Syndromic testing: multiplexing

The Good, the Bad and the Unexpected

Dr Darren Jardine
Principal Scientist
Microbiology

Presentation outline

- **Multiplex PCR**
- **Syndromic Multiplexes – Current status**
  - Advantages / Disadvantages
  - Respiratory Infections
  - Gastroenteritis
  - Herpes Multiplex
- **Syndromic Multiplexes – Future directions**
  - Bacterial vaginosis
  - Dermatophytes

What is a Multiplex PCR?

Multiplex real time PCR.

- Detection of more than one target in a single PCR reaction/tube
  - A multi-pathogen test
  - Any assay that includes an Internal control

Has many advantages and disadvantages.

Advantages:

- **Prior to the development and uptake of multiplex testing, molecular testing of individual pathogens was performed:**
  - Large number of individual assays
  - Complicated / inconsistent coding
  - Prolonged TATs
  - Results “drip-fed” as individual test were completed
  - More costly
- **Efficient identification of pathogen from a single sample**
  - One Sample – Multiple results
- **Widening the “net” can lead to the detection of pathogens the clinician was not necessarily looking for in the first instance – “Unexpected results”**

Disadvantages

- **Competition**
  - Competition for PCR mastermix between two or more different components of an multiplex assay.
    - the IC and positive target
    - Samples containing >1 target.
    - Assays that detect and type simultaneously
  - The stronger target can be preferentially amplified to the detriment of the weaker target.
    - Can result in false negative results
- **Understanding of “positive” and “negative” results**

Syndromic testing

- **Patients can often present with signs and symptoms that are indicative of a disease, but are not specific enough to clinically distinguish what makes them sick.**
- **Common signs and symptoms are called ‘syndromes.**
- **Patients presenting with:**
  - vomiting and abdominal pain
    - Gastroenteritis
  - cough, myalgia and headache,
    - acute respiratory infection respectively,
- **Both syndromes that have wide and diverse infectious causes, which may be viral, bacterial or fungal.**

Syndromic testing

- **Acute respiratory tract infections**
- **Gastroenteritis**
- **Meningitis**
- **Genital infections**
- **Vesicular rashes**
- **Conjunctivitis**

Acute respiratory tract infections

- **Upper respiratory infections – Potential targets**
  - Influenza A and B,
  - Parainfluenza 1, 2, 3, 4,
  - Respiratory Syncytial Virus A & B,
  - Adenovirus groups B, C, E, some A, D,
  - Rhinovirus / Enterovirus,
  - Metapneumovirus,
  - Parechovirus,
  - Coronaviruses,
  - Bordetella pertussis / parapertussis,
  - Mycoplasma pneumoniae
- **Upper respiratory infections – Specimens**
  - Throat / Nasal / Nasopharyngeal swabs

Acute respiratory tract infections

- **Lower respiratory infections / Pneumonia – Potential targets**
  - Mycoplasma pneumoniae, Chlamydophila pneumoniae and psittaci, Legionella pneumophila and longbeachae,
  - Haemophilus influenzae, H. parainfluenzae & H. haemolyticus
  - Streptococcus pneumoniae
  - Staphylococcus aureus
  - Bordetella pertussis / parapertussis
  - Coxiella brunetii
  - Mycobacterium tuberculosis complex
  - Aspergillus fumigatus
  - Pneumocystis jirovecii (PCP)
  - Cryptococcus neoformans
- **Lower respiratory infections / Pneumonia – Specimens**
  - Sputum, BAL, Bronchial washings

Acute respiratory tract infections

- **Advantages**
  - Assists in the specific and differential diagnosis of acute respiratory tract infections.
  - Enables the clinician to instigate earlier targeted treatment of viral or bacterial infections avoiding inappropriate antibiotic therapy.
  - Widening the “net” can lead to the detection of pathogens the clinician was not necessarily looking for in the first instance – “Unexpected results”

Respiratory Multiplex testing: The St Vincent's experience

Prior to 2014

- Respiratory viruses
- B.pertussis/parapertussis
- Atypical pneumonia (Referred tests)

