Microbial Interspecies Associations in Fracture-Related Infection

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Objectives: Describe co-occurrence or clustering of microbial taxa in fracture-related infections to inform further exploration of infection-related interactions among them.

Design: Retrospective review.

Setting: Level 1 trauma center.

Patients/Participants: Four hundred twenty-three patients requiring surgical intervention for deep surgical site infection between January 2006 and December 2015.

Intervention: None.

Main Outcome Measurement: Connection between microbial taxa.

Results: Methicillin-resistant *Staphylococcus aureus*, methicillin-sensitive *Staphylococcus aureus*, and coagulase-negative *Staphylococcus* represented the majority of monomicrobial observations (71%). Gram-negative rods, gram-positive rods, and anaerobes presented more frequently in polymicrobial infections. *Enterobacter*, vancomycin-sensitive *Enterococcus*, and *Pseudomonas* are present in polymicrobial infections with the highest frequencies and represent the top 3 most important nodes within the microorganism framework, with the highest network centrality scores.

Conclusions: The present study indicates that there are common microbial taxa (*Enterobacter*, *Enterococcus*, and *Pseudomonas*) that tend to co-occur with other microbes greater than 75% of the time. These commonly co-occurring microbes have demonstrated interactive relationships in other disease pathologies, suggesting that there may be similar important interactions in fracture-related infections. It is possible that these microbial communities play a role in the persistently high failure rate associated with management of infection after trauma. Future studies are needed to study the intermicrobial interactions that explain the frequency at which taxa co-occur. Understanding and potentially disrupting these intermicrobial relationships could inform improvements in the treatment of established infections and in the prevention of infection in high-risk patients.

Key Words: infection, trauma, biofilm

Level of Evidence: Prognostic Level IV. See Instructions for Authors for a complete description of levels of evidence.

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BACKGROUND

Surgical site infection (SSI) after trauma remains one of the most challenging complications faced by orthopaedic surgeons.[1–7] Management of acutely infected wounds is primarily surgical, relying on thorough surgical debridement of all foreign bodies and devitalized bone/tissue, and maintenance of mechanical stability of the limb.[8] Failed treatment for infection after fracture, which requires additional unplanned surgery and occurs in approximately 30% of patients, remains unacceptably high.[9–11] Outcomes after infections are poor, requiring unplanned surgical procedures and prolonged morbidity, loss of function, and potential loss of limb.[1,12–15] The persistence of these problems and unsatisfactory outcomes suggests that current management principles are not adequate and that further improvements are needed.

Multispecies interactions and polymicrobial biofilm formation (both on foreign material and bone or soft tissue) are poorly understood but are increasingly thought to be important for postinfection clinical outcomes.[16–18] It is increasingly clear that co-occurring microbes can mutually influence their behavior and physiology in profound ways that may directly impact infection outcomes.[19] Although the default interpretation is that different microbes mostly compete with each other for space and resources, neutral, synergistic, or actively cooperative interactions can also occur. Interactions such as these can modulate virulence factor expression and antibiotic sensitivity, altering bacterial dispersal, foraging, reproduction, chemical warfare, and defense.[20–22] This has been increasingly studied in the context of diseases such as cystic fibrosis and chronic soft tissue wounds where interactions between *Pseudomonas*

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TABLE 1. Organism Prevalence

