LEADING ARTICLE

A molecular revolution in the study of intestinal microflora

E Furrie

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Bacterial colonisers of the colon comprise several hundred bacterial species that live in a complex ecosystem. Study of this complex ecosystem has been carried out, until recently, by traditional culture techniques with biochemical methods to identify organisms. The development of molecular techniques to investigate ecological microbial communities has provided the microbiologist with a vast array of new techniques to investigate human intestinal microflora. Metagenomics, the science of biological diversity, combines the use of molecular biology and genetics to identify and characterise genetic material from complex microbial environments. The combination of metagenomics and subsequent quantitation of each identified species using molecular techniques allows the relatively rapid analysis of whole bacterial populations in human health and disease

Bacteria permanently colonise the whole length of the gastrointestinal tract with by far the highest concentration of organisms found in the large intestine. These bacterial colonisers of the colon comprise several hundred bacterial species that live in a complex ecosystem with estimates of 10^{12}–10^{14} organisms per gram of faecal material.[1 2] Anaerobic bacteria predominate, with bacterial numbers increasing progressively through the colon.[3] The study of this complex ecosystem has been carried out, until recently, by traditional culture techniques using viable counting of colonies and biochemical methods to identify organisms.[1 3 4] There are a number of advantages and disadvantages in using culture methods to investigate diverse and complicated ecosystems that grow in challenging environments (see table 1). The development of molecular techniques to investigate ecological microbial communities has provided the microbiologist with a vast array of new techniques to investigate human intestinal microflora (reviewed by Macfarlane and Macfarlane[3]). The relative pros and cons of molecular analysis are outlined in table 2.

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Much of the previous more traditional microbiology carried out on inflammatory bowel disease (IBD) has focused on the search for a causative bacterial agent, with many and varied candidates being proposed.[5–9] It has now been generally accepted that analysis of the microbial ecosystem and changes in the balance of organisms at initiation and during disease yields far more relevant information than hunting for the proverbial ''needle in the haystack''. This change has partly been driven by the general ineffectiveness of targeted antibiotic therapy to treat IBD[10–14] and the potential of probiotics as therapy for IBD, allowing re-establishment of homeostasis present in healthy gut.[15–17]

In order to develop these alternative therapies it is essential to determine what comprises a healthy colonic ecosystem and how this balance of organisms is altered during various states and stages of IBD. As a large majority of bacterial species present in the colon are effectively unculturable,[18 19] it is impossible for detailed examination of the colonic microflora to be achieved using traditional culture techniques. The increased ease in which molecular analysis can be carried out by most microbiologists has led to an explosion in sequencing of ribosomal DNA (rDNA) from different bacterial species and strains from many different environments. This has allowed the construction of relevant sequence databases.

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The rDNA gene has regions of consensus that are identical for all bacteria, and regions of variability that are specific for particular groups and species.[2 18 20] Within these variable regions there are also small areas of hypervariability that may be unique for different strains of the same organism.[20] Therefore, rDNA sequences can be used to identify different species and strains of particular species within complex mixed bacterial communities using array technology. Only high throughput molecular techniques that can examine multiple organisms from multiple donors, both healthy and IBD, can provide an accurate picture of the complexities of these bacterial communities.[19]

Metagenomics has been defined as the science of biological diversity; it combines the use of

Correspondence to:
Dr E Furrie, Department of Immunology, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK; e.furrie@dundee.ac.uk

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Abbreviations: IBD, inflammatory bowel disease; rDNA, ribosomal DNA; qPCR, quantitative real time polymerase chain reaction

Table 1 Advantages and disadvantages of classical culturing techniques to identify and examine intestinal species

