pathogen-reduced platelet products has been reported but is extremely rare[9,10]. This case suggests that pathogen reduction technology should still be accompanied by rigorous infection control precautions during downstream processing, transport and storage.

In case 2, the possibility of an occult but developing bloodstream infection present prior to transfusion was also considered as a potential explanation for the patient's post-surgical sepsis. As in case 1, assessment of plasma samples collected before and after transfusion clarified the sequence of events and demonstrated that *P. aeruginosa* was only detectable post-transfusion. Evidence for a septic transfusion event was further corroborated by temporal measurement of procalcitonin, a host inflammatory biomarker with specificity for bacterial infection[13,14]**.** Post-transfusion procalcitonin levels above the upper limit of detection in the context of normal pre-surgical levels provided further evidence that a septic transfusion event had occurred. The absence of detectable *P. aeruginosa* in the transfused PRBC segments suggested that platelets or plasma may have been the source of contamination, although neither sample type was available for confirmation.

Discovery that the implicated pathogen in case 1 represented a novel species of *Acinetobacter* highlights the ability of sequencing-based diagnostics to extend our understanding of pathogens responsible for transfusion-related infections. Notably, the standard clinical microbiology workflow at the study site hospital involving culture, biochemical testing and matrix associated laser desorption ionization (MALDI) time of flight (TOF) mass spectrometry was unable to distinguish this *Acinetobacter* as a novel species.

All three cases notably demonstrated that mNGS afforded high resolution taxonomic identification without a need to isolate a pathogen in culture. This allowed for post-hoc analyses of banked clinical specimens obtained both pre- and post-transfusion that were either unsuitable or unavailable for culture. For instance, even though no cultured isolates were available in case 2, direct mNGS of leftover blood products allowed for precise identification of the most closely related *Pseudomonas aeruginosa* strain, which incidentally was recovered in 2013 from a patient's wound in Massachusetts, USA.

Confirming transmission of a pathogen during septic transfusion events is essential for hospital infection control, but in some cases is not possible because culture fails to identify a

microbe. This problem was highlighted by case 3, in which blood cultures from patient D remained negative despite the development of post-transfusion septic shock. Patient D was receiving a prophylactic antibiotic with activity against *Klebsiella*, which likely inhibited bacterial growth in culture, precluding definite confirmation of a septic transfusion event related to that experienced by Patient C. Culture-independent mNGS not only confirmed the presence of *K.* in the blood of both patients but also established that it was identical to the isolate derived from transfused platelet product. This unfortunate fatal transfusion case demonstrated the capability of mNGS to provide definitive confirmation and characterization of septic transfusion events in cases where culture fails to yield an isolate.

Acinetobacter and *Pseudomonas* are environmentally ubiquitous and common contaminants of mNGS and 16S rRNA gene sequencing library preparation reagents[11,16], and as such can add considerable complexity to investigations in which accurately assessing the abundance of transfused pathogens belonging to these genera is critical. To address this, we employed two complementary approaches that may be broadly useful for future investigations: 1) assessing compositional changes of species within the relevant genus as a proxy for environmental contamination, and 2) focusing analysis exclusively on the exact species implicated in the transfusion event. In case 1, for example, these approaches clarified that the novel *Acinetobacter* species was present only in the residual transfused product and in the patient's plasma following transfusion, but not in the other co-components nor in the patient's bloodstream prior to the event.

Rapid assessment of septic transfusion events is critical to ensure related contaminated products can be swiftly quarantined, and probable sources of contamination identified. We found that mNGS and pathogen analysis could be reliably performed in under 48 hours, faster than the turnaround time for blood culture at many institutions. Cost, time and infrastructure requirements currently make sequencing impractical at many healthcare institutions, however

new platforms such as the Illumina iSeq and Oxford Nanopore Minion will undoubtedly increase the broad applicability of this technology for rapid hospital epidemiologic investigations.

As genomic approaches become more widely used for investigating transfusion-related infections, rapid exchange of pathogen genomic information via open access databases could accelerate identification of related cases, enhancing infection control efforts of emerging outbreaks. Indeed, the findings described here have contributed to a multicenter United States Centers for Disease Control and Prevention investigation which has identified the novel *Acinetobacter sp*. from Patient A in related cases from Utah and Connecticut[15].

In summary, transfusion-related sepsis continues to cause excess mortality and morbidity despite the introduction of pathogen-reduction technologies. We found that culture-independent mNGS complemented current best available methods for investigation of transfusion-related sepsis by extending traditional whole genome sequencing-based phylogenetics of cultured isolates and by permitting longitudinal assessment of pathogen abundance pre- and post-transfusion from direct clinical specimens. Broader implementation of mNGS for both investigation and prevention of transfusion-related infections may enhance existing practices.