September 2014

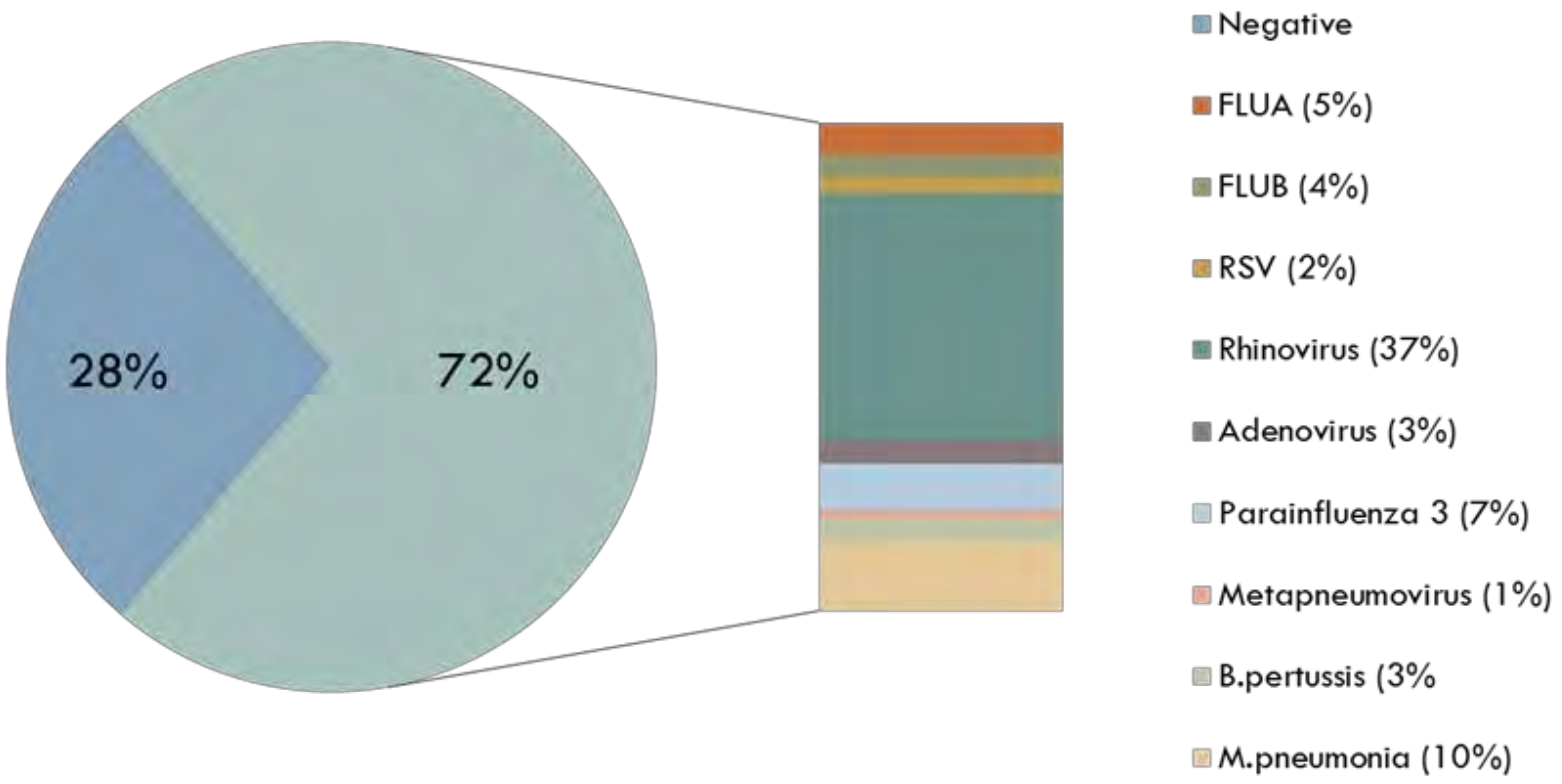
- Respiratory Pathogens (16plex)

Savings:

- Staff time
- $54K/annum

Clinical benefits – "Unexpected results"

Suspected Whooping cough



Pitfalls of Syndromic Testing

- **What does a Negative result mean?**
  - Testing for certain pathogens with an inappropriate specimen can lead to misleading clinical information.
    - A “negative” PCR result for LRTI pathogens on a URT sample (throat/nasal swab) does necessarily mean that they do not have a LRTI infection.

Pitfalls of Syndromic Testing

- **What does a Positive result mean?**
  - Not all positive PCR results for an organism will mean that it is the pathogen responsible for the symptoms.
  - Normal flora
    - *Pneumocystis jirovecii* pneumonia (PCP)
    - Haemophilus influenzae
    - Streptococcus pneumoniae

Pneumocystis pneumonia (PCP)

- **Caused by *Pneumocystis jiroveci.***
- ***Pneumocystis jiroveci* can be present in healthy individuals in the general population.**
  - Molecular detection of *P. jirovecii* in lung fluids does not mean that a person has PCP.
  - PCR is best utilised for excluding the diagnosis of PCP
  - Quantitative PCR may be used to distinguish true infection from colonisation.
    - Indeterminate zone of clinical significance

Gastroenteric infections

- **Viruses – Potential targets**
  - Noroviruses, Rotavirus, Astrovirus, Adenovirus group F and G, Sapovirus and Enterovirus
- **Bacteria – Potential targets**
  - Salmonella species, Shigella species, Shiga toxin 1 and 2, Campylobacter coli & jejuni, Yersinia enterocolitica, Aeromonas species, Vibrio specis, Plesiomonas species.
  - Clostridium difficile (toxinigenic)
- **Parasites – Potential targets**
  - Giardia duodenalis (lamblia), Cryptosporidium, Entamoeba histolytica, Diantamoeba fragilis, Blastocystis hominis, Cyclospora cayetanensis

Pitfalls of Syndromic Testing

- **What does a Positive result mean?**
  - Not all positive PCR results for an organism will mean that it is the pathogen responsible for the symptoms.
  - Enteric pathogens
    - *Dientamoeba fragilis* and *Blastocystis* species

Enteric Parasites

- ***Dientamoeba fragilis* and *Blastocystis* species**
  - The role of as gastrointestinal pathogens is highly controversial.
  - With the recent introduction of enteric PCR with primers for these targets, it was observed that these organisms were more common than previously thought
    - Up to 20% of all faeces received in the laboratory
  - Their pathogenicity is yet to been established in humans.
  - Symptoms are often falsely attributed to the presence of these organisms leading to overtreatment.
  - This can result in possible harm due to disruption of normal gut flora.