Organism	#Observations	Monomicrobial	Polymicrobial
Gram-positive cocci	413	188 (45.5%)	235 (56.9%)
Staphylococcus aureus (all)	223	135 (60.1%)	88 (39.5%)
MRSA	115	71 (61.7%)	44 (38.3%)
MSSA	108	64 (59.3%)	44 (40.7%)
CNS	85	34 (40.0%)	51 (60.0%)
Streptococcus (all)	25	3 (12.0%)	22 (88.0%)
Enterococcus (all)	78	6 (7.7%)	72 (92.3%)
VSE	66	5 (7.6%)	61 (92.4%)
VRE	12	1 (8.3%)	11 (91.7%)
Graunilicitella	1	0 (0.0%)	1 (100%)
Micrococcus	1	0 (0.0%)	1 (100%)
Gram-negative rods	237	46 (19.4%)	191 (80.6%)
Enterobacter	84	17 (20.2%)	67 (79.8%)
Pseudomonas aeruginosa	45	9 (20.0%)	36 (80.0%)
E. coli	30	11 (36.7%)	19 (63.3%)
Serratia	29	5 (17.2%)	24 (82.8%)
Klebsiella	20	2 (10.0%)	18 (90.0%)
Proteus	13	1 (7.7%)	12 (92.3%)
Acinetobacter	11	1 (9.1%)	10 (90.9%)
Sphingomonas	3	0 (0.0%)	3 (100%)
Chryseobacterium	1	0 (0.0%)	1 (100%)
Stenotrophomonas	1	0 (0.0%)	1 (100%)
Gram-positive rods	60	11 (18.3%)	49 (81.7%)
Clostridium	18	2 (11.1%)	16 (88.9%)
Propriobacterium	17	4 (23.5%)	13 (76.5%)
Corynebacterium	12	3 (25.0%)	9 (75.0%)
Bacillus	12	2 (16.7%)	10 (83.3%)
Arcanobacterium	1	0 (0.0%)	1 (100%)
Anaerobes	57	3 (5.3%)	55 (96.5%)
Peptostreptococcus	25	0 (0.0%)	25 (100%)
Citrobacter	11	2 (18.2%)	9 (81.8%)
Aeromonas	5	1 (20.0%)	4 (80.0%)
Bacteroids	5	0 (0.0%)	5 (100%)
Morganella	4	0 (0.0%)	4 (100%)
Prevotella	2	0 (0.0%)	2 (100%)
Fusobacterium	2	0 (0.0%)	2 (100%)
Hafnia	1	0 (0.0%)	1 (100%)
Lactobacillus	1	0 (0.0%)	1 (100%)
Providencia	1	0 (0.0%)	1 (100%)
Fungi	5	0 (0%)	5 (100%)
Candida albicans	5	0 (0.0%)	5 (100%)
Total	772	238	534

aeruginosa and *Candida albicans* as well as *P. aeruginosa* and *Staphylococcus aureus* are well established.[23,24] Although the spectrum of infecting organisms in orthopaedic surgery have been described,[25,26] the importance of both competitive and mutually beneficial interactions between microbial community members has not yet been explored. The purpose of this study was to evaluate the co-occurrence or clustering of microbial taxa in fracture-related infections and to motivate further exploration of infection-related interactions between commonly co-occurring microbes. Our hypothesis was that specific microbes would tend to co-occur in fracture-related infection, suggesting the presence of potentially clinically relevant intermicrobial interactions.

METHODS

Study Design and Procedures

This is a secondary analysis of a cohort of infections previously identified.[25,26] In that study, patients were screened for fracture-related infection using current

procedural terminology codes in the trauma patient database at a Level I trauma center occurring from December 2006 through December 2015, yielding 3899 patients. Patients were excluded if they had not undergone surgical treatment of an extremity, acetabular or pelvic ring fracture with a plate, nail or joint fusion, or if the debridement was performed for reason(s) other than infection (such as traumatic wound) (n = 3445). Infections with recurrences were counted only once. This resulted in 423 unique infections.

All patients included in this study had deep SSI as defined by the Centers for Disease Control and Prevention (CDC) pre-2016 criteria and had undergone surgery to treat infection within 12 months of the index fixation date.[27] Culture-negative infections were those that satisfied the CDC criteria but had negative intraoperative cultures. Cultures were obtained from all patients who underwent surgical treatment for fracture-related infection. It is our standard practice to obtain both aerobic and anaerobic deep tissue cultures during surgical debridement. Bacterial taxonomic designation and antibiotic susceptibility tests were performed by the hospital microbiology laboratory using standard methods.

TABLE 2. Eigenvector Centrality Scores for Each Organism Examined

Organism	Centrality
Gram-positive cocci	
Staphylococcus aureus	
MRSA	0.892
MSSA	0.911
CNS	0.926
Streptococcus	0.664
Enterococcus (all)	
VSE	0.999
VRE	0.573
Graunilicitella	0.153
Micrococcus	0.143
Gram-negative cocci	
Enterobacter	1
Pseudomonas aeruginosa	0.943
E. coli	0.843
Serratia	0.749
Klebsiella	0.916
Proteus	0.635
Acinetobacter	0.684
Sphingomonas	0.164
Chryseobacterium	0.061
Stenotrophomonas	0.207
Gram-positive rods	
Clostridium	0.730
Propriobacterium	0.529
Corynebacterium	0.637
Bacillus	0.662
Arcanobacterium	0.166
Anaerobes	
Peptostreptococcus	0.881
Citrobacter	0.678
Aeromonas	0.424
Bacteroids	0.614
Morganella	0.387
Prevotella	0.153
Fusobacterium	0.250
Hafnia	0.103
Lactobacillus	0.0548
Providencia	0.097
Fungi	
Candida albicans	0.568

