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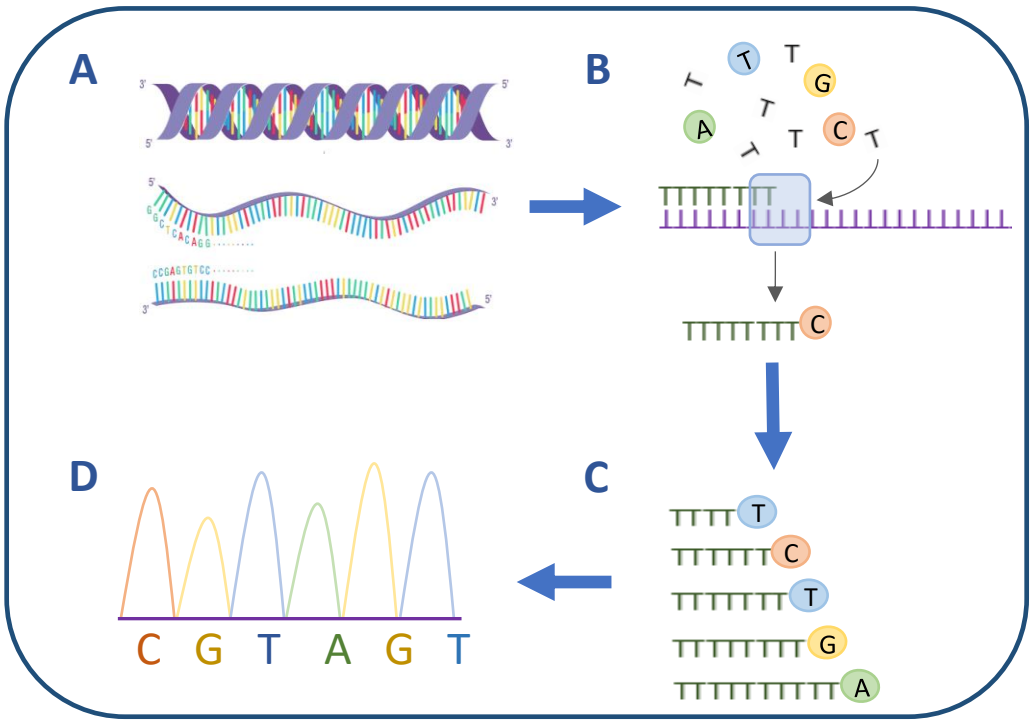
Sequencing at Micropathology Ltd

Sequencing technology

We use ABI 3500xL genetic analysers in combination with ABI BigDye v3.1 technology to perform our in-house DNA sequencing. This technology, referred to as Sanger sequencing, is a variant of the chemistry first described by Fred Sanger in 1977 and is based on the protocol described below:



(A) A sequencing reaction mix containing amplified template DNA, oligonucleotide primers and a mixture of 'normal' dNTPs and fluorescently tagged terminator dNTPs is heated to 95^{O}C to denature the double stranded DNA template.

(B) The temperature is reduced and short, complementary primers bind to the now single-stranded template. A subsequent extension step at an enzyme-specific temperature allows DNA polymerase to replicate the template. The inclusion of

chain-terminating fluorescently tagged nucleotide analogues leads to the random termination of growing DNA molecules.

(C) Appropriately concentrated reaction mixtures will produce a pool of molecules containing representatives that have been terminated at every position of the original template amplicon.

(D) This pool of DNA strands of varying lengths is then separated chromatographically in hair-thin capillaries and the sequence read by analysing the emerging fluorescent tags as they pass the capillary terminus.

We routinely employ sequencing technology to aid the identification, genotyping and drug resistance testing of some pathogens, as described below:

Identification of unknown pathogens

There are certain circumstances under which traditional diagnostic tests for specific infecting organisms cannot be applied. This occurs most frequently where infections are culture negative or caused by organisms which grow slowly, are fastidious in their growth requirements or are identified only rarely in human infection.

We perform both **16S rRNA** analysis, a genetic locus common to all bacterial species that can be used for phylogenetic typing, and a similar test targeting the **18S rRNA** locus found in fungi. Although these regions are universal to a wide range of organisms, there is sufficient nucleotide sequence variation to enable identification at the genus and sometimes species level. Bacterial and fungal phylogenetic typing is accomplished by searching against the NCBI BLAST Local Alignment Search Tool.

