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Research paper

High-throughput sequencing of microbial diversity in implant-associated infection

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ABSTRACT

Few molecular studies have shown that the number of bacterial species in implant-associated infection may have been underestimated. To determine the actual microbial diversity in implant-associated infection, a high-throughput sequencing method was adopted to sequence the DNAs extracted from the tissues of infected and uninfected patients. Principal component analysis (PCA) and β diversity showed an obvious divergence of infected and uninfected groups, and that the overgrowth of *Proteobacteria* (80.87%), *Firmicutes* (13.41%) in the positive deep infection group (P.d, via biopsy) and *Proteobacteria* (91.68%) in the positive surface infection group (P.s, via swabs) might be the causative factors in implant-associated infection. Moreover, Venn results indicated that a mean of 330 common operational taxonomic units (OTUs) was obtained in all groups, of which 113, 109, 45, 20, 13 and 12 OTUs belonging to *Proteobacteria*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, *Gemmatimonadetes* and *Chloroflexi* were identified. In conclusion, many traditional "pathogenic bacteria" were identified as the common bacteria in operation sites, and the disruption of their complex interaction caused infection; therefore, further work is need to illustrate the aetiology of implant-associated infection using in-depth systems-level analyses.

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1. Introduction

Implant-associated infection is caused by polymicrobial flora originating from the external environment, local skin flora, the enteric tract, the vagina and the oral mucosa, and is difficult to diagnose and treat because of the rapid formation of biofilms (Arciola et al., 2004; Arciola et al., 2005; Martin et al., 2010). Therefore, it is crucial to know the actual microbes in infected tissues and relate them to the bone graft materials, pathophysiology and therapy of implant-associated infection (Arciola et al., 2008; Arciola et al., 2007; Arciola et al., 2012).

To date, biofilms are increasingly appreciated as being important in the pathogenesis of persistent infections, particularly in chronic mucosal or skin-surface infections (Arciola et al., 2008; Arciola et al., 2007; Stewart and Costerton, 2001; Stewart and Franklin, 2008; Zimmerli and Moser, 2012). However, medical microbiologists have relied on culture techniques to elucidate the complexity of infections for decades, and these culture methods can only be used to identify the "culturable" bacteria associated with such biofilms growing relatively quickly and easily in laboratory media (McGuckin et al., 2003; Thomson, 2000). It is technically difficult to separate and identify more than three to six species because of their various nutritional, pH, temperature and oxygen requirements; moreover, interspecies competition and different concentrations of bacteria within a plate also increase the difficulties of screening (Davies et al., 2004; Whitley, 2008). To establish the role of bacteria in wound healing, it is necessary to define the full panoply of organisms within a wound, and high-throughput sequencing methods may be a useful means of alleviating this problem because they can detect almost all of the DNA signatures of microorganisms within a specific environment, even some of them with low copy numbers or a dormant metabolic state (Kobayashi et al., 2006; Wang et al., 2011; Zhang et al., 2012).

To eliminate the overestimate of the importance of species that are easily cultured and the underestimate of fastidious organisms that

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may be highly prevalent and important in implant-associated infection, high-throughput sequencing analyses were used in the present study, proving basic data for the understanding of the relationship between the presence of bacteria and delayed wound healing.

2. Materials and methods

2.1. Ethical statement

The study was approved by the Ethical Committee of the second affiliated hospital of Nanchang University. All participants provided written informed consent and all methods were carried out in accordance with the approved guidelines.

2.2. Patients and bacterial isolates

In the second affiliated hospital of Nanchang University, 26 consecutive patients (38 implants) undergoing revision arthroplasty between June 2014 and Feb. 2015 were retrieved. For all patients, 2 g Cefotiam were used 30 min before and 24 and 48 h after the operation to prevent infection (patients who used other antibiotics were excluded from the sample), and the infection status of all patients were determined within 12 days. Among these patients, twenty of the 38 implants (53%) were believed to be infected on the basis of preoperative diagnosis as described below, 18 implants (47%) were believed to be aseptic and 12 implants were refused during the procedure of sampling and DNA extraction.

Preoperative diagnoses were performed on the basis of clinical symptoms, local clinical findings, physical findings and serologic testing. Briefly, patients were believed to have infection if they had local pain, swelling or redness with evidence of positive serologic testing, such as C-reactive protein and sedimentation rate and/or a positive culture of aspirated joint fluid, and the patients believed not to have infection were considered to be aseptic and were culture-negative.

