LABORATORY DIAGNOSIS OF COMMUNICABLE DISEASES —PITFALLS AND PROSPECTS

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This is the first in a series of articles from the Centre for Infectious Diseases and Microbiology – Public Health (CIDM-PH) describing the clinical and microbial epidemiology of communicable diseases; and new developments in the identification and typing of pathogens of public health importance. The CIDM-PH is a research group based at Westmead Hospital that is closely allied to clinical and laboratory service divisions of CIDM and the Institute of Clinical Pathology and Medical Research (ICPMR).

Part one of this article provides an overview of current laboratory diagnostic methods, and part two illustrates how these tests are applied in practice. A glossary of terms is included to assist readers.

PART 1:

AN OVERVIEW OF CURRENT LABORATORY DIAGNOSTIC METHODS

The diagnosis of communicable diseases involves laboratory tests that either detect the presence of a potential pathogen (live organisms, antigen or nucleic acid) or a host response to a pathogen. Sensitivities, specificities (and so predictive values) vary between test categories, individual assays and laboratories. Interpretation requires knowledge, expertise and an understanding of the technical idiosyncrasies of assays. The turnaround times for results can vary from hours to weeks. This article is offered as a guide for the users of diagnostic laboratories, both clinicians and public health practitioners. Like any form of evidence, laboratory test results should be interpreted in light of other relevant information and questioned if they seem implausible.

SETTING THE SCENE

Clinical microbiology and serology are cottage industries compared with the rapid, automated systems used in clinical chemistry and haematology. Methods that rely on microbial culture are often too slow to influence the choice of treatment or disease outcome; serological testing often provides only a retrospective diagnosis—more useful for describing the epidemiology than a diagnosis. Microscopy is rapid, but neither sensitive nor specific, unless combined with an immunological stain for a particular antigen.

The development of rapid nucleic acid amplification tests (NAAT) has partially changed this scenario. NAAT are most widely used to detect organisms that grow slowly or not at all *in vitro*, require special facilities or are dangerous to handle. There is reluctance to replace the culture of rapidly growing bacteria with NAAT, because isolates are required for antibiotic susceptibility testing or subtyping. This could also change, with development of real-time multiplex methods that allow simultaneous amplification and identification of many microbial gene targets and could potentially provide all the data needed for patient management, disease surveillance and outbreak investigation.

The introduction of new methods does not overcome the need for careful interpretation of results, which requires an understanding of microbial virulence, microbial-host interactions—colonization, infection versus disease, and opportunistic infection—and the patient's current and relevant past medical and social history. Serological test interpretation must also take into account the timing of serum collection in relation to symptom onset, potential cross-reactions and the type of antibody being assayed. The purpose, limitations, sensitivity, specificity and predictive values of the test and the pretest probability of a particular diagnosis must be considered. Thus the interpretation of a test depends on whether it was done for population screening or case detection in someone who is asymptomatic but at risk due to, for example, exposure to an illness. A negative result rarely, if ever, excludes a diagnosis and a positive result should not be accepted as diagnostic proof in the absence of plausible clinical features or a history of exposure.

CULTURE—OPEN-ENDED OR SELECTIVE?

Despite new developments, culture remains the mainstay of diagnosis for many (mainly bacterial) infections. Bacterial culture can be broadly categorised according to the type of specimen collected and type of media used.

Culture of normally sterile specimens on enriched, non-selective media

Specimens that are normally 'sterile', for example blood, CSF and other fluids from enclosed body spaces, are routinely cultured in enriched, non-selective media on the assumption that anything isolated from a patient with a clinical infection is likely to be significant. Enriched liquid media, such as those provided in commercial, automated blood culture systems, will support the growth of most rapidly growing pathogenic bacteria—*Staphylococcus* species, *Streptococcus* species, *Neisseria meningitidis, Listeria moncytogenes,* enterococci, *E.coli,* salmonella, other Gram negative bacilli, and some highly fastidious species, such as *Brucella* species—which are responsible

for most cases of community and hospital-acquired septicaemia and meningitis.

