DIAGNOSTIC PARASITOLOGY

The diagnosis of parasitic infections is a holistic integrated science involving inductive and deductive inference using multiple clinical/paraclinical parameters covering host-parasite biology, morphology, physiology, biochemistry and immunology. Diagnostic parasitology seeks to identify the aetiological (causative) agent of disease thus enabling appropriate management (treatment/control) options. Diagnosis is based on a sound working knowledge of the taxonomy of living organisms, but the identification/characterization/classification of individual parasite taxa (species/strains/genotypes) is not the primary aim. Often, the identity of the actual parasite species is not required by the clinician, e.g. knowledge that gastro-intestinal nematodes have been implicated as the cause of neonatal scours may be sufficient to commence treatment with an anthelmintic.

Diagnostic parasitology is not a dry esoteric exercise conducted by technicians/scientists in remote laboratories, but rather a detailed reasoning exercise conducted by the clinician who interacts with the patient and is ideally cognizant of most relevant facts and uses a variety of technologies and support services to gain further knowledge to make a differential diagnosis. While the mental processes involved in clinical reasoning and differential diagnosis are difficult to categorize (involving many cognitive and metacognitive processes), there are certain things that are obviously known to the clinician which can be used to make some common-sense predictions.

- The clinician should always know the host species involved - medical practitioners should recognize their patients as humans, and veterinary practitioners should be able to identify the animal species they are working on. This simple information already allows clinicians to consciously/subconsciously access information on host specificity, host range, host distribution, host susceptibility/resistance, etc.

- The clinician should be able to determine why the patient has presented – symptoms can be described, signs can be observed. This allows an assessment of how serious and urgent the situation may be, as well as helps to pin-point the tissue/organ systems involved. Most parasites have predilection sites of infection and exhibit tissue tropism and this information helps to facilitate diagnosis, to assess disease development and progression, and to predict possible outcomes (prognosis).

- Samples can then be collected for further examination, and it is a poor clinician who does not know that they are up to their armpits in faeces, urine, vomitus, sputum, blood, tissue aspirates, tissue biopsies, etc. In selecting specific samples, the clinician is seeking further information to help rule in or rule out certain aetiological agents. It is also up to the clinician to request the most appropriate tests to be conducted (e.g. full blood count, differential, haematocrit, liver enzymes, worm egg count, etc.).

However self-evident, all of this host-parasite information helps in the clinical reasoning process so that the clinician can make a working diagnosis to begin disease management. In addition to providing detailed information about the specific case in hand, it also allows the clinician to predict the mode of transmission of the parasite so that preventive measures can be implemented to avoid further cases. For example, many parasites in the digestive tract causing enteritis and diarrhoea are transmitted by the faecal-oral route; many parasites in the circulation causing fever and anaemia are vector-borne; and many parasites in visceral organs causing lesions and organ malfunction are transmitted by carnivorism. Other horizontal transmission strategies used by parasites include venereal (sexual), transdermal (percutaneous) and air-borne (respiratory) transmission. Water-borne and food-borne transmission usually involves faecal-oral or predator-prey transmission. Some parasites also exhibit vertical transmission between generations, including transplacental and transmammary transmission. Knowledge of the mode of transmission allows affirmative action to be taken to minimize the spread of infections within host populations. Clinicians not only have a responsibility for their individual patients, but also a broader responsibility for the general community to identify disease clusters and prevent outbreaks by recommending appropriate control strategies.