Statistical Analysis

Descriptive statistics using Student *t* test for continuous data and χ^2 test for categorical data were used to describe the propensity for each organism to occur in a polymicrobial or monomicrobial infection. To understand the propensity of certain microorganisms to coexist in an infected traumatic wound, a network analysis methodology was used. Cultures were examined from a single institution, and all relationships were captured via an adjacency matrix. The eigenvector centrality metric was used to evaluate the connectedness of all organisms to each other, providing a score that is determined by the centrality of each species' node relative to the sum of the centralities of the nodes to which it is connected. Eigenvector centrality is a measure of the influence of a node in a network. This score is a proportion, ranging from 0 to 1, with 1 representing high degree of influence in the network. In the context of this study, higher eigenvalue centrality scores are attributed to organisms that are connected to many other organisms that are, in turn, connected to still more organisms.

RESULTS

Microorganisms identified by cultures of 423 patients with infected traumatic wounds were investigated. One hundred eighty-five patients had polymicrobial infections (36.6%) with ≥2 microbial taxa present. A total of 772 microorganisms were classified representing 35 distinct classifications (Table 1). *Staphylococcus aureus* was the most commonly cultured microbe and present in 28.9% of all samples (51.6% methicillin resistant and 48.4% methicillin sensitive). Methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *Staphylococcus aureus* (MSSA), and coagulase-negative *Staphylococcus* (CNS) represented the majority of monomicrobial infections (combined n = 169, 71.0%) (Table 1). Gram-positive cocci, as a group, represented the majority of monomicrobial infections (79.0%) (Table 1, see **Figure**, **Supplemental Digital Content 1**, http://links.lww.com/JOT/B634). Gram-negative rods, gram-positive rods, anaerobes, and fungi presented far more frequently as part of polymicrobial infections (Table 1, see **Figure**, **Supplemental Digital Content 1**, http://links.lww.com/JOT/B634). *Enterobacter* and vancomycin-sensitive *Enterococcus* (VSE) represented the most common microbes in polymicrobial infections (accounting for 24.0% polymicrobial infections) (Table 1). Infections occurring after open fracture were polymicrobial in nature at a higher rate than