Enteric Parasites

- ***Dientamoeba fragilis* and *Blastocystis* species**
- **Guidelines from the RCPA - November 2015**
  - consider using a multiplex PCR without these targets
  - Where PCR is used, its diagnostic the report should contain a comment highlighting the questionable pathogenicity of these two organisms.
  - https://www.rcpa.edu.au

Herpes Multiplex Assays

- **Herpes Multiplex assays**
  - Herpes simplex virus type 1 (HSV-1),
  - Herpes simplex virus type 1 (HSV-2),
  - Varicella zoster virus (VZV),
  - Cytomegalovirus (CMV)

Herpes Multiplex Assays

- **Herpes Multiplex assays**
  - HSV-1,
  - HSV-2,
  - VZV,
  - CMV

- **Vesicular rashes – Potential targets**
  - HSV-1, HSV-2, VZV, Enteroviruses
- **Genital ulcers – Potential targets**
  - HSV-1, HSV-2, *Haemophilus. ducreyi*, and *T. pallidum*
- **Genital lesions– Potential targets**
  - HPV, HSV-1, HSV-2 and *C. trachomatis*
- **Ocular Infections - Potential targets**
  - Adenovirus, HSV-1, HSV-2, VZV, CMV, *C. trachomatis, N, gonorrhoeae,* Toxoplasma
- **Viral Meningitis – Potential targets**
  - HSV-1, HSV-2, VZV, CMV, HHV-6, EBV, Enteroviruses, Parechoviruses

Herpes Multiplex Assays

- **Herpes Multiplex assays used to aid in the diagnosis of:**
  - Viral Meningitis
  - Genital infections
  - Vesicular rashes
  - Viral conjunctivitis

Herpes viruses:
The St Vincent's experience

ST VINCENT'S HOSPITAL MELBOURNE

Prior to 2011

- HSV 1&2 (Roche Lightcycler)
- VZV and Adenovirus (In-house assays)
- CMV and Enterovirus (referred tests)

Dec 2011

- Viral conjunctivits (Easyplex) HSV/VZV/Adeno
- Herpes 6 (Easyplex) HSV/VZV/CMV

Sep 2014

- Herpes, Adenovirus and Enterovirus (Highplex)

Consolidation of assays – Herpes viruses, Adenovirus, Enterovirus

ST VINCENT'S HOSPITAL MELBOURNE

Savings:

- Staff time
- $20K/annum

Clinical benefits

- CSF specimens (Herpesviruses and Enteroviruses)
- Ocular specimens (Herpesviruses and Adenoviruses)
- Genital specimens – “Unexpected results”

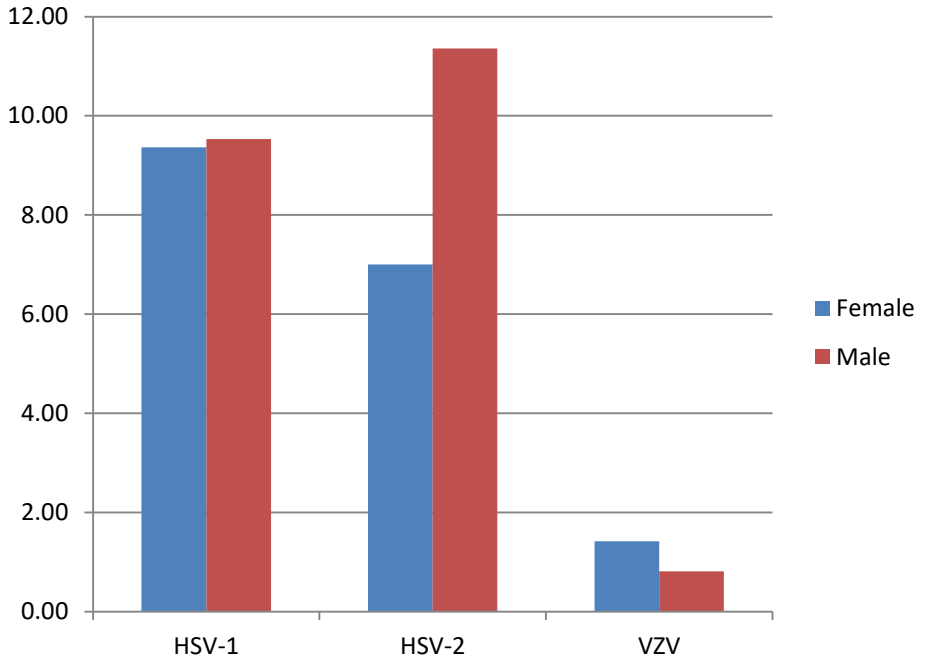
Genital Herpes

- **Genital HSV-1 infection**
  - Oral sex
  - No previous exposure to HSV-1
- **Genital HSV-2 infection**
  - Associated with frequent symptomatic recurrences
- **Varicella zoster virus**
  - Primary infection – chickenpox
  - Reactivation - herpes zoster (shingles)
  - Herpes multiplex assays contain primers/probes to VZV
  - a small but significant number of cases of presumed genital HSV infection are caused by VZV.

Herpes multiplex testing of Genital specimens

2010 - 2013

- 1978 genital specimens tested
  - -1485 Female
  - -493 Male



Pitfalls of Syndromic Testing

- **What does a Negative result mean?**
  - Syndromic testing is only useful if the patient has the “Syndrome”
  - Testing for certain pathogens in the absence of symptoms can lead to misleading clinical information.
    - A “negative” HSV PCR result on a patient that has no symptoms does mean that they do not have herpes.
    - A “negative” Syphilis PCR result on a patient that has no symptoms does mean that they do not have Syphilis.
  - Specimens must be collected at the time of clinical presentation
  - Specimens must be collected appropriately to ensure that the genetic material of the pathogen is present

Future applications for Syndromic testing

- **Bacterial vaginosis**
  - Imbalance of vaginal flora
    - Reduction of vaginal *Lactobacilli* and an overgrowth of other commensal anaerobic bacteria.