TYPES OF DIAGNOSTIC TESTS

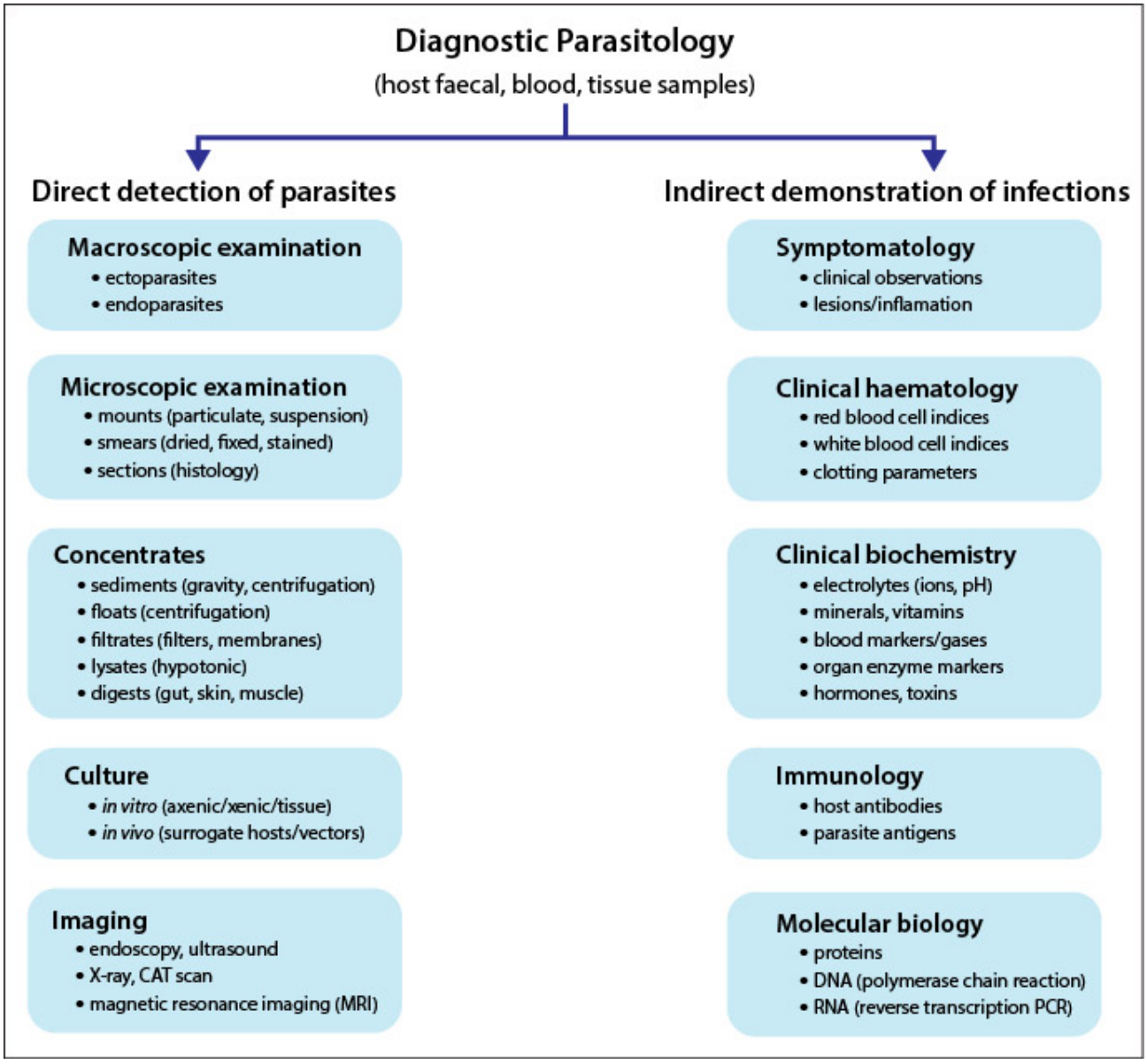
A variety of medical and veterinary tests are used to diagnose parasitic infections and diseases. Different techniques are employed to either directly detect parasites in host tissues/fomites or provide presumptive evidence of infection by the demonstration of specific clinical, biochemical, immunological or molecular parameters.

