Queensland Health Pathology Service

Specimen Processing, Microscopy

Narelle George, Supervising Scientist
Division of Microbiology, QHPS-Central
Overview

- Role of the Microbiology Laboratory in the Clinical Setting
- Functions of the Microbiology Laboratory
  - Specimen Processing
  - Microscopic Examination
Role of the Microbiology Laboratory in the Clinical Setting

- **Diagnosis**
  - Isolation and identification of bacterial pathogens

- **Therapy**
  - Antimicrobial susceptibility testing
  - Reporting of significant pathogens with appropriate sensitivity results

- **Consultative Service**
  - Provision of interpretative comments
  - Advice regarding collection of appropriate specimens
How are Laboratory functions Achieved?

- Diagnosis
  - Specimen collection
  - Specimen receipt and processing
  - Microscopic examination
  - Culture of clinical material
  - Isolation of potentially pathogenic organisms
  - Identification of organisms
  - Antimicrobial Susceptibility Testing
  - Conditional reporting of AST results
  - Addition of interpretative comments
How are Laboratory functions Achieved?

- Diagnosis
  - Specimen collection
  - Specimen receipt and processing
  - Microscopic examination
  - Culture of clinical material
  - Isolation of potentially pathogenic organisms
  - Identification of organisms
  - Antimicrobial Susceptibility Testing
  - Conditional reporting of AST results
  - Addition of interpretative comments
SPECIMEN PROCESSING
AIMS

- Understand the key elements in Specimen Processing
- What constitutes an inadequate specimen
- Procedures for urgent specimens
- Rationale for microscopic examination
- Type of microscopic examinations
- Culture of clinical specimens
Key Elements

- Clock in and accession samples
- Evaluate criteria for acceptance
- Recording the macroscopic appearance of the sample (including viscosity, colour pH)
- Preparation and staining of smears for direct microscopic examination of the sample
- Allocation and Inoculation of culture media according to specimen type
Receipt of Samples

- Comparison of the patient and sample details on specimen and request slip
- Record any relevant clinical information from specimen onto request slip eg collection time, specimen type
- Application of a unique laboratory identification number
- Transfer specimen and patient information to a log book or computer
Specimen Acceptance

- Rubbish in - Rubbish out
- What constitutes an inadequate sample
  - unlabelled
  - leaking
  - too old (>24 hours)
  - received in formalin/alcohol
  - no request slip
Specimen Rejection

- Log and accession specimen
- Contact clinician with details of problem
- Issue a written report indicating reason for rejection of sample
- Keep the specimen
Urgent Specimens

- Dedicated list or record
  - time of request
  - patient details and test required
  - requesting clinician
  - telephone contact
  - details of person receiving request and telephoning results
  - time results telephoned
  - document results - prelim report
Macroscopic Examination

- Perform for all fluid specimens
  - CSF, body fluids, sputum (saliva), faeces

- Assess
  - colour, consistency, viscosity, turbidity, pH

- Why?
  - Useful information to assist microscopy
  - Guide culture procedures (faeces)
  - Guide to culture interpretation (respiratory)
Microscopic Examination

- Indicate the presence of inflammation
  - degree and type of leucocytosis
- Indicate the presence of infectious agents
- Assess the types of microorganisms
  - anaerobic infection
  - guide to initiation of antimicrobial therapy
Types of Microscopic Exams

◆ Wet preparation
  ▼ saline, iodine, india ink, lactophenol blue

◆ Advantages
  ▼ detection of motile infectious agents
  Trichomonas (vagina), Strongyloides/Giardia (faeces)
  ▼ accurate assessment of the WBC:RBC
  ▼ visualise key morphological features
  capsules in India Ink, spiral shape of treponemes in darkground microscopy
Stained Smears

- Biological stain demonstrate internal detail
- Types of
  - Gram stain
  - Ziehl Neelsen / Kinyoun (Acid Fast)
  - IH or Trichome (parasitic)
  - Giemsa (Diff Quik)
  - Calcoflour White (fluorescent fungal stain)
Gram stain

- Most commonly used stain in Microbiology
- Several variations to the method
  - alcohol fixation vs heat
  - different decolourisers: acetone, acetone/alcohol, iodine/alcohol
  - counterstains: safranine vs carbol fuchsin
- Problems with this staining technique
  - over-decolourisation
  - carry over of organisms from one slide to another
  - thicknesses requiring varying decolouration times
Bacterial Characteristics

- Gram stain
- Cellular morphology
Bacterial Cell Walls

GRAM POS

GRAM NEG
Acid Fast Stains

- Detect presence of
  - mycobacteria
  - parasitic elements (Cryptosporidium)

- Two forms
  - hot stain (ZN)
  - cold stain + detergent (Kinyoun)

- Problems
  - under decolourisation
  - inadequate heating giving poor resolution
Smear Preparation