- **Normal Flora**
  - Complex ecosystem
  - Lactobacilli dominate
  - pH 4.0 to 4.5
- **Bacterial vaginosis**
  - Bacterial load *G vaginalis* & CGNR significantly ↑
  - Lactobacilli ↓
  - Elevated pH >4.5

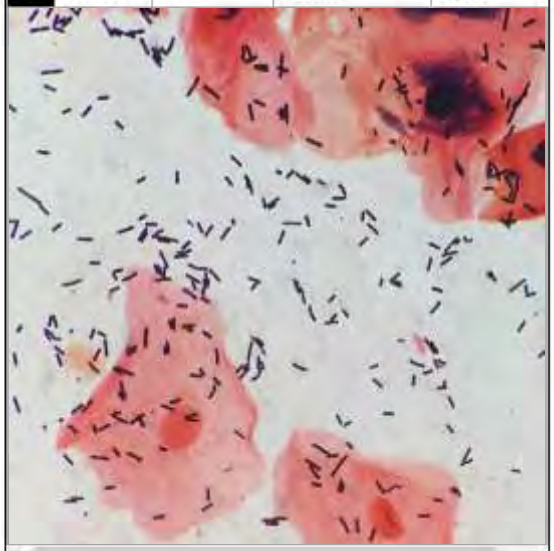
Molecular detection of Bacterial vaginosis

- **What does a Positive result mean?**
  - Not all positive PCR results for an organism will mean that it is the pathogen responsible for the symptoms.
  - Multiplex PCR allows for the detection of the various bacterial species present in a sample.
    - Balance between “good” and “bad” bacteria
    - It is not the presence of the various species that determines BV, but the
      - Relative proportions of these species
      - Clinical picture.