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Authorship Contributions

C.L., E.C., J.K. and S.M. wrote the paper

J.K. and L.L. performed phylogenetic analyses

C.L., E.C., J.K., L.L. and PC performed additional data analyses

A.N., A.N., D.Y., M.M., M.Z., N.T. and S.B. directed clinical aspects of the investigations

C.T. and J.D. directed molecular aspects of the investigations

A.L., B.P., C.L., E.C., P.H., and J.Q. performed sample extractions and metagenomic library preparation

M.P. served as clinical research coordinator

M.T. and R.S. performed sequencing and quality control

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Figure Legends

Figure 1. *Acinetobacter* septic transfusion investigation

A) Maximum likelihood phylogenetic tree based on SNP alignments demonstrates relatedness of the novel *Acinetobacter species* isolated from both patient A and the residual transfused platelet product relative to closely related species within the *Acinetobacter calcoaceticus/baumannii* (ACB) complex. **B)** Abundance of *ACB* complex in the transfused platelet product and co-components (left panel) and in patient A's plasma (right panel), determined by culture-independent metagenomic sequencing and measured in reads per million, rpM.

Figure 2. *Pseudomonas* septic transfusion investigation

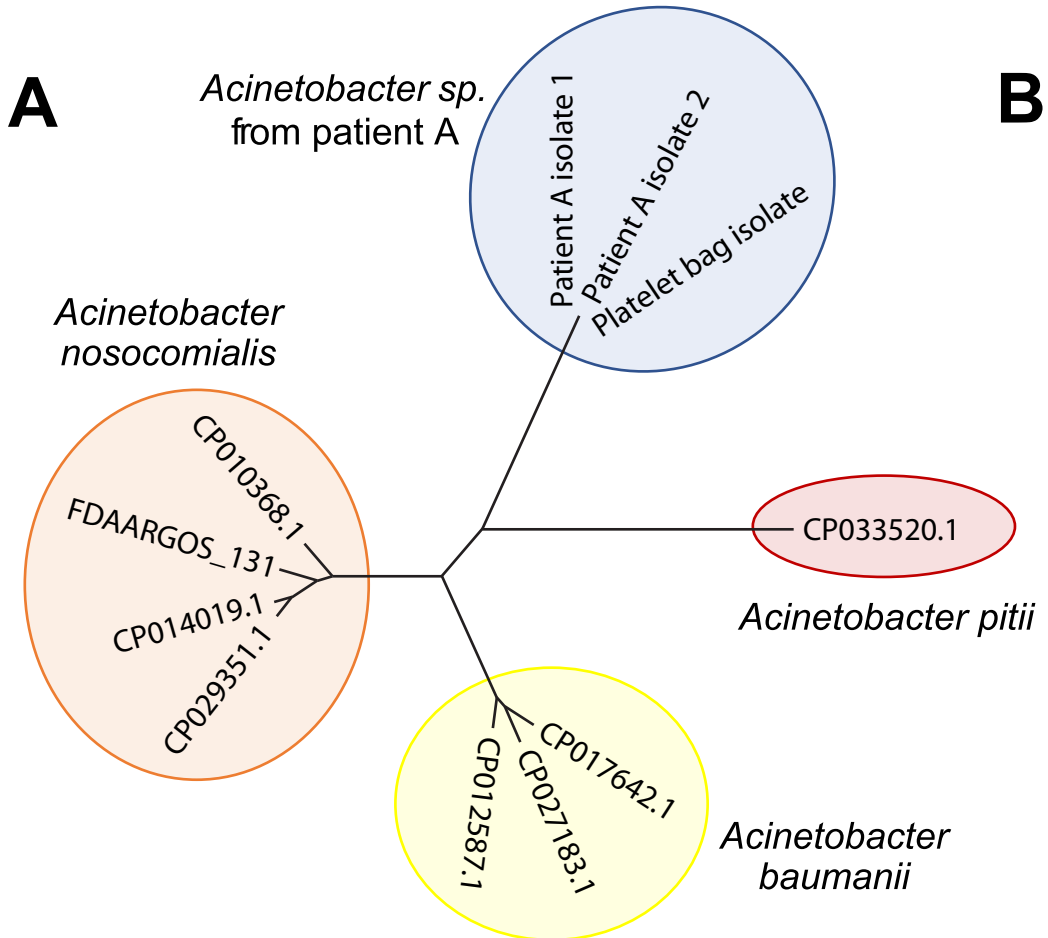
Abundance of *Pseudomonas aeruginosa* in patient B's plasma throughout the course of the fatal septic transfusion event, determined by culture-independent metagenomic sequencing and measured in reads per million (rpM, lower panel). Procalcitonin level (ng/mL) over the course of the septic transfusion event is plotted in the upper panel.