Advantages	Disadvantages
Relatively inexpensive	Slow, time consuming, and labour intensive
Widely available	Samples require immediate processing
Allows quantification of bacterial populations	Extensive expertise and specialised equipment needed to isolate strict anaerobes
Can provide a good indication of ecosystem complexity, if carried out by skilled and experienced microbiologist!	Restricted to culturable organisms
Physiological studies are possible	Selection of growth media can greatly affect results. Not all viable bacteria can be recovered
Biochemical studies are possible	Once isolated, bacteria then require identification using a number of techniques

molecular biology and genetics to identify and characterise genetic material from complex microbial environments. A full metagenomic approach is a comprehensive study of nucleotide sequence, structure, regulation, and function, providing a picture of the dynamics of complex microbial communities.[21 22] This approach can identify the diversity, but not the relative numbers, of each species residing in that particular environment. This analysis requires the production of a metagenomic library that, in theory, contains all the genetic material present in the initial sample but in a form that can be readily analysed by the researcher. The completeness of this library is entirely dependant on the initial extraction of total genetic material from the primary source.

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There is potential for significant bias in the metagenomic approach as different bacteria are more or less susceptible to lysis, with Gram positive organisms being particularly resistant.[23 24] Therefore, DNA extraction must be optimised for construction of an effective library. A second source of potential bias is during manipulation of the genetic material to construct the library. Each extracted piece of DNA must be able to insert into the vector (fosmid) with equal efficiency to give a library representative of the original material. Once the library is constructed each individual clone must be analysed using a DNA probe. Correct selection of this probe is critical for the balanced analysis of the library.

In this issue of *Gut*, Manichanh and colleagues[25] describe how they constructed a mixed universal probe by amplifying DNA extracted directly from healthy faecal samples using universal bacterial specific primers to optimise the hybridisation potential of the probe, and maintain, on analysis, the diversity of the original material used to construct the library ***(see page 205)***. With all array analysis it is essential to check the results obtained in the array using a quantitative molecular technique. There are two molecular options, either fluorescent in situ hybridisation or quantitative real time polymerase chain reaction (qPCR). These techniques, unlike the metagenomic approach, require the target organism (and sequence) to be known. Construction of specific fluorescent probes (in situ hybridisation) or primers (qPCR) allows quantitative analysis of organisms previously identified in the metagenomic library.

In situ hybridisation has previously been used to determine sites of colonisation and quantify bacteria in IBD.[26] This technique has the advantage of allowing analysis of specific bacterial species in situ on mucosal tissue and faecal samples, enabling the spatial relationship between different organisms in a particular environment to be investigated.[27] It can also be developed into a high throughput assay by coupling in situ hybridisation with flow cytometry and the potential of analysing up to seven different bacterial specific fluorescent probes in unison.[28 29] qPCR has generally been the method of choice for quantitative confirmation of array analysis, particularly quantitation of specific gene expression, but it can also be used to determine numbers of specific bacteria using primers designed to anneal to species unique areas of the rDNA gene.[17 24 30 31]

''The combination of macroarray technology (metagenomics) and the subsequent quantitation of each identified species using molecular techniques allows the relatively rapid analysis of whole bacterial populations in human health and disease''

Table 2 Advantages and disadvantages of using molecular techniques to investigate intestinal bacterial populations

Advantages	Disadvantages
High throughput and relatively short learning time with most techniques	Difficult to standardise extraction of genetic material from each species equally. Severe bias possible in mixed populations
Anaerobic handling and expertise not required	Can be very expensive
Samples can be frozen for later analysis	Selection of primers and probes can introduce severe bias in detection
DNA can be transported easily between laboratories	Many methods are not quantitative so confirmatory analysis is necessary
Unculturable species are detectable	Impossible to model ecosystem
In theory, down to one molecule of target DNA can be quantified.	Some methods are very insensitive

The combination of macroarray technology (metagenomics) and the subsequent quantitation of each identified species using molecular techniques allows the relatively rapid analysis of whole bacterial populations in human health and disease. It removes the problem of organisms that are either difficult or impossible to culture, and further introduces the possibility of analysing gene expression in these organisms, directly from their natural environment, thereby removing any bias introduced through manipulation and repeated culture passage.

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