Normal Flora

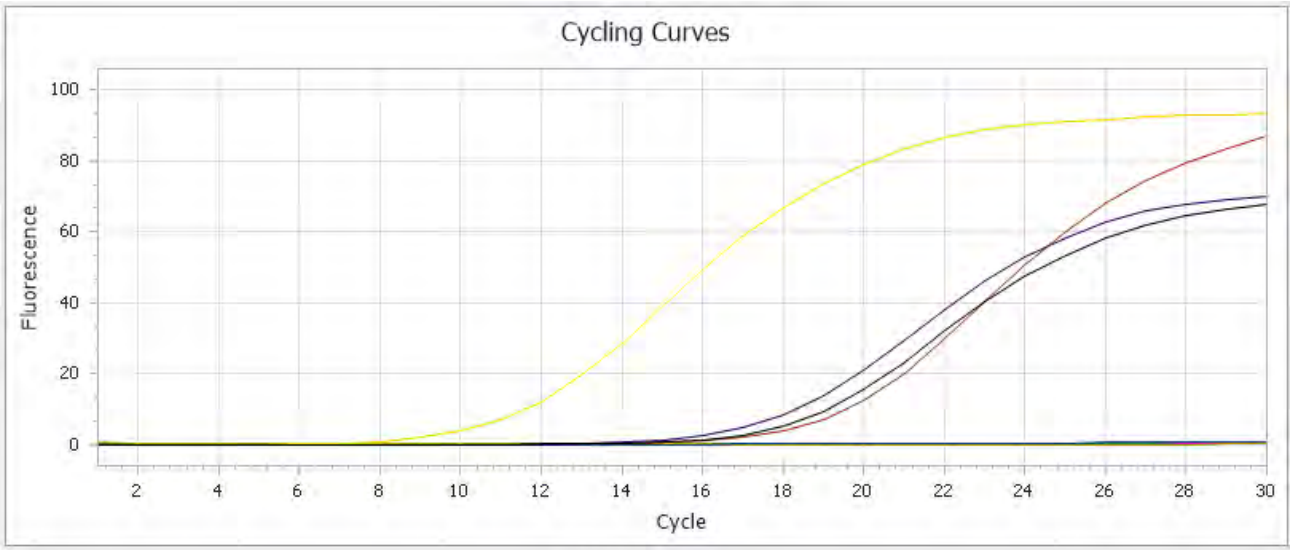
Results

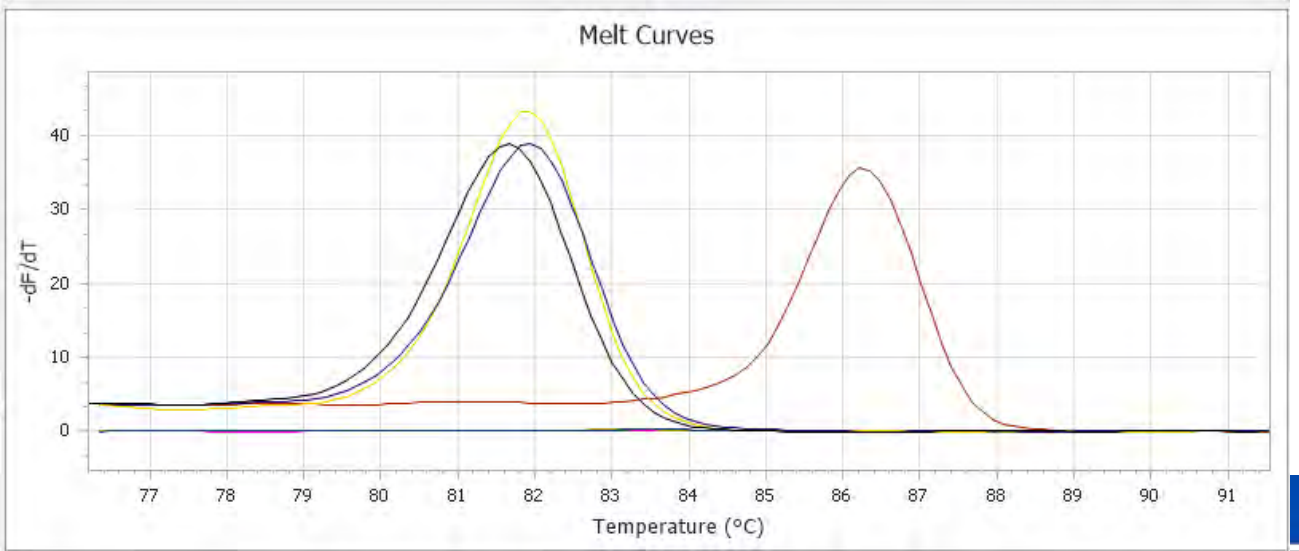
No.	Sample	Gene	Call	Concentration
A13	M463174	Trichomonas		
C13	M463174	Candida		
E13	M463174	A.vaginae		
G13	M463174	G.vaginalis	Present (1%)	6,288
I13	M463174	L.iners		
K13	M463174	L.crispatus	Present (99%)	1,116,045
M13	M463174	NONO	Present	17,361
O13	M463174	SPIKE	Present	10,000