Direct detection techniques include:

- macroscopic examination of host surfaces, orifices or fomites (for visible parasites);
- microscopic examination of host faeces, blood or tissues (for microscopic parasites);
- physical concentration of parasites from samples (qualitative or quantitative);
- *in vitro* culture of parasites from samples (axenic, monoxenic or tissue culture);
- *in vivo* culture of parasites from samples (by laboratory animal inoculation); and
- medical imaging of parasites in tissues (by endoscopy, ultrasound, X-rays, CT scans or MRI)

Indirect demonstration techniques include:

- detecting specific disease symptoms or signs (by clinical observations);
- detecting specific host blood parameters (by clinical haematological tests);
- detecting specific host plasma proteins/enzymes (by clinical biochemical tests);
- detecting specific host antibodies or parasite antigens (by immunological tests); and
- detecting specific parasite proteins or nucleic acids (by molecular biological tests).

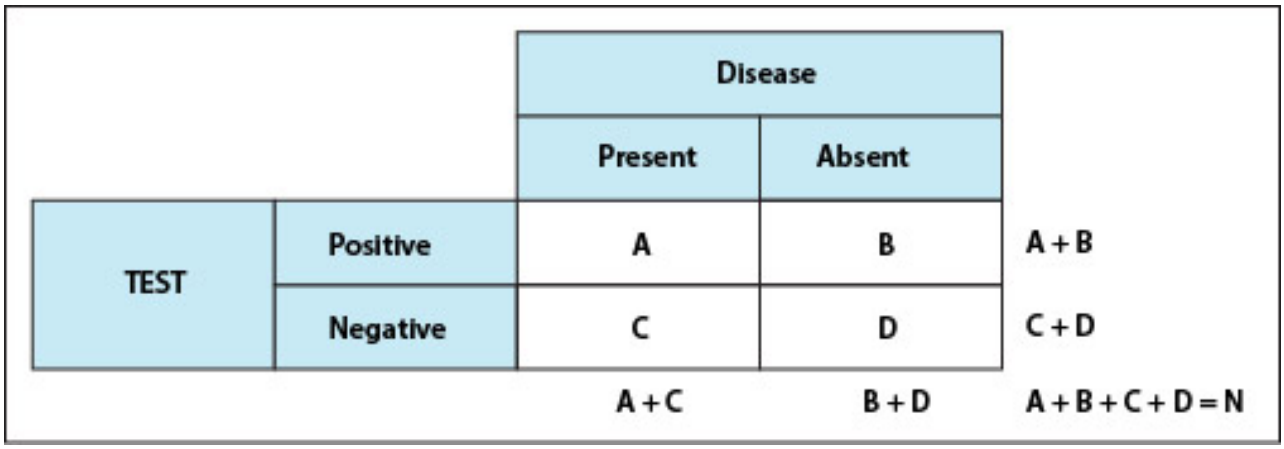


Parasitic diseases vary greatly in their presentation and exhibit marked fluctuations in their dynamics (quantitative measures) and kinetics (time courses). Thus there is no single type of test appropriate for the diagnosis of specific infections. A plethora of tests have been developed over many years to differentially detect different life-cycle stages of different parasites or different host responses to different parasites. Clinicians often request a small battery of relevant tests to be conducted to either exclude or include parasites as the aetiological agent of disease. Ideally, diagnostic tests should exhibit the following desirable characteristics:

- safety (pose little risk to patient and test operator);
- cost efficiency (be cheap and affordable);
- time efficiency (provide a rapid result);
- simplicity (by easy to perform);
- durability (be robust with stable long-lived reagents);
- reproducibility (give the same results within test conditions);
- accuracy (discriminate between infected and non-infected individuals);
- specificity (detect the correct parasite, and give few false positive reactions); and
- sensitivity (detect low level infections, and give few false negative reactions).