Figure 3. *Klebsiella* septic transfusion investigation

Abundance of *Klebsiella pneumoniae* in plasma from patients C (upper panel) and D (lower panel) during the course of related septic transfusion events, as determined by culture-independent metagenomic sequencing and measured in reads per million (rpM). Patient C, who did not survive the event, had post-transfusion blood cultures return positive for *K. pneumoniae* that was highly related (1 SNP across the 4.2 Mb core genome) to the *K. pneumoniae* isolated from the residual transfused platelet product. Patient D, who was receiving antibiotics with activity against *Klebsiella* prior to transfusion, survived, but had negative post-transfusion blood

cultures, precluding definitive confirmation of a related second septic transfusion event in the absence of culture-independent metagenomic sequencing.

Figure 1


A
Acinetobacter sp.
from patient A
Patient A isolate 1
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Platelet bag isolate
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CP010368.1
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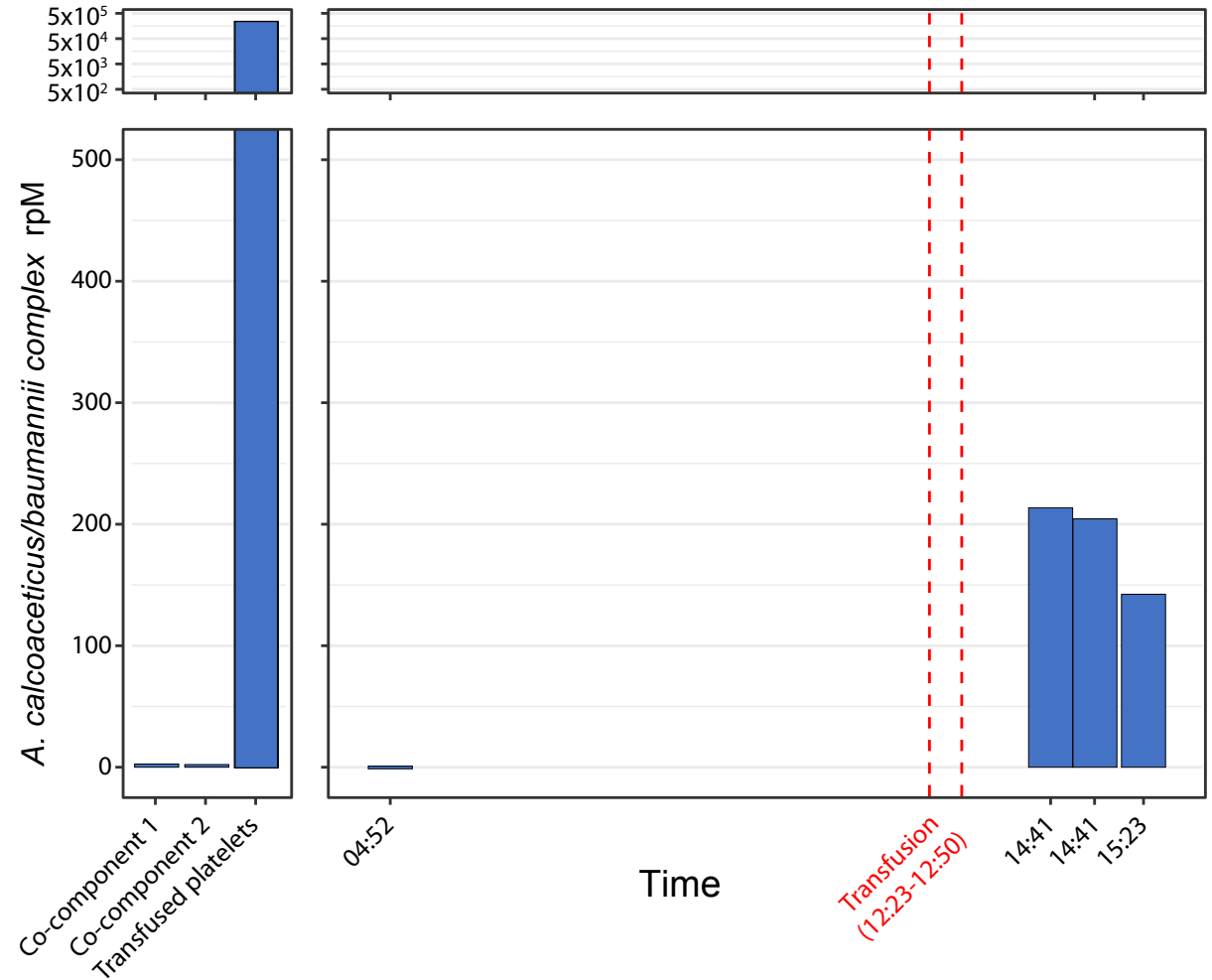
Acinetobacter
baumanii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A. calcoaceticus/baumannii complex rpM
Co-component 1
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04:52
Time
Transfusion
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14:41
15:23