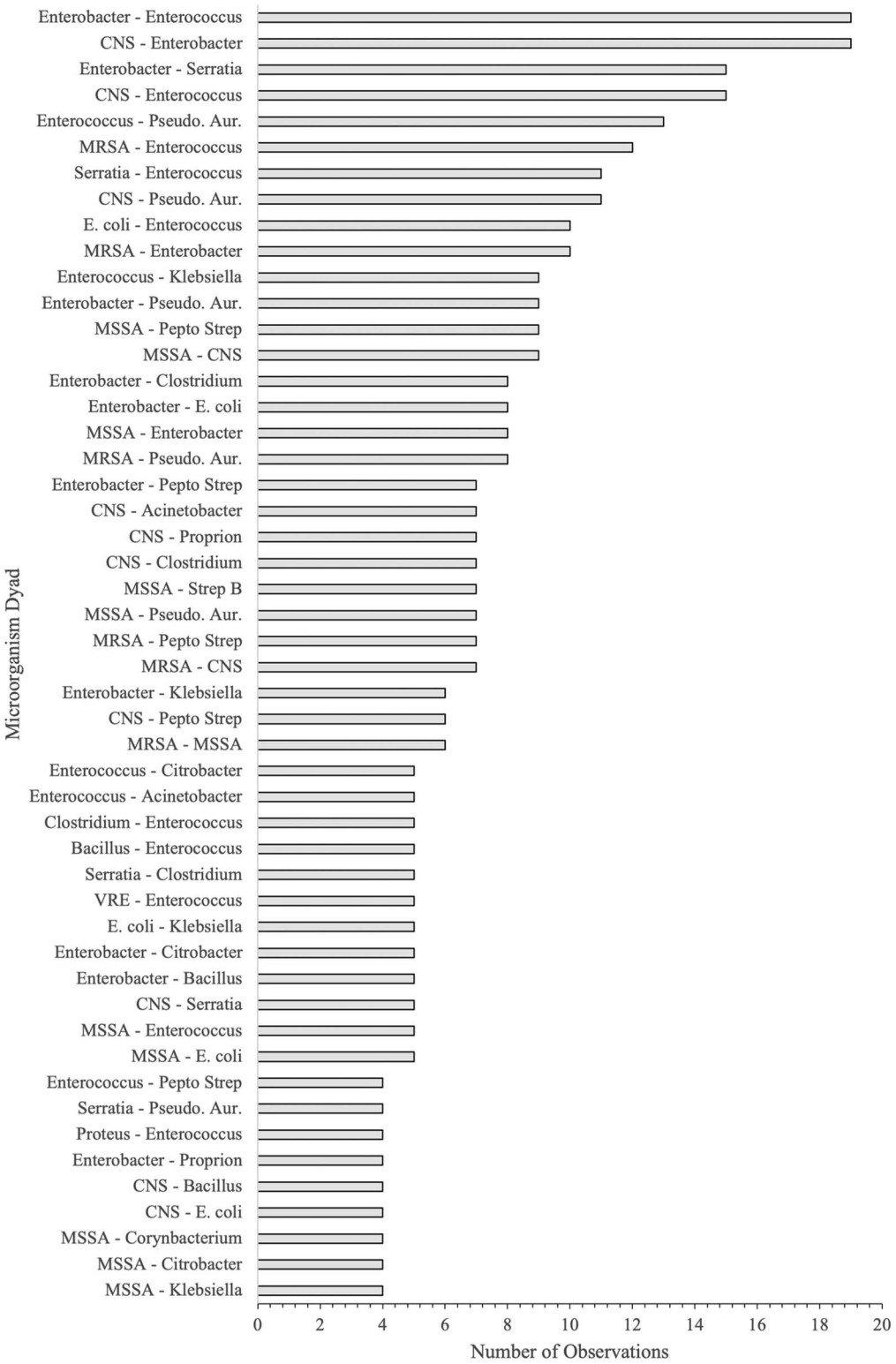

FIGURE 1. Frequency of microorganism dyadic relationships (gray scale).

those occurring after closed fracture (45.6% vs. 35.7%, $P = 0.04$). There was no association between open fracture severity as reflected in Gustilo type and frequency of monomicrobial versus polymicrobial infection (see **Table**, **Supplemental Digital Content 2**, http://links.lww.com/JOT/B635).