INTERPRETATION OF DIAGNOSTIC TESTS

Few diagnostic tests are absolutely perfect; they may give erroneous results for a variety of reasons, both technological and biological. Inappropriate samples may be tested, samples may be stored or transported improperly, reagents may be too old, test conditions may be violated, and many human errors may have been made by the test operator (pipetting errors, steps accidentally omitted, time delays, etc.). Many tests cannot identify recently acquired infections (e.g. during the incubation or pre-patent period), many cannot differentiate between chronic infections and previous exposure (e.g. antibodies persist long after infection has resolved), many cannot detect low level infections (e.g. parameter below detectable threshold), and many tests cannot identify specific infections (e.g. cross-reactivity between parasites). It is therefore important that users of any particular test know how good it is, as determined by objective quantitative assessment. Test efficacy is quantified by comparing results with disease status in a reference population (disease status being the 'gold' standard). Regrettably, gold standards are not always available so test efficacy may be poorer than reported. Bayes' theorem is used to quantify test accuracy, sensitivity, specificity, and predictive values. Four outcomes are possible when testing the host population: as shown in the following 2x2 matrix:



Outcomes (and consequences):

A = true positive:	test correctly diagnoses disease, facilitating treatment.
B = false positive:	test falsely diagnoses disease, resulting in unnecessary treatment.
C = false negative:	test falsely rules out disease, allowing disease progression, death
D = true negative:	test correctly rules out disease, suggesting other cause.

Prevalence (proportion positive or diseased at a particular point in time)

Disease prevalence	= (A+C)/N	[total diseased over total population]
Test prevalence	= (A+B)/N	[total test-positive over total population]

Accuracy (probability of correct test result)

Test accuracy	= (A+D)/N	[total true results over total population]

Sensitivity (probability of positive test in diseased person)

Test sensitivity = A/(A+C) [true positives over total diseased]

Ideally, test will have low rate of false negatives, thus a negative test often rules out disease

[SNNOUT = SeNsitive test, Negative test rules OUT diagnosis]

When a disease is very serious and missing it will have dire consequences, select a sensitive test.

Specificity (probability of negative test in non-diseased person)

Test specificity = D/(B+D) [true negatives over total non-diseased]

Ideally, test will have low rate of false positives, thus a positive test often rules in disease

[SPPIN = SPecific test, Positive test rules IN diagnosis]

When a disease is suggested by other data, select a specific test to rule in a diagnosis.

Predictive values (probability of disease in test-positives, probability of non-disease in test-negatives)

Positive Predictive Value (PPV) = A/(A+B) [true positives over total positives]

Negative Predictive Value (NPV) = D/(C+D) [true negatives over total negatives]

Most governments have regulations where numeric scores for these parameters (expressed as proportions or percentages) must be included in the product information accompanying commercial test kits, so users (laboratory scientists or clinicians) know the limitations of the test selected. Most pathology laboratories also conform to best practice standards advocated by impartial agencies which recommend the adoption of standard operating procedures and ongoing compliance with quality assurance/control programs (through routinely testing positive reference samples and negative controls) to ensure test performance is within acceptable standards. International test standardization is particularly important when considering import/export certification and quarantine requirements in our global society.

THRESHOLD OF DETECTION

The term sensitivity often has an ambiguous meaning. Bayes theorem uses a mathematical expression to measure the diagnostic or clinical sensitivity to be the probability of a obtaining a positive test result in an infected individual. Alternatively, the analytical sensitivity of a test can mean the smallest detectable number of parasites (or antibodies, or DNA); also known as the threshold limit of detection (LOD), below which the test gives a negative result although some parasites may be present (but not in detectable numbers). The LOD can be quantified both in terms of how much sample should be examined and how few parasites could be detected. It becomes a numbers game!

How much of a sample can be examined?