Figure 2

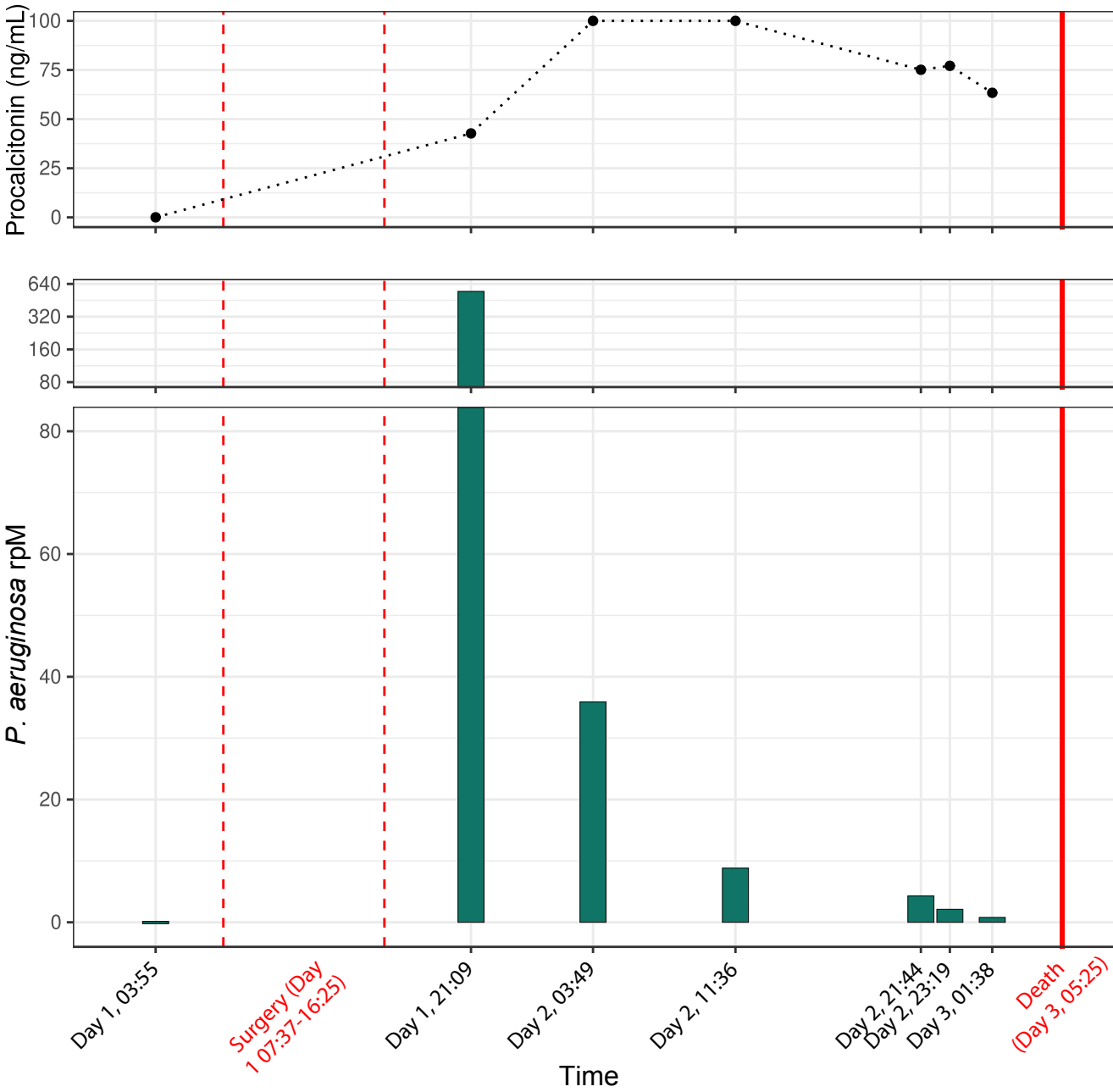
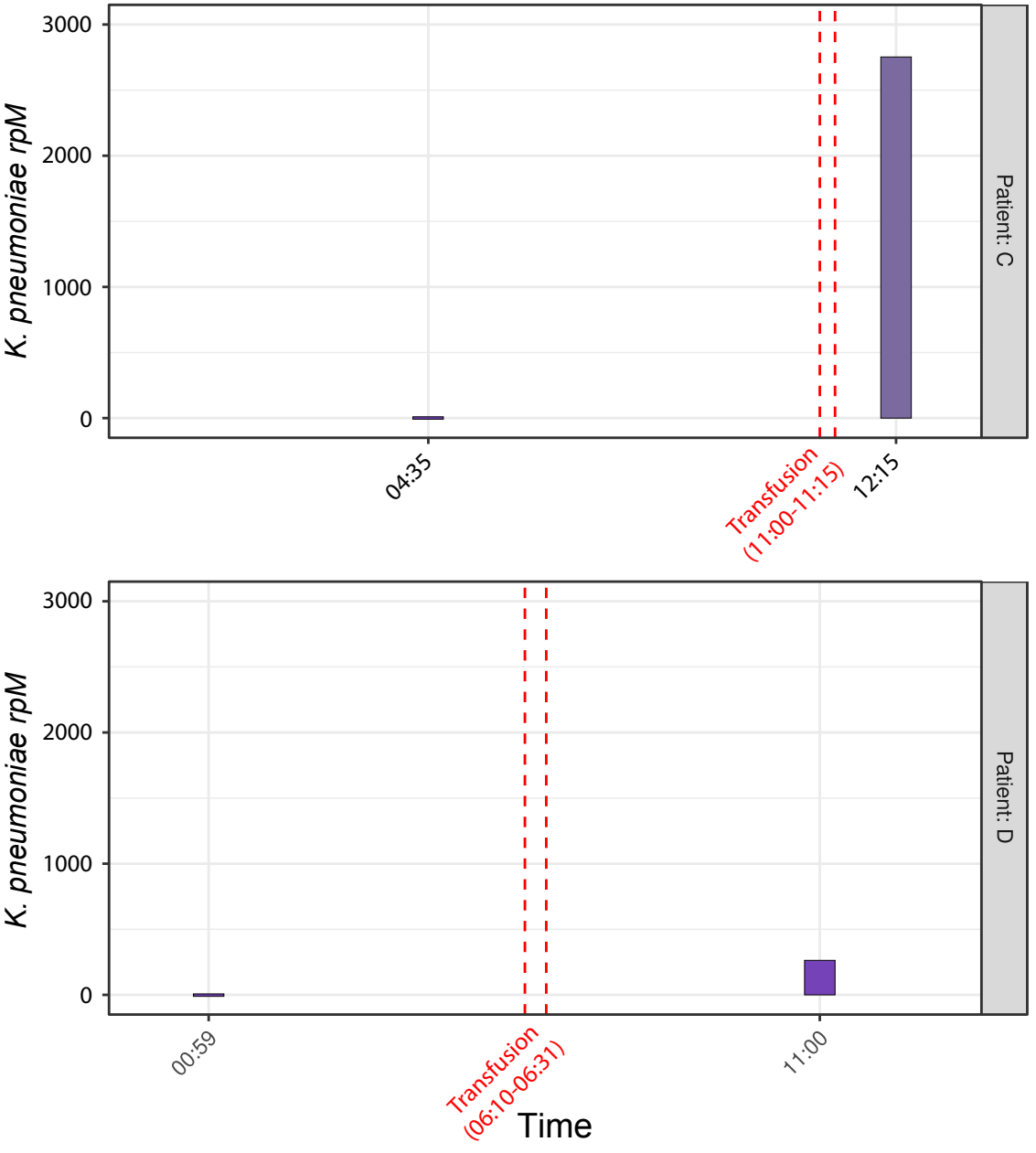

Procalcitonin (ng/mL)
100
75
50
25
0
640
320
160
80
P. aeruginosa rpM
80
60
40
20
0
Day 1, 03:55
Surgery (Day 1 07:37-16:25)
Day 1, 21:09
Day 2, 03:49
Day 2, 11:36
Day 2, 21:44
Day 2, 23:19
Day 3, 01:38
Death (Day 3, 05:25)
Time

Figure 3


3000
2000
1000
0
K. pneumoniae rpM
Patient: C
04:35
Transfusion
(11:00-11:15)
12:15
3000
2000
1000
0
K. pneumoniae rpM
Patient: D
00:59
Transfusion
(06:10-06:31)
11:00
Time