Blood cultures will usually signal as positive in automated systems within 8 to 24 hours or occasionally longer, depending on the bacterial species, number of organisms in the specimen and whether they have been damaged by exposure to antibiotics. A Gram stain will give clues to the organism's identity, but full identification and antibiotic susceptibility testing takes another 1–3 days, or more, if further testing is required. Interpretation is usually straightforward, but contamination with skin commensals (normal flora) such as coagulase negative staphylococci, diphtheroids, or environmental bacteria, is common. Careful clinical assessment is needed, because these same organisms can cause septicaemia, for example in patients with indwelling devices or who are immunosuppressed.

Culture of specimens with normal flora

a) On selective media (such as faecal specimens)

Specimens with a copious normal flora, such as faeces, are cultured on media designed to inhibit growth of these bacteria relative to target pathogens. Several different media are needed. Most laboratories routinely culture only salmonella, shigella and campylobacter from faeces, which takes 48–72 hours or more if further identification or typing is required. Additional selective media (for example, *Vibrio* species) or antigen or NAAT (see below) for specific pathogens (for example, rotavirus, norovirus, *Cryptosporidium, Giardia* etc) or toxins (for example *E. coli* shigatoxin; *C. difficile* toxins) can be added if there is a relevant history or the specimen is watery or bloody, but this greatly increases the cost and time to diagnosis. Added to the fact that there are, undoubtedly, many still unknown and probably unculturable enteric pathogens, this means that no pathogen is identified in a high proportion of cases of infective diarrhea.

Despite many technical and interpretive obstacles, this is an area in which real-time multiplex NAAT, targeting a wide range of microbial species and virulence genes, would greatly enhance diagnosis and epidemiological investigation of diarrhea.

b) On nonselective media (such as respiratory specimens)

Assessing the bacterial causes of respiratory infection is difficult, because the most common bacteria (for example *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis)* can occur as normal respiratory flora, and colonization with antibiotic-resistant bacteria often precedes hospital-acquired pneumonia. Selective media can be used only for a few bacteria (for example *M. tuberculosis, Legionella* species), and viruses. The quality of the specimen is crucial—it must be from the lower respiratory tract (expectorated or induced sputum, bronchalveolar lavage) and uncontaminated, as far as possible, with saliva. The specimen is assessed by microscopy for the presence of pus cells and the predominant bacterial type and discarded if unsatisfactory. A positive result is generally only reported if a) the specimen was satisfactory and b) both Gram stain and culture show a single predominant bacterial type/species, in excess of normal respiratory flora.

ANTIGEN DETECTION

Rapid antigen tests, such as immunfluorescence (IF) or enzyme immunoassay (EIA) have been used for many years, especially for slowly growing organisms such as viruses, *Chlamydia trachomatis* and *Legionella pneumophila,* and are generally highly specific. The sensitivity of IF depends on the skill of the microscopist and the quality of the specimen, which can be assessed by noting the presence of epithelial cells. Respiratory virus IF is useful for rapid diagnosis of infections due to influenza or respiratory syncytial virus, for which infection control measures may be required. If no virus is identified, the specimen can be cultured. The urinary antigen test for *Legionella pneumophila* serogroup 1 has greatly improved the speed, sensitivity and convenience of diagnosis of legionellosis, and serum testing for hepatitis B surface antigen (HBsAg) is used routinely to identify chronic hepatitis B virus carriers. Antigen tests alone are often relatively insensitive because they do not involve amplification and many (for example for *C. trachomatis*) have now been replaced by NAAT.

NUCLEIC ACID AMPLIFICATION TESTS (NAAT)

NAAT, of which the polymerase chain reaction (PCR) is the most widely used, have revolutionised diagnostic microbiology. Briefly, they involve the use of two primers: short, single stranded nucleic acid sequences, which are complimentary to segments at each end of the target deoxyribonucleic acid (DNA) (for example, a gene that identifies a microbial species, a virulence factor such as a toxin, or an antibiotic resistance marker). When mixed with a supply of the four nucleotide DNA building blocks and DNA polymerase, these primers amplify target DNA, which is then identified, often with a labelled probe specific for the target gene. Primer design is facilitated by the availability of rapidly expanding Internet databases of microbial genome and individual gene sequences as well as free software to identify appropriate sequences unique to the target of interest.

