

# Diagnostic molecular microbiology and its applications: Current and future perspectives

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## Abstract

Infectious diseases are a major cause of morbidity and mortality throughout the world. Although the field of diagnostic microbiology has substantially evolved over the past few decades, it is still heavily reliant on cultures and serology, which while being cost effective is largely time consuming and sometimes less sensitive and specific. Molecular biology on the other hand has developed with rapid and confirmatory diagnosis. However, the merger of molecular diagnostics in routine diagnostic microbiology labs has been slow paced mostly due to higher costs and lack of infrastructure. Molecular methods have progressed beyond identification to detect antibiotic marker genes, fastidious bacteria, and uncultivable microbes. It has found a scope in mycology and parasitology, in which the basic conventional techniques may sometimes be unable to make a definitive diagnosis of the concerned pathogens. In the field of virology where culturing may be impractical in some clinical settings, it has developed various multiplexing procedures which can detect and quantitate the viral copies present in the crude specimen. Besides the field offers different types of permutations and combinations like direct sequencing for finding the variants, whole genome sequencing, epidemiological testing like plasmid profiling, RFLP etc. which can point out the infection and different types of PCR customisation such as nested, multiplex and Real Time (RT) PCR. In the present review, we have described the various molecular typing techniques and their application in microbial testing with a briefing of the tests that have been already standardised with relevant sample acceptance and rejection criteria for commonly encountered pathogens and pointed towards future directions converging automated closed DNA, RNA extraction/amplification platforms, Next Generation Sequencing (NSG), Microarrays and Digital PCR (dPCR) into its arsenal.

## Introduction

Infectious disease is the main cause of mortality and morbidity worldwide. The different types of infection caused by various types of pathogens including bacteria, viruses, parasites and fungi are ever increasing [1]. Increasing age, diseases such as cancer, immunosuppressions etc are contributing factors. Although the era of antibiotic, antiviral, antimycotic, and antiparasitic has led to alleviation from these infectious agents, the rampant use of these agents has led to the emergence of multi drug resistant (MDR) pathogens which if not diagnosed and contained early, could spread to large geographical areas [2]. The role played by diagnostic systems is the 'golden standard' for enacting rapid treatment regimes. Routine clinical & microbiological procedures such as cultures, serology, and microscopy still remains the procedures of choice in terms of diagnosis and are also cost effective [3]. However the routine microbiological cultures are by themselves, not confirmatory tests for the accurate diagnosis of the pathogen. Even with cultivable bacteria, cultures fail to reveal an organism in many patients with sign and symptoms consistent with infectious disease. Due to the above reasons, there is a large scope for molecular biology procedures in the diagnostic clinical microbiology laboratory.

With the advent of PCR technology about 30 years ago came the era of molecular diagnostics. It is due molecular testing that the phenotypes exhibited by a pathogen can be genetically confirmed [4]. It ensures rapid diagnosis at a cost effective price, thus increasing the diagnostic arsenal for pathogen identification. This review enlists the various types of molecular typing methods which have been inculcated in molecular microbiology diagnostic labs. It also describes the need for more advanced molecular methods for increasing the sensitivity and specificity of diagnosis and also the routine protocols for various bacterial, viral, fungal, and parasites that have been already standardised

at the genus and species levels with a briefing on sample acceptance and rejection criteria for the enlisted pathogens.

## Molecular typing techniques

The advent of nucleic acid amplification techniques has led to the advancement of molecular typing techniques [5]. It offers the advantage of rapid confirmatory diagnosis. Since the start of the field of medical microbiology, the main techniques have relied on the identification of phenotypic characters like biochemical characterisation, morphological view, and cultures. The inert nature of the DNA molecule makes it the most suitable marker for confirmatory diagnosis [6]. The various types of molecular techniques have been reviewed in the following sections.