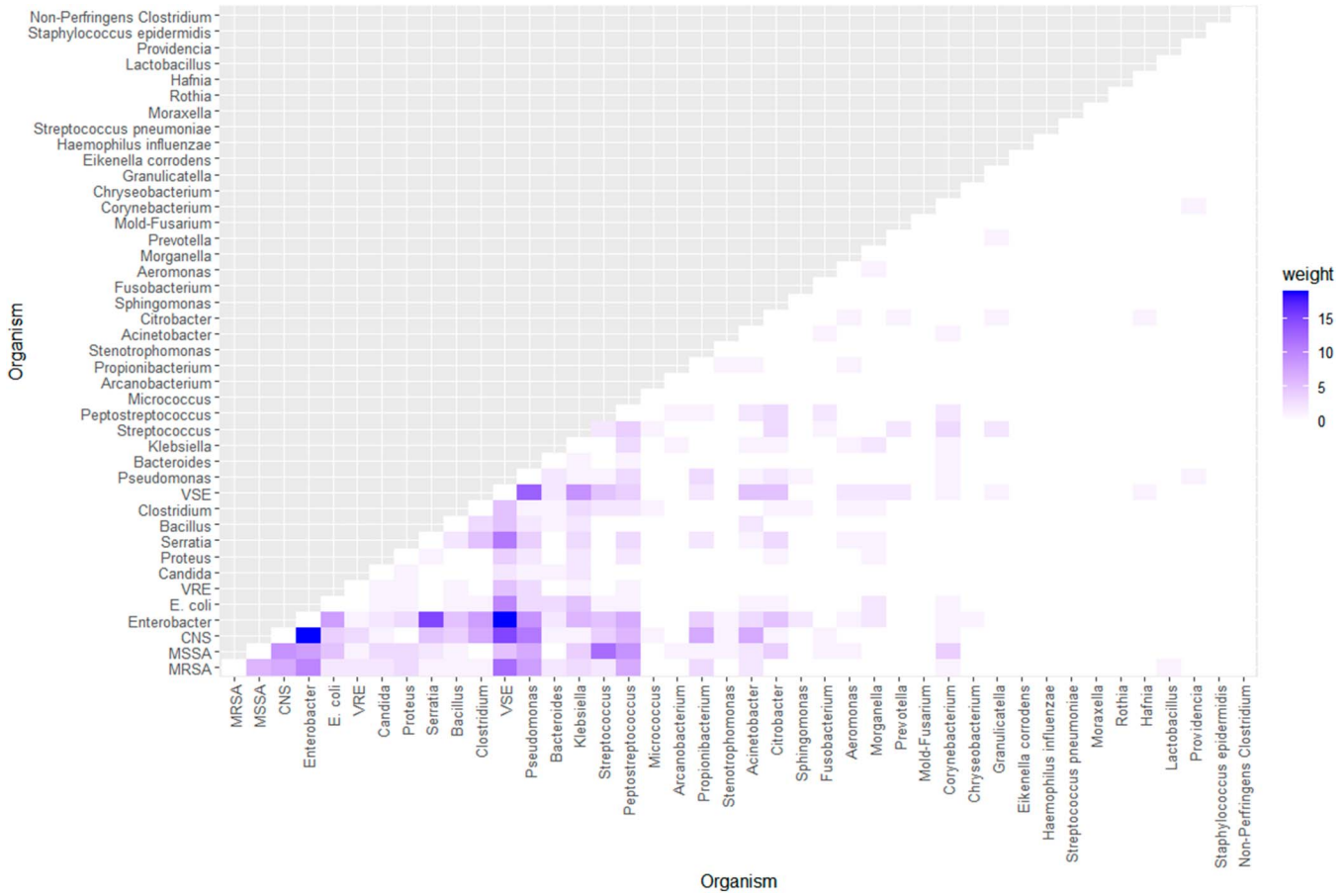

FIGURE 2. Heatmap plot demonstrating the strength of the relationship between microbial taxa (gray scale). full color online

Unique microorganism pairings were observed from single occurrence instances to as many as 19 instances (Fig. 1). The most common pairs present in polymicrobial infections were *Enterobacter*/*Enterococcus*, CNS/*Enterobacter*, *Enterobacter*/*Serratia*, and CNS/*Enterococcus*. Beyond the frequencies of dyadic relationships, the network analysis (see **Figure**, **Supplemental Digital Content 3**, http://links.lww.com/JOT/B636, Figs. 2 and 3) and resulting eigenvector centrality scores (Table 2) demonstrate that *Enterobacter*, VSE, and *Pseudomonas* represent the top 3 most important nodes within the microorganism framework and share a strong relationship (19_{obs}) with one another. VSE and *Enterobacter* demonstrate centrality scores >0.95, whereas *Lactobacillus*, *Chryseobacterium*, and *Providencia* report centrality scores approaching zero.

DISCUSSION

Clinically important behavioral interactions between co-occurring microbial species have been demonstrated in several fields including cystic fibrosis lung infections, chronic wounds, otitis media, and urinary tract infections.[28] However, to date, these microbial interactions have not been studied in fracture-related infection. Identifying microbial taxa that tend to co-occur represents a critical first step toward developing a better understanding of clinically relevant interspecies interactions that occur in the context of fracture-related infection. The frequent microbial co-occurrences that we observed in this analysis may represent the result of an evolutionary or survival benefit to the microorganisms in question. These hypothesis-generating findings warrant closer study in several areas. Next steps include (1) targeted study of interactions between species identified in this analysis and (2) examination of longitudinal patient specimens using both culture-based and molecular diagnostic tools. Microbial interactions and ecology as well as biofilm maturation can be studied using high-fidelity confocal 3-dimensional microscopy of co-occurring species on orthopaedic implants in vivo (as described in Drescher et al[29]) and in ex vivo specimens. Similar research is also needed in other forms of implant-related infection such as periprosthetic joint infection, integrating traditional culture-based diagnostic tools with more novel microbial assessment strategies, such as next-generation sequencing.