Diagnosis

High L.crispatus
Bacterial balance (0 to 10) = 0.06
Normal Flora

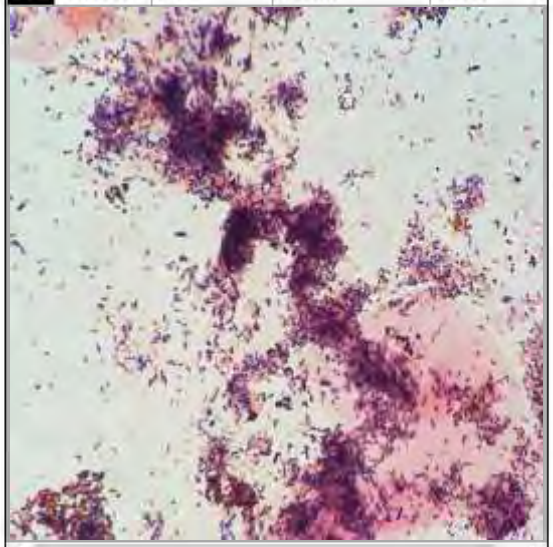




Bacterial Vaginosis

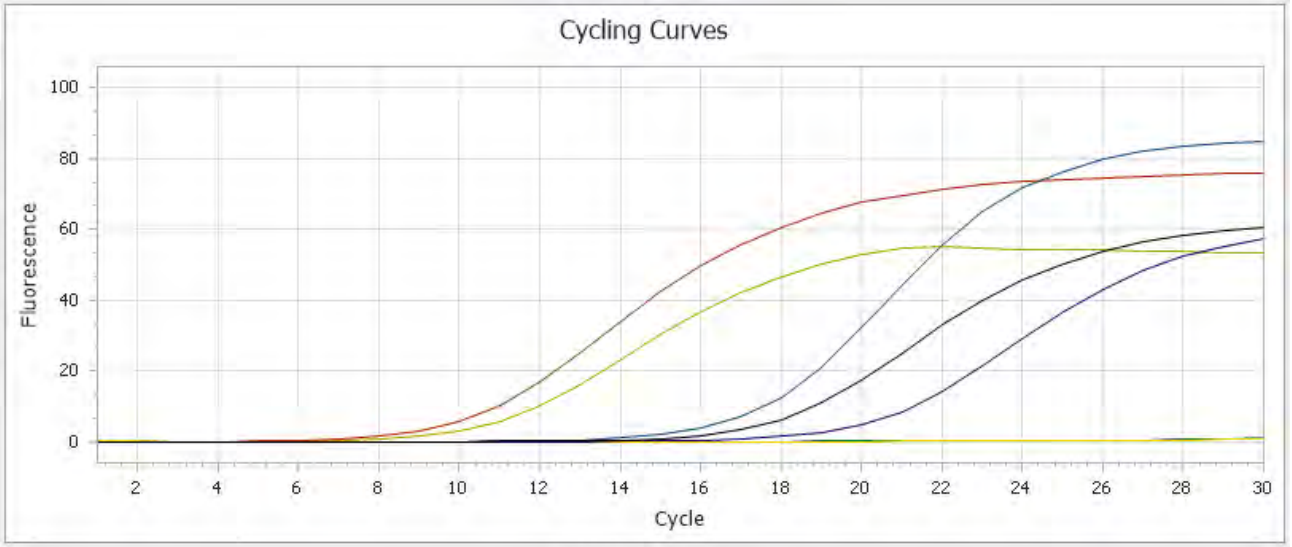
Results

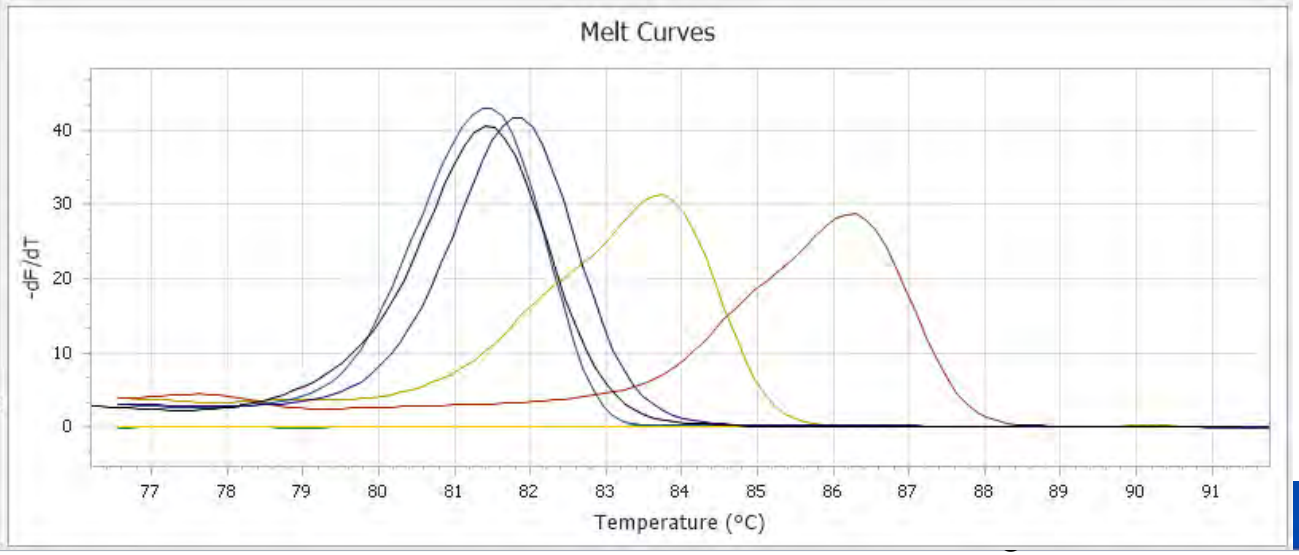
No.	Sample	Gene	Call	Concentration
A8	M463311	Trichomonas		
C8	M463311	Candida		
E8	M463311	A.vaginae	Present (42%)	1,086,210
G8	M463311	G.vaginalis	Present (58%)	1,515,132
I8	M463311	L.iners	Present (1%)	14,452
K8	M463311	L.crispatus		
M8	M463311	NONO	Present	2,177
O8	M463311	SPIKE	Present	10,000



Diagnosis

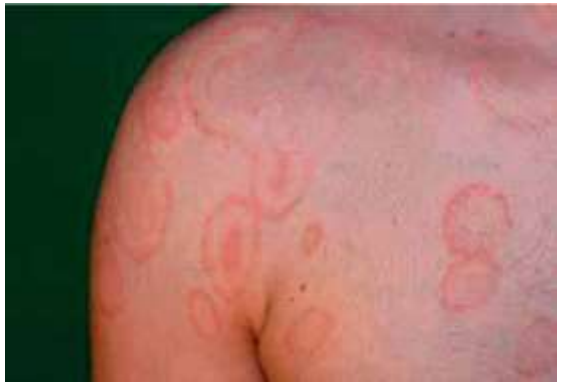
High bacterial load
Bacterial balance (0 to 10) = 9.94
Flora consistent with Bacterial Vaginosis

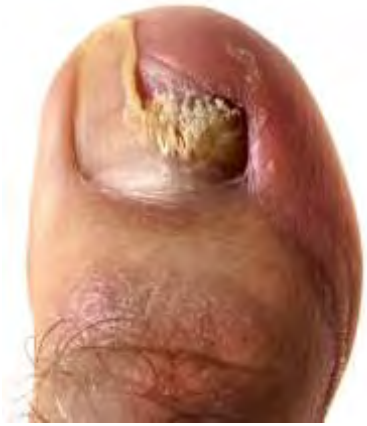


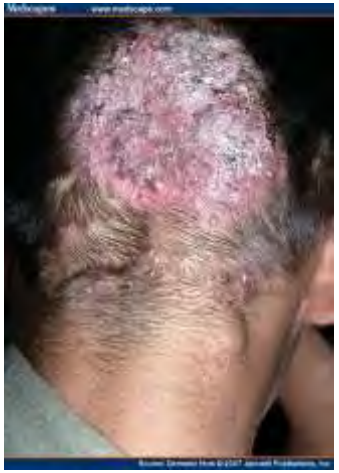


Future applications for Syndromic testing

- **Dermatophytes**
  - A pathogenic fungus that commonly causes skin, hair and nail infections in both humans and animals.
  - They obtain their nutrients for growth from material containing keratin.
  - Commonly referred to as tinea or ringworm.