Depending on the host species, you could collect faecal samples weighing up to several hundred grams (g) or millilitres (mL) or more. How much of the sample can you, or should you, physically process and examine? A single faecal smear contains around 0.001 g of faeces and it takes at least 10 minutes to examine thoroughly under a light microscope. To detect just one parasite (say, one worm egg) in 1 g of faeces, theoretically you would need to look at 1,000 smears which would take 10,000 minutes (= 167 hours = 7 days). Clearly, this is impractical. The minimum LOD achieved by looking at just one faecal smear is therefore $1/1{,}000^{th}$ of 1 egg/g; the equivalent of 1,000 eggs/g. In reality, worm egg counting techniques aim to detect as few as 60 eggs/g of faeces. They achieve this not by examining more sample or by increasing examination time, but by concentrating and/or harvesting parasites from samples. Similarly, a blood sample collected into a haematological tube may be as much as 5 mL, but many haematological tests rely on the examination of one thin blood smear (approximately 2 µL) for 10 minutes. To process the whole 5 mL sample, you would need to prepare 2,500 smears and examine them for 25,000 minutes (= 416 hours = 17.4 days). Even when one blood smear is examined, it is unusual to examine every single blood cell, more often 100 high-power microscope fields are examined, each containing around 10^2 cells. If you detected just one parasite in those 100 fields, the threshold LOD would be 1 parasite in 100 x 10^2 (= 10^4) cells, that is, 0.01% parasitaemia. This may be improved by examining more fields per smear, or by concentrating parasites from samples.

The LODs of tests may be enhanced by several orders of magnitude by concentrating parasites through centrifugation, filtration, culture, cell sorting or DNA amplification (improvements of 10^2-10^3 times for coprological tests, 10^3-10^4 for haematological tests, and 10^4-10^6 for biochemical and molecular biological tests). To compare conventional and contemporary techniques, it is necessary (but difficult) to convert the units of one type of test to those of another. Early PCR tests reported LODs enigmatically as 50 ng to 10 fg (spanning 6 orders of magnitude), but these quantities simply referred to the amount of DNA template provided in the sample without any way of reconciling how many parasites they might represent. Theoretically, a positive PCR test could indicate the presence of DNA from a single parasite. However, molecular tests do not detect whole parasite genomes but use small primers to amplify partial gene sequences (often 500 bases). Given that one nucleotide base weighs on average 650 Daltons (Da) and that 1 g = 6.022 x 10^{23} Da, then a gene fragment of 500 bases can be calculated to weigh ~5 x10^{-19} g (= 0.5 ag). By comparison, the average parasite genome size

is ~ 500 megabases (Mb) [protozoa range from 10 Mb to 100 Gb; helminths from 100 Mb to 1 Gb] which can be calculated to weigh 5 x 10^{-13} g (= 0.5 pg). [Interestingly, primers looking for 500 bases in a 500 Mb genome means you are effectively only looking for 0.0001% of the parasite]. The lowest reported LOD of 10 fg DNA could therefore theoretically represent anywhere from 0.001 of a parasite (0.1% of a whole 500 Mb parasite genome) to 20,000 parasites (maximum number of copies of 500 b gene fragment). Clearly, these values are meaningless and cannot be used to express LOD in terms of parasite load (concentration) without knowing what sample sizes were used for DNA extractions, what aliquot sizes were used for PCRs, how DNA amounts were measured, how much host DNA may have been included, etc.

More recently, experimental studies have used serial dilutions of DNA extracted from samples spiked with known numbers of parasites and found LODs to range from 1-6 oocysts/g faeces (for *Cryptosporidium*), 1 parasite/µL blood (for *Plasmodium*), 1 parasite/mL (for *Leishmania*), etc. In some instances, unit conversions are required to compare old and new tests: e.g. what percentage parasitaemia does 1 parasite/µl correspond to? The mean cell volume of a human erythrocyte is ~90 fL (= 90 x 10^{-12} mL), so there may be up to ~10^{10} erythrocytes in 1 mL of blood (realistically, there will be fewer as the whole blood volume is not composed of cells – actual blood counts show there are up to 5 x 10^9 red blood cells/mL of blood, plus 8 x 10^6 white blood cells /mL). If you can detect 1 parasite/µL (= 10^3 parasites/mL), then the threshold LOD would correspond to 10^3 parasites in 10^{10} erythrocytes, the equivalent of 0.00001% parasitaemia. Clearly, the analytical sensitivities of modern molecular tests are better than those of conventional tests that rely on the detection of whole organisms. While the detection of whole organisms does allow additional morphotypic features to be examined, molecular tests are becoming more sophisticated in detecting specific genotypes and facilitating phylogenetic analyses.