Sensitivity depends on, among other things, the number of copies of the gene in an organism, the number of organisms in the specimen, the efficiency of DNA extraction and the presence of inhibitors in the specimen. False negative results are most likely to be due to inadequate specimen quality or amount. False positive results are usually due to contamination with "foreign" DNA, which is usually detectable by use of "no DNA" controls. The presence of dead organisms (for example after treatment), or colonisation, may complicate interpretation of a positive result.

Many NAAT are commercially available in kit form, although they are still relatively expensive. The field is progressing rapidly. Soon it will be possible to simultaneously and rapidly test a single specimen for tens or even hundreds of target gene markers. This will potentially enable the application of NAAT testing for:

- detecting numerous potential pathogens in a faecal or respiratory sample
- distinguishing nonpathogenic from pathogenic bacterial strains (for example, of *E.coli*) by detecting the prevalence of virulence genes
- detecting epidemiologically important antigenic markers such as influenza virus haemagglutinins or *Salmonella* serotypes
- detecting any of a large number of antibiotic or antiviral resistance genes.

SEROLOGICAL TESTS

Serology is widely used for the diagnosis of acute or chronic infection and identification of immune status. There is a wide range of different types of serological assay, which measure either a) the ability of the antibody to bind to a specific antigen (for example enzyme-linked immunoassay, EIA, which it the commonest type used) or b) a functional antibody effect (for example complement fixation, viral neutralisation, or inhibition of viral haemagglutination). Each type of assay and each specific antibody test must be evaluated against some "gold standard" diagnostic method. Antibody assays usually measure IgG or IgM, less commonly IgA or total antibody. Results of serological assays are usually expressed semiquantitatively as titres (the reciprocal of the highest doubling dilution of serum in which the result is positive) or qualitatively as positive/reactive, negative or equivocal. For semiquantitiative tests, a fourfold change in titre between serum specimens, tested in parallel (in the same run) is regarded as a significant change. Changes in antibody levels are more difficult to assess with qualitative tests, such as EIA. The intensity of colour change resulting from the presence of antibody is expressed as an optical density (OD) and interpreted as positive if it exceeds a predetermined "cut-off" set by the manufacturer (which may be an absolute value or relative to a negative control or background reading). Some laboratories report these values without appropriate calibration, which can give an erroneous impression of changing antibody levels.

IgG, which is formed following initial infection or immunisation, usually remains present for long periods, sometimes for life, and so is used to determine immune status or evidence of past (or current, if chronic) infection. For diagnosis of recent or acute infection, serum should be collected as soon as possible after the onset of symptoms, before IgG antibody is detectable or reaches its peak level. Another specimen collected 10-14 days later (sometimes longer) and tested in parallel with the first, showing seroconversion or a significant increase in the level of IgG, confirms a serological diagnosis of recent infection. Unfortunately, the first specimen is often not collected early enough and similar (stationary) levels of IgG are found between paired sera.

In general, IgM and IgA are detectable sooner than IgG, but remain present for a relatively short time—typically 4–6 weeks—after the onset of infection, and so are used as evidence of recent infection either a) early in the course of infection, before IgG is detectable or b) after IgG has reached a stationary level. Unfortunately, there is wide variation in the length of time for which IgM and IgA persist and occasionally they persist for years. Cross-reactions and false positive IgM results are not uncommon and IgM may reappear with reactivation of latent infections; for example, infection due to herpes viruses.

An alternative strategy for diagnosis of recent or initial infection, which is particularly important when assessing a suspected vertically transmissible infection (an infection transmitted from a mother to her baby) during pregnancy, is to measure IgG avidity. This is based on the fact that the longer the interval since infection first occurs, the more avid IgG becomes—that is, the more strongly it binds to its corresponding antigen. In practice a serum specimen is divided into two portions, one of which is treated with a concentrated urea solution, which separates antigen/antibody complexes in inverse proportion to the degree of IgG avidity. The two portions are tested in parallel (usually by EIA) and the readings showing the amount of antibody present are compared. The ratio between them (the avidity ratio) indicates whether the infection occurred a relatively long time ago (usually more than 3 months) if the ratio is high (the actual cut-off ratio varies for each antibody), or more recently if it is low. A low avidity does not always indicate recent infection (it occasionally remains low for many months), but a high avidity ratio has a high negative predictive value for recent infection.