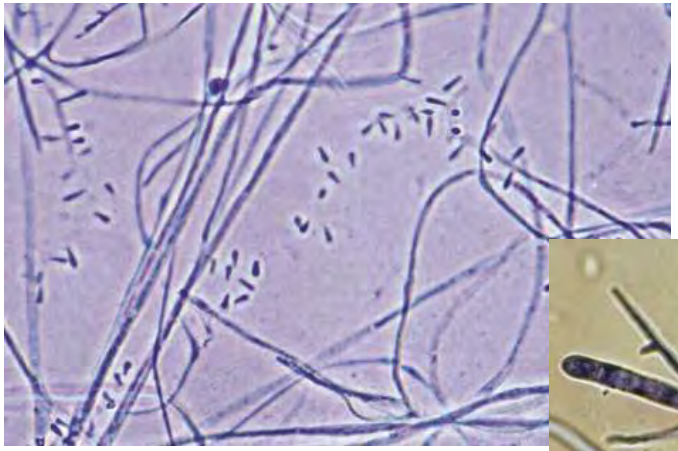






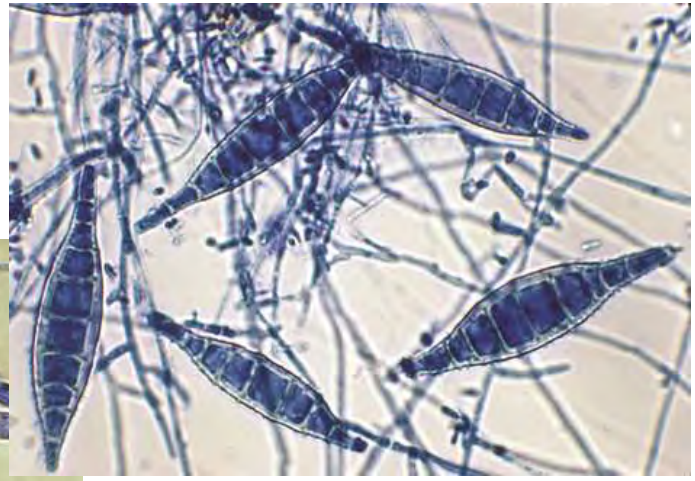
Common Dermatophytes

- **Trichophytonon (rubrum, mentagrophytes)**
- **Epidermophyton floccosum**
- **Microsporum (audouinii, canis)**



Trichophyton sp

Epidermophyton floccosum



Microsporum sp

Current Procedure for Dermatophytes

Primary Sample

- Blankophor slide and DERM plate set up.

Culture

- Plates incubated in O_2 at 26^{o}C for 3 weeks
- Plates checked twice a week for positive growth

Identification

- Identification via colony morphology, Maldi-Tof and short-tests
- Short tests incubated in O_2 at 26^{o}C for 1-2 weeks

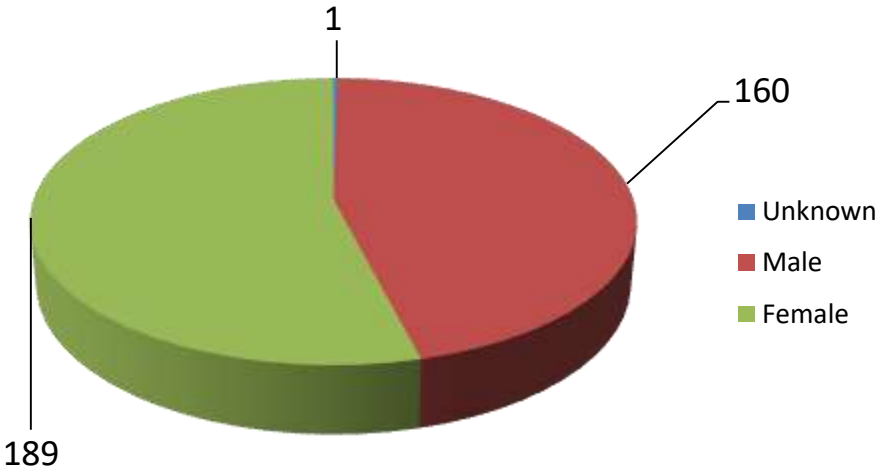
Study design

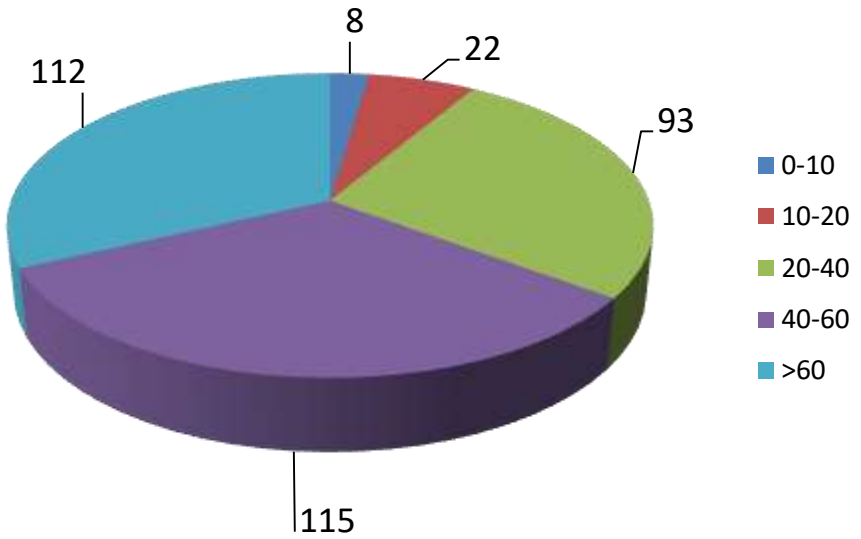
- A Prospective study running in parallel with microscopy and culture.
- 350 samples tested in parallel
  - Microscopy vs PCR
  - Culture vs PCR
- Replace current culture methods with Molecular assay?
- Study focused on dermatophytes only
  - Trichophytonon spp, E. floccosum , Microsporum spp

