Short Communication: Evidence That Microbial Translocation Occurs in HIV-Infected Children in the United Kingdom

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Abstract

Microbial translocation (MT) from the gut is implicated in driving immune activation, increasing morbidity and mortality in HIV. We used bacterial 16S rDNA PCR, Sanger sequencing, and high-throughput sequencing to identify microbial DNA in the bloodstream of HIV-infected children in London, United Kingdom. Blood samples were collected from sequential children attending the HIV clinic at Great Ormond Street Hospital, London. DNA extraction, broad range 16S rDNA PCR, and standard Sanger sequencing were carried out. A subset of positive samples was analyzed by high-throughput sequencing (Roche 454 platform). Of 105 samples collected from sequential children, nine were positive using broad range 16S rDNA PCR (8.6%; 95% CI 4.4–16%). From three amplicons, 16S rDNA sequences were identified as Streptococcus, Propionibacterium acnes, and coagulase-negative Staphylococcus. Four positive samples were analyzed by high-throughput sequencing. In the three samples in which organisms were identified by Sanger sequencing, the same species were identified. Further species, in differing proportions, were identified in all four samples. The identified organisms included known gut orders Bifidobacteriaceae, Lactobacillaceae, Bacteroidales, and Clostridiales. In immunocompetent children of equivalent age, no bacterial DNA was detected in blood using this approach. This is the first study to our knowledge using molecular techniques to identify MT in children in the developed world. Our data indicate that 16S rDNA is detectable in 8.6% of HIV-infected children. Levels of DNA were low and from multiple bacterial species. Further studies are needed to ascertain the importance of MT in HIV-infected children.

Introduction

Immune activation is central to the pathogenesis of HIV infection and is associated with increased mortality and non-AIDS-related morbidity despite antiretroviral therapy (ART). Microbial translocation (MT) across the gut is one mechanism that may drive immune activation.

Rapid CD4 cell depletion within the gut mucosa early in HIV infection is hypothesized to allow translocation of intestinal microbiotal products into the bloodstream at increased levels compared with uninfected persons. Translocation may be of viable organisms or of microbial components, which may include lipopolysaccharide (LPS) and bacterial DNA. Intact organisms and microbial components are potential immunostimulants.

MT has been reported to be higher in patients with HIV infection and has been implicated as a cause for increased immune activation and poor CD4 cell count recovery on ART. It has been hypothesized that immune activation is driven by MT as a consequence of HIV-induced gut mucosal damage. If correct, a number of potential treatment strategies could be employed to reduce MT and improve outcomes. There are clinical trials underway with this specific aim.

Most evidence for increased microbial translocation in HIV is based on detection of increased levels of LPS and bacterial 16S rDNA. The latter is detected through a broad-range quantitative polymerase chain reaction (qPCR). However 16S rDNA-PCR is vulnerable to contamination from exogenous and endogenous bacterial DNA. Without sequencing the amplicons, these results are therefore potentially artifactual and indeed thus far sequencing has largely yielded results compatible with environmental contaminants and not recognized gut commensals.

Materials and Methods

We set out to determine if bacterial 16S rDNA was detectable in the bloodstream of HIV-infected children in London,
Discard EDTA blood samples were collected from sequential children attending the HIV outpatient clinic at Great Ormond Street Hospital, London. At the time of the study 124 HIV-infected children were being seen regularly, 97 of whom were receiving ART. Of the children 57% were aged 12–17 years and 43% were under 12 years; 78% were of black African ethnic origin, 11% were African/white, and the remainder classified themselves as white, Afrocaribbean, or other. Of those on ART 88% were on triple therapy, with 6% on dual therapy, 4% on monotherapy, and 1% on four drugs. Of the children 47% had a CD4 cell percentage above 30% at the last clinic visit, with 34% having a CD4 cell percentage between 21% and 30%, 16% between 11% and 20%, and 3% under 10%; 64% of children had a viral load <50 copies/ml, with 32% between 50 and 100,000 copies/ml and 4% with a viral load >100,000 copies/ml.

The commercial QIAmp DNA mini kit (Qiagen, Crawley, UK), with an additional bead-beating step to ensure complete lysis of bacterial cells, was used to extract DNA from 200 μl of EDTA blood within 48 h of collection. Broad range 16S rDNA PCR was carried out as previously described using two primer sets.26,32 16SFa/16SFb (GCTCAGATTGAACGCTGG/GCTCAGAYGAACGCTGG) and 16SR (TACTGCTGCTT CCGTA) amplified the V1 and V2 region of the 16S rDNA gene.26 and 785F (GGATTAGATACCCBRGTAGTC) and 1175R (ACGTCRTCCCDCCTTCCCT) amplified the V5 and V6 region. The sensitivity of the broad-range 16S rDNA PCR has previously been demonstrated to be 10–100 colony-forming units per PCR reaction.26 Amplicons derived from positive samples were sequenced using the Big-Dye v.3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) and analyzed on the 3130 genetic analyzer (Applied Biosystems). The sequences obtained were compared to those on the GenBank database using the BLAST program available at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The sequence was classified to species level if there was >98% homology with two or more GenBank sequences from the same species, submitted by independent laboratories, and the percentage identification was lower for all other species. A subset of samples found to be positive by broad range 16S rDNA PCR was analyzed by high-throughput sequencing. Attached to the 16S rDNA primers were standard 454 Titanium adapters and an individual barcode sequence for each sample. The library was pyrosequenced on a 454 FLX Titanium (Roche) platform according to the manufacturer’s recommended protocol.

