Investigating molecular diagnosis of tuberculosis using transrenal DNA. The nucleic acid extraction step.

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Background

Tuberculosis (TB) is one of the most prevalent infectious diseases in the world. It is caused by the bacterium *Mycobacterium tuberculosis (Mtb)* and results in >2 million deaths a year. While global awareness has improved, diagnostic methods remain very poor especially in the parts of the developing world where HIV has resulted in a TB epidemic.

The most important role for biotechnology in improving health in developing countries has been set at improving infectious disease diagnosis[1]. But diagnostic methods for diseases like TB have remained limited with poor clinical utility partly because the most commonly used clinical sample, sputum, is highly heterogeneous and difficult to extract DNA from.

Recently detection of the *M. tuberculosis* DNA in the urine of patients with TB has been reported[2]. However results have been variable and studies have not approached this systematically.

Before studying this phenomenon in more detail we are investigating optimal extraction methods for purifying DNA from urine. Furthermore we are interested in purifying the free trans-renal DNA (trDNA) that passes through the kidneys from part of the circulating nucleic acids in plasma and serum (CNAPs).

Aims

Test different methods for purifying free DNA from urine for

- **Sizes of DNA recovered**
- **Removal of PCR inhibitors**

Methods

Urine from 10 healthy male patients was obtained and mixed with 40 mM EDTA and 3500 bp TB 16S/vector amplicon to 1000 copies / µl of extract (experiment A) or 71 bp and 450 bp TB 16S amplicon to 100000 or 10000 copies / µl of extract (experiment B) respectively.

DNA was purified from the respective urines using a number of different extraction procedures.

1. Phenol chloroform
2. Poly ethylene glycol (PEG method[3])
3. Promega (Wizard Plus[4])
4. Biomereux (NucliSens®)
5. Magnapure (Bacterial DNA robot extractor, Roche)
6. Xenomics (Urine extraction protocol)
7. Nexttec™genomic isolation kit for blood

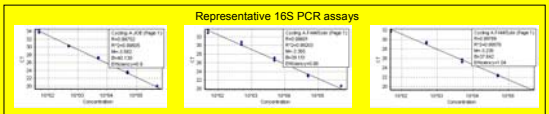
Samples were measured for recovery and inhibition (See T. Novak poster 171) using different Mtb16S PCR reactions (Table 1) with the Rotorgene 3000 (Corbett research).

Components	Final Concentration	Primers	Sequence
Biogene 10X Buffer	1X	Mtb_16S_F	CAAGTCGAACGGAAAGGTCT
$MgCl_2$	3 mM	Mtb_16S_R	ACCCAGTTTCCCAGGCTTAT
tRNA	250 ng/µl	Mtb_16S_Rs	GCAGATCACCCACGTGTTAC
Primers	600 nM	16S Probe	FAM-CCCGTTCGCCACTCGAGTATCTC-BHQ1
Probe	80 nM	16S Comp Probe	HEX-CGCCGAACTGGGTCGACCTTC-BHQ1
dNTP	200 µM	**Temperature**	**Duration**
Biogene Hot Taq	0.1 U/rxn	95°C	8 min
dH_2O	To 12.5 µl	95°C	5 sec
		55°C	5sec
		72°C	10 sec
			X 45

Table 1. TB 16s Reactions

Mtb16S PCR assays
A) Mtb_16S_F, Mtb_R & 16S Comp Probe
B) Mtb_16S_F, Mtb_Rs & 16S Probe
C) Mtb_16S_F, Mtb_R & 16S Probe

Representative 16S PCR assays



Mtb16S A competitor | Mtb16S B | Mtb16S C

Results, Experiment A: Recovery & Inhibition

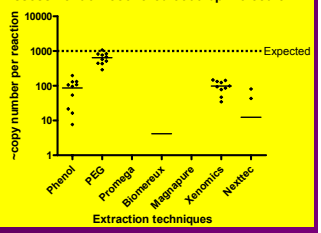
•Purpose: to assess a number of extraction methods for purifying free DNA (3500 bp) from urine

Recovery of spiked sample

•Xenomics and PEG methods yield the most detectable target amplicon using Mtb16S competitor A.

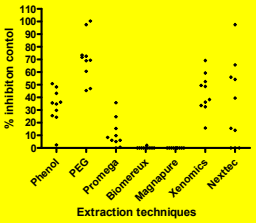
•However this "real time PCR" assessment will also be susceptible to any sample inhibition.

Assessment of recovered 3500 bp molecule



Effect of inhibition

Inhibition assessment

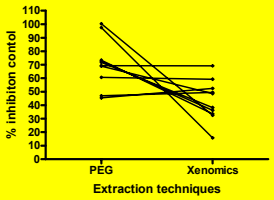


•Xenomics and PEG methods also remove the most inhibitors (@ 10,000 copies/ rxn).

•Many DNA extraction methods may not be suitable for urine if PCR methods are to be used on the extracts.

Inhibition is not sample specific

Paired Inhibition assessment



•Xenomics and PEG methods do allow some inhibition.

•However this appears not to be just due to the sample but a combination of extraction method and sample.

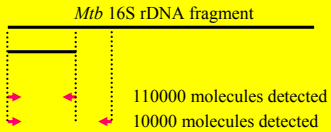
Results, Experiment B: smaller molecules

•Purpose: to assess Xenomics extraction methods ability to purify smaller DNA molecules

•Strategy:

DNA spiked into urine:
10,000 copies 450 bp
100,000 copies 71 bp

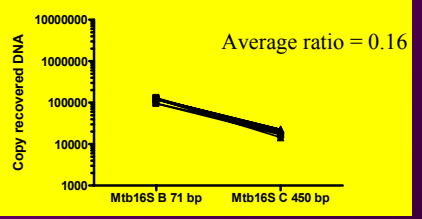
qPCR reactions
Mtb 16S B
Mtb 16S C



•Extraction of both molecules with equal efficiency will result in a C:B ratio of ~0.09

•Increase in this ratio will indicate reduced efficiency in the purification of the 71 bp molecule

Xenomics extraction



•Xenomics method extracts 71 bp and 450 bp molecules with similar efficiency.

Conclusion

- **Xenomics extraction method is capable of purifying a range of different sized free DNA molecules from urine.**
- **We are currently assessing the PEG method for purification of smaller molecules.**
- **We will use the Xenomics method (along side the PEG method) to fully characterise the phenomenon of *Mtb* trDNA in patients with TB.**

References:

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