Samples were collected using biopsy samplers (one swab for deep sites) and sterile swabs (one swab for superficial sites) in the bioclean operating room with a laminar air-flow system in an individual sterile container and then immediately transported to the microbiology department (in all cases, antibiotics were withheld until fluid and tissue cultures had been taken), and part of each sample was loaded onto each of a chocolate agar plate (Oxoid, Basingstoke, UK), blood agar plate (Oxoid, Basingstoke, UK) and China blue agar plate (Oxoid, Basingstoke, UK) at 37 °C for 24 to 48 h. Plates on which no colonies formed or the clones were identified as "commensal" bacteria were judged as negative, and plates on which clonal forms identified as pathogenic bacteria grew were judged as positive. The bacteria were identified using the automated microbial identification system VITEK2 Compact (bioMerieux, France).

Then, the infected and uninfected patients were divided into the following groups: positive deep infection (n = 7, P.d), positive surface infection (n = 6, P.s), negative deep infection (n = 7, N.d) and negative surface infection (n = 6, N.s) according to their infected sites (none of the patients belonged to more than one group), then all of the samples that had been collected from any one site were mixed for high-throughput sequencing (Table 1).

2.3. Extraction of genomic DNA and high-throughput sequencing

Genomic DNA from each sample was extracted using a TIANamp Genomic DNA kit (TIANGEN) combined with bead beating as previously published (Yu et al., 2015). The extracted genomic DNA was used as the template to amplify the V3 region of 16S rRNA genes using the primer pair 515F/806R with the barcode. PCR reactions, pyrosequencing of the PCR amplicons and quality control of raw data were performed as described previously with minor modification (Xu et al., 2015).

Table 1
The infectious bacteria isolated from orthopedic inpatients.

No.	Age	Sex	Isolated strain	No.	Age	Sex	Culture
P.d-1	37	Female	*S. epidermidis*	N.d-1	77	Female	Negative
P.d-2	62	Female	*E. cloacae*	N.d-2	60	Male	Negative
P.d-3	23	Male	*S. aureus*	N.d-3	41	Male	Negative
P.d-4	87	Female	CNS*	N.d-4	21	Male	Negative
P.d-5	55	Male	*K. pneumoniae*	N.d-5	39	Male	Negative
P.d-6	21	Female	*S. aureus*	N.d-6	76	Male	Negative
P.d-7	41	Male	CNS*	N.d-7	39	Female	Negative
P.s-1	35	Male	*S. aureus*	N.s-1	45	Male	Negative
P.s-2	62	Female	*S. marcescens*	N.s-2	48	Male	Negative
P.s-3	27	Male	*K. pneumoniae*	N.s-3	65	Male	Negative
P.s-4	77	Female	CNS*	N.s-4	20	Male	Negative
P.s-5	73	Female	*E. coli*	N.s-5	64	Female	Negative
P.s-6	55	Female	*P. aeruginosa*	N.s-6	61	Female	Negative

CNS*: coagulase negative *Staphylococcus*; P.d, (positive deep infection group, N = 7); P.s, (positive surface infection group, N = 6); N.d, (negative deep infection group, N = 7); N.s, (negative surface infection group, N = 6).

2.4. Bioinformatics and multivariate statistics

Paired-end reads from the original DNA fragments were merged using FLASH to merge paired-end reads when at least some of the reads overlapped the read generated from the opposite end of the same DNA fragment, and paired-end reads was assigned to each sample according to the unique barcodes (GenBank accession number SRX1661037).

Then, sequence analysis was performed using the UPARSE software package and the UPARSE-OTU and UPARSE-OTUref algorithms. In-house Perl scripts were used to analyze α (within samples) and β (among samples) diversity. Sequences with ≥97% similarity were assigned to the same OTUs. One sequence was picked as a representative for each OTU, and the RDP classifier was used to annotate taxonomic information for each representative sequence. Cluster analysis was preceded by weighted UniFrac distance using QIIME software package.

3. Results

3.1. Isolates from implant-associated infection

In the present study, infectious pathogens, such as *Staphylococcus epidermidis*, *Enterobacter cloacae*, *Staphylococcus aureus*, coagulase-negative *Staphylococcus* (CNS), *Klebsiella pneumoniae*, *Staphylococcus marcescens*, *Escherichia coli* and *Pseudomonas aeruginosa*, were identified from positive patients in deep/surface tissues using the culture method (Table 1). Although no pathogenic bacteria were identified from the "negative" group, samples taken from this group were also subjected to high-throughput sequencing.

3.2. The α diversity of the microbial community in implant-associated infection

To compare the microbes in implant-associated infection, 16S rRNA amplicon sequencing analysis was used to sequence the V4 hypervariable region, and the sequencing data were filtered to obtain valid data. All of the effective tags of all samples were clustered and those sequences with over 97% similarity were considered as one OTU. In total, 260,672 usable raw sequences (39,108 unique sequences) and 2300 OTUs were obtained from all samples with an average of 575 OTUs per group (supplementary material 1). Moreover, the Chao1 index and the Shannon index were almost saturated and the rarefaction curve of every sample could enter the plateau phase (supplementary material 2).

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