How much space does a parasite occupy in a sample?

Let us assume that a sample of 1 g = 1 mL = 1 cc = 10^{12} µm^3. One intact stereotypical spherical protozoan cyst measuring 10 µm in diameter occupies a volume = 4/3 πr^3 = 524 µm^3. The cyst therefore occupies 0.00000005% of the 1 g sample. For comparison, one stereotypical spherical helminth egg measuring 100 µm in diameter occupies a volume = 4/3 πr^3 = 524,000 µm^3. The egg therefore occupies 0.00005% of the 1 g sample. One stereotypical spherical arthropod measuring 1,000 µm (1 mm) in diameter occupies a volume = 4/3 πr^3 = 524,000,000 µm^3. The arthropod therefore occupies 0.05% of the 1 g sample. Obviously, looking for one parasite developmental stage can be like looking for a needle in a haystack. Various techniques are employed to improve the chances of detection: such as concentrating parasites from samples, separating them from confounding material, and making them more readily observable by using histochemical or immunological stains. By comparison, the amount of host sample occupied by specific parasite molecules (proteins or DNA) may be extremely miniscule, but their detection relies on a huge range of factors in addition to their concentration, notably their reactivity under test conditions.

How many parasites does it take to cause disease?

This is the quintessential question, one for which we have few answers. By definition, parasites cause harm to their hosts, but the harm may not be sufficient to even be noticed by the host. Parasites cause disease by:

- stealing nutrients (ingestion, absorption..)
- destroying cells (ingestion, lysis…)
- migrating through tissues (tunneling, tracking..)
- lodging in tissues (obstruction, space-occupying lesions..)
- provoking host reactions (toxicoses, inflammation, hypersensitivity..)

At what stage does the resultant perturbation in structure/function of host tissues (= pathology) become manifest as disease, with recognizable symptoms and signs? Parasitic infections are dynamic processes, with parasites numbers changing markedly over time (increasing during multiplicative phases, and decreasing during senescent phases or due to host immunity). The relationship between parasite numbers and disease expression is further complicated by how susceptible the host is to infection and disease, and how virulent the parasite is in terms of invasiveness, appetite, growth and multiplication. Protozoan parasites usually undergo massive multiplication in host tissues often giving rise to acute disease syndromes (rapid onset, short duration). Helminth parasites do not multiply but accumulate in host tissues often giving rise to chronic disease syndromes (slow onset, long duration). Arthropod parasites vary in their abundance, some multiplying in host tissues and others accumulating on host surfaces. All types of parasites can cause severe disease if the cumulative damage to the host overwhelms homeostasis and repair processes. Attempts to quantify pathogenic doses for some parasites have been made in experimental animal models, but the results often cannot be extrapolated to natural field infections. Theoretically, disease may ultimately result from infection by one parasite, but in reality it takes more to establish clinical infections.

The minimum level of detection of a diagnostic test should therefore be better than the number of parasites required to cause disease, otherwise the test would give unacceptable numbers of false negative results, with potentially disastrous consequences. Most tests, especially modern molecular tests, have a very low threshold level of detection and they can detect small amounts of parasitic material in host samples. However, this can also bring about problems in interpretation, because detection does not unequivocally imply causation. Subclinical parasitic infections occur in many hosts, so although parasites may be detected, it does not mean that they caused the disease. They were simply fortuitous or opportunistic findings in hosts succumbing to disease due to other causes. Concomitant infections by more than one parasite species are also common in clinically-affected hosts, as many parasites utilize the same modes of transmission that have resulted in infection. Which parasite was responsible for disease, the most abundant, the most virulent, or various combinations of those present?

The interpretation of diagnostic test results is therefore not straightforward, with many potential problems in sensitivity, specificity, diagnostic threshold and clinical threshold confounding differential diagnosis. It must be remembered that the clinician makes the diagnosis based on a wealth of evidence gleaned from observation, history, physical examination and laboratory investigation.