## Restriction analysis

The microbial DNA consists of various sites containing sequences which are repetitive in nature. These repeats are tandemly repeated after constant intervals [6]. Also restriction enzymes or endonuclease (R.E) a class of DNA-cleaving enzymes isolates from bacteria are used to cleave these DNA at a particular and specific sequence which results in the fragmentation of DNA molecule. This technique thus can detect the fragmentation pattern of the target and compare it with the in house pathogenic strains and handout results whether the isolated pathogens are similar or dissimilar in origins. Thus, in this technique

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the DNA is cut with the restriction enzymes [7].

### Plasmid profiling

In these technique plasmids which are extrachromosomal DNA elements are cut with restriction enzymes (RE). Transfer of plasmids is very common between members of the family *Enterobacteriaceae*. It is mainly useful in epidemiological outbreaks in which the plasmids are cut with the same RE which may result in same restriction patterns from plasmids isolated from various pathogens in the outbreak area thus showing the profiles in outbreak associated strains [8]. It has been carried out in opportunistic multidrug resistant *Pseudomonas aeruginosa* isolated from wound infections, which carried a pattern of resistance to various antibiotics [9]. A similar study was used for screening of *Neisseria gonorrhoeae* [10] and *Shigella spp.* [11] among others [12,13]. It has been also widely carried out to identify multidrug resistant isolates present in sewage associated with health care centres [14]. A study carried out used this technique for the study of the emergence of drug resistance in diarrheagenic *E. coli* in paediatric population in a developing country [15,16].

### Restriction fragment length polymorphism (RFLP)

RFLP has been mainly used in multi drug resistant *Mycobacterium tuberculosis* outbreaks for simultaneous identification and differentiation in which polymorphisms present in DR locus which is characterised by repetitive sequence interspersed between non repetitive sequences has been hybridised by a probe known as spacer oligotyping or spoligotyping. It can differentiate *M. bovis* from *M. tuberculosis*, a distinction which is often difficult to make by traditional methods [17]. In parasitology PCR-RFLP has been used to diagnose and differentiate Old and New World *Leishmania* species [18] and also for differentiating *Fasciola hepatica* and *Fasciola gigantica* [19]. In a study carried out in outbreak in Japan characterization of enterohemorrhagic *Escherichia coli* O111 and O157 Strains were done by RFLP [20]. In case of the highly transmissible zoonotic infection Brucellosis, caused by the slow growing bacterium *Brucella spp.* this technique has found importance [21].

### Pulse field gel electrophoresis (PFGE)

With the advent of RFLP, there arose a need to perform high resolution separation on agarose gel to look out for similarity in banding patterns obtained after RE digestion. In 1982, Schwartz introduced the concept that DNA molecules larger than 50kb can be separated by using two alternating electric fields. This led to the technique of PFGE. It is based on the digestion of bacterial DNA with RE that recognises few sites along the chromosome, generating large DNA fragments (30-800Kb). The basis for PFGE separation is the size-dependent time-associated reorientation of DNA migration. When visualised electronically bacterial isolates with identical or very similar band patterns are more likely to be related genetically than with more divergent band patterns. PFGE has been used for the detection of *Listeria monocytogenes* and *Campylobacter spp.* which are food borne pathogen difficult to culture [22,23]. It is also widely applied to the diagnosis of eukaryotic DNA like parasites which are dense and complex in nature [24].

### PCR amplification of gene target

This method is the basis of all types of PCR technology. In this method designed primers which amplify a unique region of microorganisms are used. Thus, this method is target specific.

### Multiplex PCR

Multiplexing is one of the most widely applied techniques in the field of diagnostic microbiology. It is a variant of PCR, which enables simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers. Its main use is in the field of virology in which primers are designed to detect different types of viruses from a sample [25]. Different types of panels are available from brands such as Biomuriex, Roche, life technologies, Qaigen etc., which are named syndromically e.g. Respiratory panel, Gastrointestinal panel, Meningitis panel etc. Multiplexing is also useful for differentiating at the species levels e.g. Multiplex PCR for identification of *Campylobacter coli* and *Campylobacter jejuni* from pure cultures and stools [26], rapid detection of Methicillin Resistant *Staphylococcus aureus* (MRSA) from blood culture bottles [27], acquired carbapenemase resistance [28]; and in parasite detection, e.g. for differential diagnosis of helminths like Taeniasis and Cysticercosis [29].