Sequences were processed and analyzed using QIMIE.33 16SFa/16SFb/16SR and 785F/1175R reads were discarded if they contained ambiguous bases, if the quality score was <25, if the run of homopolymer bases was >6, if there was a mismatch in primer sequence, and if the barcode could not be corrected. Operational taxonomic units (OTUs) were clustered at 97% sequence similarity and chimeric sequences were removed using USEARCH.34 Representative OTUs were assigned taxonomy using the RDP classifier35 at a minimum support threshold of 80% again the Greengenes database.36

Discard samples were used in accordance with the guidance of the National Patient Safety Agency and National Research Ethics Service, which assessed this project to be within the remit of assay performance evaluation.

### Results

Samples were collected from 105 sequential children attending the outpatient clinic representing 85% of the clinic population.

Nine samples were found to be positive using broad range 16S rDNA PCR representing a positivity rate of 8.6% (95% CI 4.4–16%). From three of these amplicons, 16S rDNA sequences were obtained and identified as Streptococcus species, Propionibacterium acnes, and coagulase negative Staphylococcus. Direct Sanger sequencing failed in the other six samples, probably due to a mixture of 16S rDNA sequences from different bacterial species.

Three samples that were successfully sequenced and one sample on which direct sequencing failed (samples A, B, C, and D) were then analyzed by high-throughput sequencing. This identified several orders of bacteria known to be associated with the human gut.31,30,37 The relative proportions of these bacteria are shown in Fig. 1. Two of four samples were positive for Bifidobacteriales, Bacteroidales, and Clostridiales, whereas all samples were positive for Lactobacillales, Burkholderiales, Bacillales, and Pseudomonadales.

Of the nine patients in whom microbial DNA was detected, five (55%) had an undetectable viral load; four (45%) had a CD4 percentage above 30% and two (22%) had a CD4 of less than 10%.

### Discussion

There has so far been few data generated investigating the question of MT in HIV-infected children,15,22,38,39 with this being the first study to our knowledge using molecular techniques to identify MT in children in the developed world, and the only one to have successfully sequenced bacterial DNA using conventional and high-throughput techniques. In adult populations standard sequencing has been carried out in only a handful of studies7,20 and has yielded results compatible with possible contamination such as Serratia spp.

![FIG. 1. Bacteria orders recovered from HIV+ blood using 16S rDNA high-throughput sequencing. Proportions of the total recovered sequences from each sample after quality filtering and assigning taxonomy to reads generated by high-throughput sequencing. Samples are labeled A, B, C, and D.](image-url)
and Rahnella spp. Previous work in this field has largely relied on the detection of LPS or surrogate markers of MT such as sCD14 (a coreceptor for LPS produced by monocytes) and LPS-binding protein (LBP). In view of ongoing debate about optimal methods to detect MT in HIV-infected people, there is an urgent need for further assay development and optimization. This need is compounded by the conflicting data generated in pediatric populations in particular, but also in adult populations. Indeed a major African longitudinal study found no evidence of increasing MT markers during untreated disease progression. Detection of known gut commensal microbial DNA in the bloodstream will accelerate our understanding of MT and its potential impact on HIV pathogenesis. These findings are compatible with MT occurring in relatively well children infected with HIV in the United Kingdom, for the most part on ART with reasonably well-controlled disease. In resource-limited settings in the context of malnutrition, enteropathy, and poorly controlled HIV infection, MT has potentially an even greater significance.

It has been difficult to demonstrate evidence of gut-associated organisms using broad-range 16S rDNA PCR and conventional Sanger sequencing techniques. Although a powerful technique for detection and identification of most bacterial species from culture-negative samples, 16S rDNA PCR does have limitations. One disadvantage of the technique is that typically a single dominant species will be identified with minority species often being outcompeted in the PCR. The addition of a cloning step can resolve mixtures of 16S rDNA sequences and identify minority species. However, the technique is very labor intensive and this limits the maximum number of sequences that can be obtained to somewhere in the order of 100. When cloning has been used in this field, it has demonstrated the presence of mixed sequences, which further emphasizes the need for more sophisticated techniques. High-throughput sequencing can generate hundreds of thousands of sequences from a single 16S rDNA amplicon, which, with subsequent bioinformatics input, can provide a comprehensive picture of all bacterial species that have been amplified, even those that are a small minority. To our knowledge this is the first study to use high-throughput sequencing to investigate microbial translocation in HIV. The potential usefulness of the technique is demonstrated by the detection of 16S rDNA sequences of common gut organisms such as Bifidobacteriales and Lactobacillales that were not detected by conventional Sanger sequencing of the amplicon.

The strengths and weaknesses of this approach to identifying bacterial DNA in whole blood are highlighted by our findings. Due to the relative paucity of bacterial DNA in the samples, high-throughput sequencing will identify low levels of multiple organisms including potential contaminants that are present in PCR reagents. However, the depth of coverage afforded by high-throughput sequencing also identifies potentially interesting organisms that could be derived from the GI tract. We cannot rule out contamination as the source of microbial DNA in the samples. However, in 191 blood samples of immunocompetent children previously evaluated using this 16S rDNA PCR technique, none was positive despite some having clinically significant bacteremia.

The microbial translocation hypothesis needs further investigation in children where data so far are limited and conflicting. Our data indicate that MT may consist of low levels of multiple organisms resident within the GI tract. Given the increased survival of HIV-infected children on ART, investigating the importance of MT in this population and in resource-limited settings is vital for the future rational design of intervention studies. If microbial translocation is indeed responsible for increased levels of immune activation and thus excess morbidity and mortality in those infected with HIV, there is the possibility of rational therapeutic intervention, including probiotics, symbiotics, nonabsorbable antibiotics, and LPS binders. There is an urgent need for further investigation using molecular techniques that can comprehensively describe the microbiota detectable in the bloodstream of those with HIV and determine whether this profile changes over time and its relationship to clinical outcome.

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**Author Disclosure Statement**

No competing financial interests exist.

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