FURTHER READING

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3. Sintchenko V, Gilbert GL. Evidence based diagnostic microbiology: Has its time come? *J Clin Pathol* 2001; 54: 441–2.
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PART 2: USE OF DIAGNOSTIC TESTS IN PRACTICE

In general, the diagnosis of specific infectious diseases involves a combination of different tests and the level of certainty can be classified as confirmed or probable, depending on the type of infection and the clinical circumstances, as illustrated by the following examples.

MEASLES

Suspected acute measles infection is usually diagnosed by serum IgM, which is moderately reliable if a properly validated EIA is used. Ideally, IgG seroconversion would be confirmed (but takes longer). In some circumstances, a rapid diagnosis is made by IF or PCR on an upper respiratory specimen or PCR on blood or urine. Viral culture can be performed if molecular typing is required to identify the likely source of infection, such as imported. Immune status is assessed by a serum IgG assay.

TUBERCULOSIS (TB)

The Mantoux or tuberculin skin test (TST), a test of cell mediated immunity is used to diagnose TB infection. It has well-known limitations, including the fact that it may be falsely positive in people previously immunized with BCG. New tests that measure gamma interferon production by the patient's cells, in response to exposure to purified *Mycobacterium tuberculosis* antigens, *in vitro*, have the advantage that, unlike TST, they can distinguish between tuberculosis infection and previous BCG (Bacillus Calmette-Guerin) immunization. Diagnosis of TB disease in a patient with a consistent clinical picture is based on: microscopy of an appropriate specimen (usually sputum), using a stain for acid fast bacilli (which will detect mycobacteria, not specifically *M. tuberculosis*); and culture, which is relatively slow. PCR is specific and slightly less sensitive than culture, but relatively expensive and generally used only when there is urgency (for example, meningitis) or significant doubt about the diagnosis (for example, atypical presentation in a high risk patient).

PERTUSSIS

PCR on a throat swab or nasopharyngeal aspirate or swab is now the diagnostic test of choice early in the course of illness (in the first 1–2 weeks) because it is more sensitive and reliable than culture. Various serological tests are used. Seroconversion is rarely demonstrated because of the subacute course of illness. Later in the course of illness, and in older children and adults, specific IgA (above an arbitrary cut-off) is now the most commonly used (and the only commercially available) method. However, there is limited validation of its predictive value.

HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION

IgG antibody assays (usually by EIA) are used for screening, case detection and diagnosis of chronic HIV infection. A positive result is confirmed by repeat EIA and Western blot (a specialised serum antibody test). Acute HIV infection, if symptomatic, can be diagnosed by demonstrating seroconversion. Quantitative PCR is performed to measure viral load (which is a measure of infectivity and the need for treatment) and to monitor the efficacy of treatment, once begun.

GLOSSARY OF TERMS

CELL MEDIATED IMMUNITY (CMI)

CMI is the host response to infection, which involves cells (such as lymphocytes and macrophages) and local soluble mediators (such as cytokines), which together combine to kill or inactivate invading organisms, usually with varying levels of assistance from specific antibodies (which are responsible for humoral immunity). CMI can be detected simply by an intradermal inoculation of antigen, which, in someone with past exposure who has had a CMI response, will stimulate a delayed type hypersensitivity reaction (a localized, raised, red lump after 48 hours). Different types of pathogen stimulate a predominantly CMI response (for example intracellular bacteria, such as *M. tuberculosis*) or a predominantly humoral (antibody) immune response (for example, many viruses) but both types of response are usually involved to varying degrees.

DEOXYRIBONUCLEIC ACID/RIBONUCLEIC ACID (DNA/RNA)

The genetic material of bacteria (as well as that of other organisms, including mammals) and many viruses is amplified in nucleic acid amplification tests (NAAT). Some viruses contain RNA not DNA, which can be detected in NAAT by first converting it to DNA with the enzyme reverse transcriptase.