This study demonstrated that there are common bacterial taxa (specifically *Enterobacter*, *Enterococcus*, and *Pseudomonas*) that co-occur with other microbes in greater than 75% of their observed fracture-related infections. Conversely, we identified other microbes, such as MRSA and MSSA, that are also common but occur both in isolation

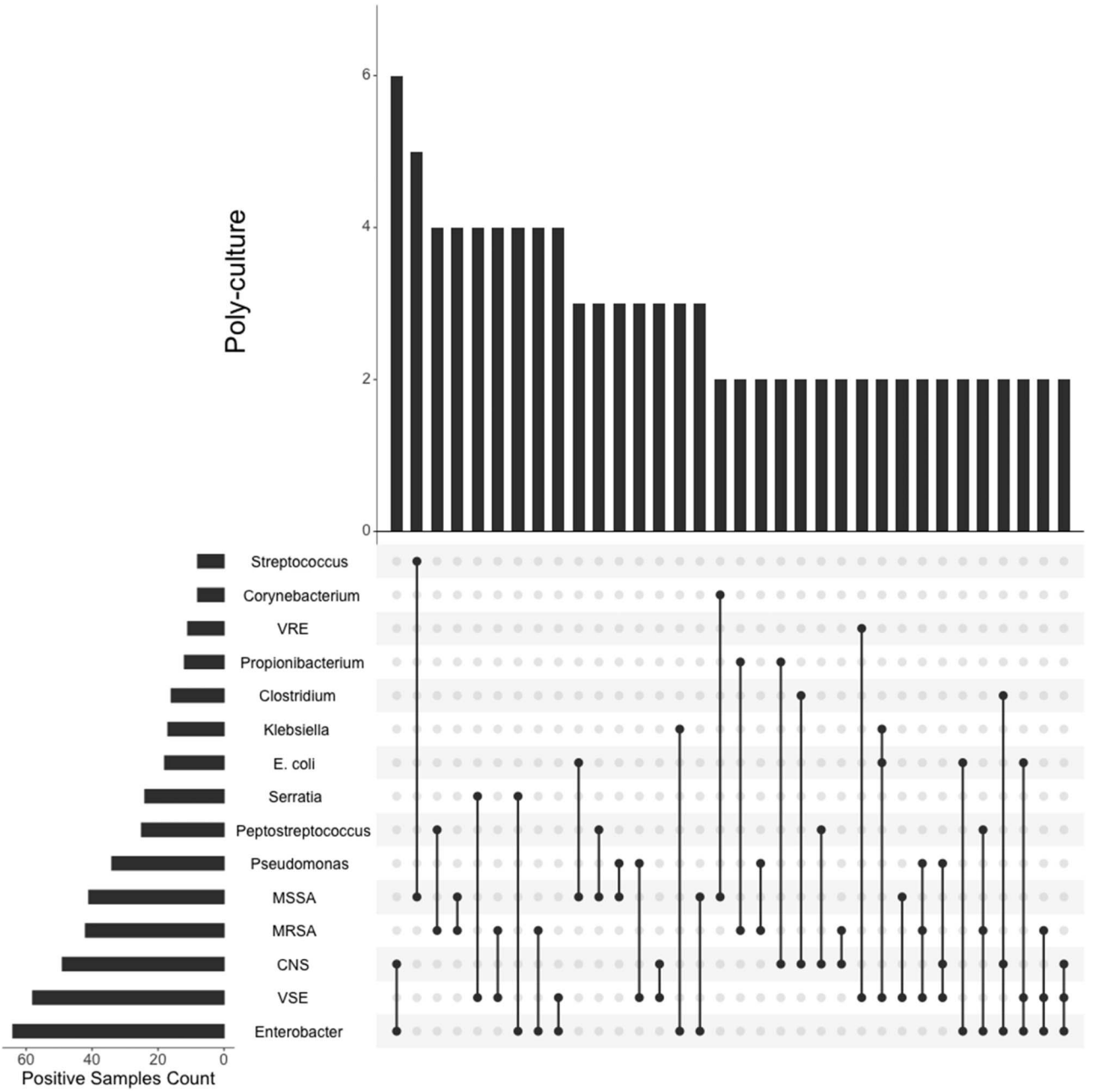

FIGURE 3. Upset plot showing taxa cultured from more than one polymicrobial infection. Bars to the left show number of polymicrobial infections in which the taxon was found. Dots indicate members of polymicrobial infections. Columns above indicate number of instances of the indicated polymicrobial infection in the dataset. Only polymicrobial cultures that occurred more than once in the dataset are included (RGB).