Method

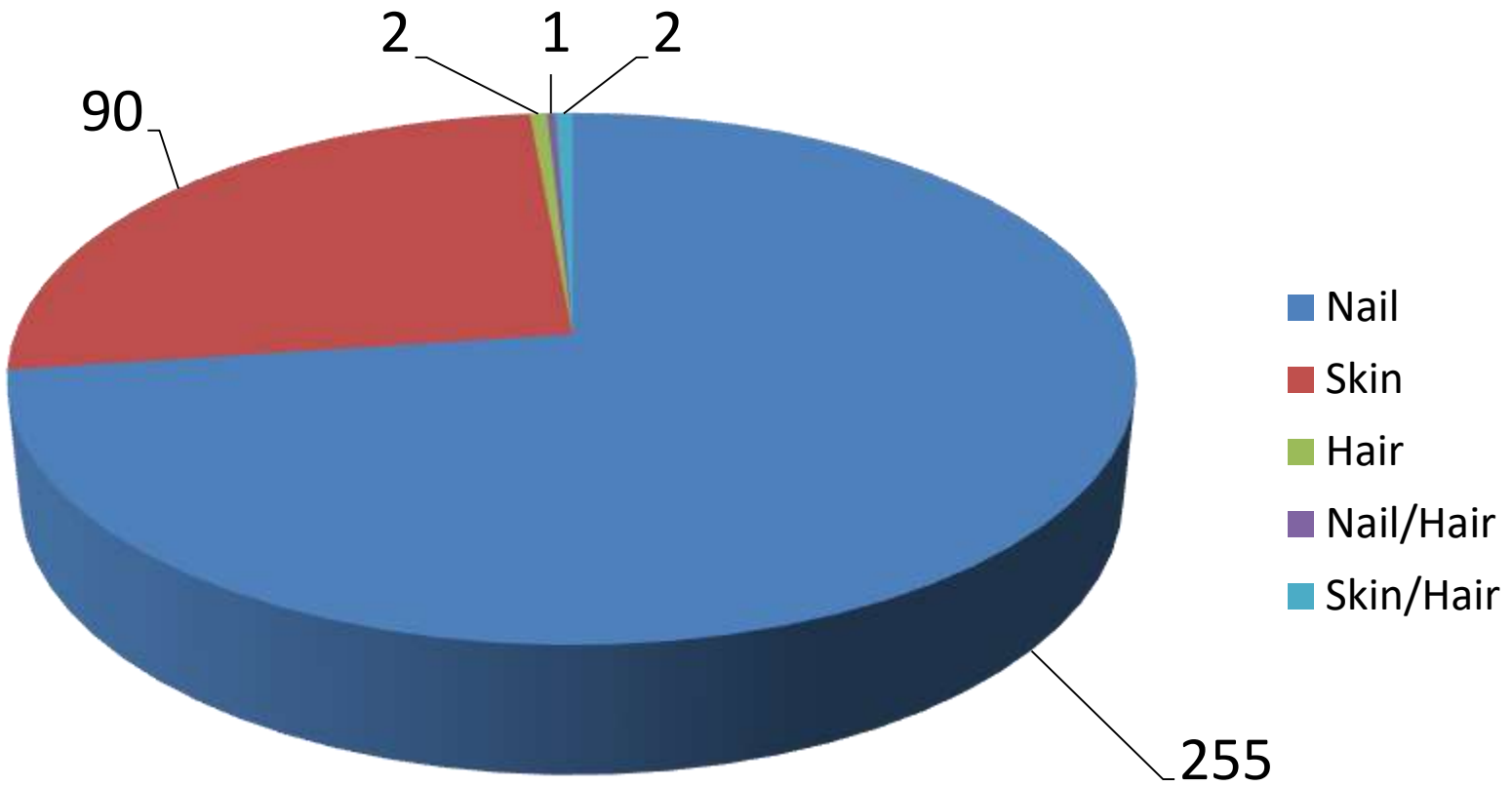
- Nails clippings, skin scrapings or hair samples were placed into a 2ml tube with the following:
  - 300ul sterile saline,
  - 180 ul MagNA Pure 96 Bacterial Lysis buffer
  - 20ul Proteinase K, recombinant PCR grade
- The contents were briefly vortexed then incubate at 65^{o}C for 30 mins.
- The samples were then briefly vortexed and then centrifuged at 13,000rpm for 10 seconds to pellet debris.
- 200ul was extracted using the AusDiagnostics MT Prep.
  - 50 µL elute
  - 10 µL extract tested using the AusDiagnostics Dermatophytes and Other Fungi assay

Part B – Gender and Age





Specimen type



Culture vs PCR

	Culture	PCR
T.rubrum	34	32*
T.mentagrophytes	4	4
T.violaceum	1	T.rubrum **
Microsporum canis	2	2

* 1 identified as *T.mentagrophytes* by PCR, 1 as Trichophyton spp alone
** 1 identified as *T.rubrum* by PCR

Discrepant ID samples were sent out to AusDiagnostics for sequencing. Results came back confirming the PCR results were correct.

Time savings:
Turnaround and Operator

- **200 requests/month**
- **2-3 plates/week (22 samples)**
- **Receipt of specimen to PCR result**
  - 3 days
- **Receipt of specimen to culture result**
  - 24 days
- **Scientist time for PCR (22 samples)**
  - 30 min
- **Scientist time for culture (22 samples)**
  - 3 hours

Summary

- **Multiplex real time PCR has many advantages.**
  - **Efficiencies through Multiplexing**
    - ***Faster***
    - ***Cheaper***
    - ***Improved workflow***
    - ***Clinical benefits***
- **Important to be aware of pitfalls associated with these assays**
    - ***Potential competition***
    - ***Use of appropriate specimen***
    - ***Understanding of "negative" and "positive" results***