We also perform phylogenetic typing for the **Mycobacteriaceae family**, as well as **Pseudomonas** and **Mycoplasma** genera. These tests target regions specific to the family/genera in question and sequence data analysis of the resulting product is usually sufficient to permit species identification. In addition to these, we employ Sanger sequencing to confirm results from various targeted assays.

Viral genotyping

Many viral species have been classified into genotypes on the basis of clustering by nucleotide sequence identity. This information is frequently of clinical relevance as treatment response or severity of infection in an individual may differ with infecting genotype. We routinely perform **Hepatitis B and C genotyping** by searching specific, curated databases for these organisms with nucleotide sequence data obtained from genotyping assays. These assays are designed to target regions which exhibit some level of conservation sufficient to allow successful amplification across the range of observed genotypes but are diverse enough to allow their distinction by sequence analysis. Genotyping of **human papilloma virus and adenovirus** are accomplished by searching against the NCBI BLAST Local Alignment Search Tool.

Drug resistance testing

Where therapeutic options for the treatment of chronic bacterial and viral infections exist, the development of drug resistance is frequently inevitable. This process is dependent upon a number of factors including patient adherence to their treatment regimen and the genetic barrier to the development of a drug resistant phenotype.

Phenotypic drug resistance is initiated by genetic mutations that lead to changes in the structure, binding capability or function of pathogen proteins targeted by the drug in question. Where sufficient data is available, particular mutations can be assigned predictive values representing their likely effect on drug susceptibility phenotype. Genotyping assays are frequently quicker and easier to perform than phenotyping but novel mutations or resistant phenotypes caused by complex genotypic combinations may not be identified.

Bacterial Drug Resistance Screening

We utilise Sanger sequencing methods to identify **macrolide/ fluoroquinolone resistant *Mycoplasma genitalium*** and **rifampicin resistant *Mycobacterium tuberculosis*.**

Viral Drug Resistance Screening

We routinely test for **resistance to multiple drugs commonly used to treat HBV, HIV-1, CMV, and HSV-1 infections.** To this end, we have adopted Illumina next generation sequencing (NGS, see below for description) technologies which promise improved detection capabilities over traditional Sanger based drug resistance screening for low-level drug resistance mutations. Our NGS screening assays can reliably detect drug resistance mutations as low as 5% frequency within the viral population (compared to traditional Sanger methods which typically detect mutations at 15-20% frequency).

Once an appropriately predictive amplicon has been sequenced, analysis can be performed using one of two pipelines. A curated database can be queried if it exists for the organism in question (e.g. HIV) or alternatively the consensus sequence can be compared to a reference list of mutations known to be associated with drug resistance.

Next generation sequencing (NGS)

During the last decade, sequencing technologies using methods that diverge significantly from traditional Sanger sequencing have proliferated. These ‘next generation’ methods can vastly increase the number of sequence reads obtained in a single run and are thus commonly described as ‘massively parallel sequencing’.

We currently utilise two Illumina MiSeq instruments which allow for paired-end next-generation short-read sequencing. Using this technology, template DNA is fragmented into short fragments and labelled with unique adaptors during library preparation. The labelled, single-stranded DNA fragments are then bound to the Illumina flow cell via adaptor hybridisation to oligos on the flow cell’s surface. A polymerase reaction then

extends the flow cell-bound oligo to generate a complement of the hybridised fragment. This is then denatured and the original template is discarded. A series of adaptor-mediated bridge amplification and wash steps clonally amplifies the complement sequences to produce clonal clusters of flow cell-bound DNA fragments in the forward orientation for sequencing by synthesis.
In this process, the DNA fragments are reverse complemented by the sequential addition of fluorescently labelled nucleotides and excitation by a light source. This generates a characteristic fluorescent signal for each clonal cluster which can be translated in parallel by the Illumina MiSeq into series of base calls for each of these 'forward reads'. The fluorescently labelled strand is discarded, the forward template strand is reverse complemented by another adaptor-mediated bridge amplification step to produce a reverse template sequence, the forward template is discarded and finally a second sequencing by synthesis step produces base calls for each of the 'reverse reads'. Together this process generates forward and reverse read sequences that are labelled with base quality information in the fastq format. Further information is available on the Illumina company website. In addition to this, we are also exploiting Oxford Nanopore long read sequencing technologies for research and diagnostic purposes.

Both Illumina and Oxford Nanopore sequencing technologies are used for research projects describing microbial population biology, genetic variation within the virulence factors of a single species, whole genome sequencing, diagnostic analysis of challenging mixed infections and the detection of very low-level drug resistance associated mutations. Please contact our laboratory at **info@micropathology.com** for further information on how we can help meet your sequencing requirements.