ENZYME IMMUNOASSAY (EIA)

EIA is the most commonly used antibody assay. A specific antigen, which is a component of a micro-organism, is combined *in vitro* (usually in the wells of a plastic microtitre plate) with test serum. A specific antibody, if present, will attach to the antigen to form an antigen/antibody complex, which is detected (after removal of any non-

specific antibody) by an antihuman antibody combined or conjugated with an enzyme. After removal of excess conjugate, remaining enzyme (indicating the presence of specific antibody) is detected by addition of a substrate, which changes color in the presence of the enzyme. EIA can be configured to detect different classes of antibody individual – IgG, IgA or IgM.

IMMUNOGLOBULIN (IG), SUCH AS IGG, IGA, IGM

Immunoglobulins or antibodies are formed, specifically, in response to foreign antigens, including microbial pathogens (and vaccines). Once programmed, the cells that produce specific antibodies can be rapidly recruited to produce more, very rapidly, if the host is again exposed to the same antigen. A specific antibody "neutralises" the organism or antigen, making it more easily engulfed by phagocytic cells (such as macrophages), which kill or inactivate them. IgM is formed first (after 5 to 10 days), after initial exposure, but usually persists for only a few weeks to months. IgG is formed a little later (after 7 to 21 days) and generally persists for life, although the level may fall in the absence of re-exposure. IgA is formed predominantly at mucosal surfaces, but is also detectable soon after exposure, usually for a short period, in serum.

IMMUNOFLUORESCENCE (IF)

The principle of IF is similar to that of EIA except that the antigen is an object that can be seen with a light microscope (for example, bacteria or a virus-infected cell monolayer) and is fixed to a microscope slide. Test serum is added and, after washing, the presence of antigen-antibody complexes is detected by adding an antihuman antibody conjugated with a fluorescent dye, the presence of which (indicating specific antibody) is detected with an ultraviolet microscope.

IMPORTED INFECTION

An infection that is acquired overseas.

MEDIA

Bacteria are cultured on artificial media, which provide nutrients for their growth. Media may be solid (nutrients are added to agar – a jelly-like substance derived from sea-weed, which is solid at room temperature) or liquid (nutrient broth). Agar plates are typically enriched by addition (to basic nutrient preparations) of horse or sheep blood, which are lysed by hemolytic bacteria and so assist identification. To facilitate the identification or isolation of a target pathogen, one of the following is added:

- specific nutrients that enhance the growth of fastidious bacteria
- selective agents that inhibit the growth of unwanted bacteria in favour of target bacteria, or
- chemical reagents that change colour in the presence of certain bacterial enzymes.

For example, antibiotics are added to more easily detect the presence of antibiotic resistant bacteria.

PREDICTIVE VALUES—NEGATIVE AND POSITIVE (NPV/PPV)

Negative and positive predictive values are quantitative estimates of the likelihood that a negative or positive test result truly is negative or positive, respectively. The values depend on the sensitivity and specificity of a particular diagnostic test and the prevalence of the condition in the population studied. (Sensitivity refers to the percentage of cases of a disease, such as an infection, that are detected by a diagnostic test, and specificity refers to the proportion of subjects not affected by a condition in whom a diagnostic test is negative.) Given a fixed proportion of tests that give false positive results, the positive predictive value (proportion of all positive results that are true positive) will be higher if the prevalence of the condition/infection in the population is high. For example, in patients with a clinical illness consistent with, say, acute toxoplasmosis, the PPV of a positive toxoplasma IgM test will be higher than in an asymptomatic subject such as a pregnant woman in whom toxoplasma IgM is tested as part of routine antenatal screening.

VERTICALLY TRANSMISSIBLE INFECTION

This is an infection that is transmitted from a mother to her fetus or infant by a mechanism that depends on the unique mother-infant relationship. Infection may occur: during pregnancy (across the placenta at any time during pregnancy or by spread from the mother's cervix into the amniotic fluid—called ascending infection—usually late in pregnancy); during delivery (for example by exposure of the infant to the mother's blood or genital secretions); or after birth (for example by breast feeding).