- use glass slides
  - single use - never recycled
- thick/thin preparations may be used for grossly purulent material (cytospin)
- for pus or mucopurulent material, select the most purulent or blood stained portions
- fix by heat or alcohol
Staining Practices

- Filter all stains
- Use small volume containers for daily use
- Do not top up - Clean container and refill
- Pour stain directly onto slide when cool.
- Cover entire slide with a thin film of stain except for the label
- Rinse well with running water after staining
- Air dry in a upright position
Cell Quantitation

◆ Perform on
  ▼ CSF, body fluids, dialysis fluids, urines

◆ Why
  ▼ WBC indicates pathological process
  ▼ WBC CSF differentiates viral/bacterial
      100-1000 viral, >1000 bacterial
  ▼ RBC indicates adequacy of fluid collection
      traumatic tap in CSF
MICROSCOPIC EXAMINATION
Slide Examination

- Macroscopically locate smear on slide
- Focus on slide using low power x10
- Apply a drop of oil
- Do not to get oil on the other objectives
- Swing to the oil immersion lens and focus on the sample. Fine adjustment only should be required
- Wipe oil lens between slides to prevent carry over of organisms from one slide to the next.
Phase Contrast

- Used to examine unstained material
- Objects dark grey against a bright background
- Light rays are refracted at different wavelengths that recombine to reduce light intensity
- Differences are maximised by passing through different thicknesses of glass in the phase lens
- This process retards the diffracted light and reduces the intensity of direct light rays.
- After passing through the phase objective, the two sets of rays are combined to give the image.
Phase Contrast Requirements

- An annular diaphragm that transmits a ring of light through the condenser
- Different annular diaphragm for each objective
- Special lenses with phase plates at the back
  - a glass disc with a circular trough etched into it
  - trough is of a depth that light passing through it is retarded 1/4 of a wavelength
- Telescopic eye pieces are required to centre the condenser and the phase objectives
Fig. 2.5 Diagram illustrating the paths of light rays in phase-contrast microscopy. (Reproduced by permission of American Optical Company.)
CASTS
You Decide

- Are there Leucocytes, Erythrocytes, and Casts

- What is the yellow object likely to be?
Fluorescence Microscopy

- Conversion of short wavelength UV into longer wavelength visible light = fluoresce
- Microorganisms stained with a fluorescent dye appear bright against a dark background
- Different fluorescent dyes
  - Auramine O stains mycobacteria - yellow
  - Acridine Orange stains - RNA red, DNA yellow green
Fluorescent dyes

- Fluorescent dyes attached to antibodies (DFA)
- Two dyes most commonly used are
  - fluorescein isothiocyanate - yellow green
  - rhodamine B - orange
- UV light source - high pressure mercury lamp 280-600 nm
  - 290 - 325 is required for fluorescein dyes
  - 310 - 350 is required for rhodamine dyes
- limited life 200 hours - keep log book
UV Illumination Systems (1)

- Incident
  - UV light directed onto the specimen from above through the condenser
  - UV radiation is directed onto a 45 degree angled mirror which reflects the correct wavelength down the objective onto the sample
  - Visible light (fluorescence) passes back through the objective to the mirror and then via a filter to the eye piece
  - The advantage of this technique is that it gives brighter fluorescence.
UV Illumination Systems (2)

- Transmitted
  - UV light is transmitted through a substage condenser onto the specimen from beneath. Visible fluorescent light passes directly up the objective to the eyepiece.
  - a 3 lens aplanatic condenser is used
Laboratory Requirements

- Darkened Room
- Teflon coated slides
- Adequate bench space
- Log or timer for UV lamp
- Appropriate filters to protect operator
Fluorescence Microscopy

- Check filters are in place particularly the secondary barrier filter to protect the eyes
- Find the plane of focus
- Use teflon coated slides and focus on the side of the opaque material
- X25/40 high dry are best
- Find areas with the appropriate fluorescence
- Confirm fluorescence is from a regular structure
Errors in Fluorescent Microscopy

- False positives
  - non-specific fluorescence due to inadequate wash
- Quenching = diminishing fluorescence due to changes in the fluorescent dye from the effects of the UV radiation
  - must continually move to a new, previously unexposed field to see objects with bright fluorescence
  - scan many fields to conclude whether the specimen is pos or neg.
**Dark Ground Microscopy**

- Enhances the detection of unstained bodies.
- Images appear brightly lit against a black background the reverse to phase contrast.
- Limited application to microbes with a diameter between 0.1 and 0.2 um.
- Useful for smaller spirochaetes (Treponemmas, Leptospiras and Borrelia) which are too thin to be seen under ordinary light or phase contrast microscopy.
- Light microscope = LR 0.2um whereas darkground has a resolving power of <0.2um.
Principle of Dark Ground