as monomicrobial infections (62% and 59% of the time, respectively) and in tandem with other microbes in polymicrobial infections. Coagulase-negative *Staphylococcus* tended to occur singularly and part of a polymicrobial infection (40% and 60%, respectively). There are several possible explanations for these observations regarding the frequency of coexisting microbial taxa in fracture-related infection, which require further study. One possibility is that common sources of contamination due to either open fracture or exposure in the operating room tend to contain 2 or more of these taxa resulting in frequent coinfection in the absence of interspecies interaction (whether competitive or cooperative). Conversely, it is also possible that the presence of one microbe increases the suitability of the infection environment for others to colonize. The modified environment may be due to metabolic byproduct secretion, biofilm matrix secretion, or other infection-related phenotypes. Given that mutually helpful growth behavior in biofilms has been observed in other

commonly co-occurring microbes in cystic fibrosis lung infections, chronic wounds, otitis media, and urinary tract infections,[28] it is possible that these microbes engage in mutually beneficial interactions. Infection model testing using methods such as 3-dimensional single-cell imaging will allow us to distinguish between these different possible explanations of the co-occurrence patterns observed here.

Recent research in nonorthopaedic fields have revealed highly clinical relevant interspecific interactions that alter microbial survival, disease progression, and susceptibility to antibiotics.[9,18,20,28,30–36] Interactions between 2 microbial species can alter virulence factor production by one or both species, thereby influencing pathogenesis, persistence, and antibiotic susceptibility.[36,37,38] For example, in a model of cystic fibrosis, coinfection with *P. aeruginosa* and *S. aureus*, cells form mixed colonies that promotes the survival of *S. aureus* in the presence of vancomycin.[39] It even seems that *P. aeruginosa* can sense the presence of *S. aureus* and move toward it in an effort to form a mixed colony.[33] Because we identified similar species that tend to co-occur in fracture-related infection, it seems likely that these interspecies interactions are also relevant to natural history and treatment strategies in our patient population. Based on these observations, some have proposed the development of treatment strategies around blocking interspecies interactions that complement current treatment strategies.

The strengths of this study include the large sample size with 423 separate infections. Additionally, patients were identified using the strictly defined CDC definition of deep SSI. Despite these strengths, the study had several limitations, including those common to retrospective study designs. It is possible that some infected patients who were initially treated at this institution were lost to follow-up and were, therefore, not included in this cohort. More importantly, we did not include perioperative antibiotic choice in this analysis, and it is possible that the microbes and microbial synergies identified in this analysis were affected by perioperative antibiotic administration. However, the data collected for this analysis occurred over 9 years, and this represents a pragmatic reflection of the microbes present after standard-of-care management of fractures and infections. More research is needed to clarify the effect of antimicrobial choice on microbial communities. The conclusions drawn from these single-center origin data also require validation across other study sites; future multicenter approaches are needed to address this. The infections analyzed in this study occurred over a 9-year period, and it is possible that there may have been changes over that period that may have affected microbial species clustering. However, the network analysis is not predicated on simultaneous data collection.[44–45] The network analysis simply analyzes the frequency and strength of connections between taxonomic nodes. The most common co-occurring microbial pair (*Enterococcus*/*Enterobacter*) occurred only 19 times (approximately 10% of polymicrobial infections). *Enterobacter* additionally participated in the top 3 most common microbial pairs, which represent 30% of all polymicrobial infections. This observation is frequent enough that it is likely to have clinical relevance. Subsequent research exploring this should be initiated.

Many of the most commonly co-occurring microbes in fracture-related infection also appear frequently as common co-occurring microbes in other disease pathologies that have demonstrated intermicrobial interactions.[31,36,38] This suggests that we may find similar clinically relevant interspecies interactions in fracture-related infections, which has the potential to improve our understanding of the natural history of specific infections and/or lead to more targeted treatment strategies. Follow-up research has been initiated using clinical specimens and high-fidelity biofilm modeling methods to explore whether clinically relevant interspecies interactions are occurring in these polymicrobial fracture-related infections. This analysis serves as a critical first step, identifying candidate microbial pairs that may display clinically relevant interactions in fracture-related infections. If there are such interactions occurring, understanding the nature and consequences of these interactions may be critical in developing more effective treatment strategies.

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