- Specimen is illuminated by oblique light
- Unless the light is refracted by objects, it will not enter the objective and thus fail to reach the eye
- Scattered rays will enter the objective and appear gleaming bright against a black background.
Fig. 2.4 Diagram showing the paths of rays through the darkground condenser and a $\frac{1}{4}$-in-oil-immersion lens fitted with a funnel stop. AB and CDC are reflecting surfaces. The surface at CC is opaque. (After E. Leitz.)
Requirements for Dark Ground (1)

- Special condenser
  - concentric reflecting mirrors
  - central mirror reflects light to outer edges
  - peripheral mirrors reflect light at an angle

- Centring and focusing critical

- Minimise loss of light by using oil on condenser
Requirements for Dark Ground (2)

- Slide thickness critical (1.0 - 1.1 mm)
- Film thickness critical
  - objects need to be in a single plane
  - coverslip slides to protect lenses
  - dilute sample if large number of objects present
  - seal edges if duration of examination is lengthy
- Lamp
  - switch on and off slowly
Polarised Light Microscopy

- Used for crystals
- Diagnosis of gout
  - sodium urate
  - pyrophosphates
- Insert a prism that split light between the light source and the specimen
- Crystals will deflect the light based upon their structure
- Crystals may appear blue/yellow against a pink background
Care of the Microscope

- Keep at a uniform temperature
- Do not move once positioned and focused
  - ▼ If moving, lift by the body and foot only
- Avoid jarring and vibrations
- Cover to protect from dust and dirt
- Daily clean all lenses and remove excess oil
- Use lens paper not tissues due to lint and scratching
Care of the Microscope

- Hardened oil may be removed with xylol but NEVER alcohol, acetone, chloroform as these will dissolve the cement holding the lenses.
- Daily clean oil from under stage and condenser lens.
- Remove any dust from the eye pieces using a fine camel hair brush.
CULTURE

- Inoculate as soon as possible after receipt
  - minimise loss of delicate organisms or overgrowth
- Inoculate fluids in a biohazard hood
- All specimens are potentially infectious
- Warm culture media prior to use
- Use in date media - check expiry labels
- Never use wet or contaminated plates
- Order of plate inoculation is important
  - inoculate nonselective enriched media first
Selection of Media

- Specimen type
- Microorganisms to be recovered
  - enriched media chocolate vs blood
- Likely presence/absence of normal flora
  - selective vs non-selective media
- Presence/absence of inhibitory substances in the specimen
- Likely concentration of organisms present
  - if low use broth or other enrichment
Specimen vs Media

- All specimens
  - non-selective (HBA)
  - selective media (MAC)

- Specialised media
  - fastidious organisms
  - mixed cultures
  - high bacterial loads

- Incubation conditions
  - O2
  - 5% CO2
  - anaerobic
Incubation Conditions

- Aerobic
  - 35°C (why not 37°C)
  - 5% CO2 to enhance growth of fastidious organisms
- Anaerobic – no or limited O2
  - Anaerobic cabinet
  - Anaerobic gas generating kits
- Microaerophilic
  - Specific for Campylobacter (faeces)
  - <5% O2, 10% CO2 balance N2
Duration of Incubation

- Primary examination
  - Minimum 16-18 hours
  - 24 hour laboratories – dual examination periods
- Secondary examination
  - Reincubate after 16-18 hours
  - Examine after an addition 18 hours
- Extended incubation
  - Fastidious or slow growing bacteria
  - Individual sealed packs to minimise drying
  - eg Bordetella pertussis, Legionella pneumophilia, Nocardia and Actinomyces (min 5 days).
Preliminary identification

- Different protocols based on colonial morphology
- Rapid manual tests (2 minutes to 2 hours)
  - oxidase, catalase
  - tube coagulase (*Staphylococcus*)
  - germ tube production (yeasts *Candida albicans*)
- Biochemical tests (4-48 hours)
  - sugar utilisation
  - enzyme production (urease, lipase, lecithinase)
- Sensitivity to specific agents (18-24 hours)
  - optochin *Strep pneumoniae*
  - bacitracin *Strep pyogenes*
- Biochemical utilisation
- Enzyme production
Antimicrobial Susceptibility Testing

♦ Determine
  ▼ antimicrobials suitable for treatment
  ▼ detect potential resistance
  ▼ provide therapeutic options

♦ Test Procedures
  ▼ disk diffusion (antibiotic in paper disk)
  ▼ agar dilution (antibiotic in agar medium)
  ▼ broth dilution (antibiotic in broth - automated)
  ▼ MIC - Etest (antibiotic gradient in plastic strip)
Reporting of AST results

- **SENSITIVE**
  - organism should respond to treatment with this agent

- **RESISTANT**
  - organism will not respond to treatment with this agent

- **INTERMEDIATE**
  - organism will only respond to high levels of this agent
Summary

† Reviewed the diagnostic functions of clinical microbiology laboratory
  ▼ Specimen Processing
  ▼ Microscopic Examination
  ▼ Inoculation of Culture Media
  ▼ Examination of Cultures

† Future lectures in this series
  ▼ Organism identification
  ▼ Antibiotics and testing