### Nested PCR

Involves two consecutive PCR reactions of 25 cycles. The first PCR uses primers external to the sequence of interest. The second PCR uses the product of the first PCR in conjunction with one or more nested primers to amplify the sequence within the region flanked by the initial set of primers. It has been designed for various bacterial [30-33], fungal [34,35], and parasitic infections.

### Real time PCR (RT-PCR)

It allows viewing the increase in the amount of DNA as it is amplified. All real time PCR systems rely upon the detection and quantitation of the fluorescent reporter, the signal of which increases in direct proportion of the amount of PCR product in a reaction. The most economical reporter is the double strand DNA specific dye SYBR green, which upon excitation emits light. However SYBR green has its limitations as it will bind to any double stranded DNA in a reaction [36]. Two most popular alternatives to SYBR green are TaqMan and molecular beacons. Both technologies depend on hybridisation probes relying on fluorescence resonance energy transfer (FRET) and quantization [37]. There are numerous laboratory-developed real time PCR tests, including assays for viruses like Dengue [38], Ebola [39] and bacteria like *Pseudomonas aeruginosa* [40].

### Direct sequencing

Direct sequencing is the most reliable method for the detection of sequences at the molecular level. The first whole genome sequenced was that of bacteriophage  $\phi$ X174. Given below is a briefing of some of the methods applied in microbial sequencing. It is noteworthy that sequencing has helped to differentiate fungi and parasites at the species level which is difficult to achieve by current morphological and phenotypic techniques [41].

### Whole genome sequencing (WGS)

WGS has changed the landscape of molecular biology with its advent in 2005 by Roche. It is the most cost effective approach for solving many epidemiologic outbreaks and confirmation of pathogens after cultures [42,43]. The near market platforms available are Roche 454, Pacific Biosciences, Ion Torrent, and IlluminaMiSeq which can perform operations in 48hrs [44]. In terms of sample preparation for pathogens Oxford Nanopore is also very reliable [45].

## Target gene sequencing

It is the most common method of sequencing used which relies on Sanger dideoxy chain terminator method. In this the primers complementary to the gene of interest are used to amplify the band containing the gene of interest and sequenced using an automated sequencer like the Applied Biosystems 8180xl genetic analyser. It is used for diagnosing multi drug resistance in microorganisms like presence of acquired carbapenemase resistance geneNDM-1 gene [28,46].

## Universal gene target

These are specific sequences used in microbial diagnostics which are highly conserved and also impart certain phylogenetic traits at the genus and species levels.

## Ribosomal RNA (rRNA)

The bacterial pathogens which are unidentifiable with routine testing, slow growers, uncultivable can be identified by amplification of DNA encoding ribosomal RNA genes followed by DNA sequencing. In bacteria there are three genes that make up rRNA functionality, *i.e.*, 5S, 16S, and 23S rRNA. The 16S has been employed for identification purposes due to it being highly conserved and having a moderate copy number depending on the genus. Besides 16S rRNA genes are found in all bacteria and accumulate mutations at a slow, constant rate over time, hence serving as “molecular clocks” [47]. The use of this technique has led to the discovery of novel clinical isolates and culture negative infections [48]. It has been widely used to identify tuberculosis and non-tuberculosis infections [49] and biothreat agents like *Brucella spp*s [50]. Recently, there have been several reports regarding the use of the large subunit (23S rRNA) which is specific for bacterial species recognition. It has been used frequently for the detection of *Stenotrophomonas maltophilia* from patients with cystic fibrosis [51].

## Heat shock proteins

The use of 16S rRNA although applied routinely bears some limitations. Strains with less than 97.5%16S rRNA gene sequence identity are unlikely to be related at the species level. However, there are a number of strains that share less than 50% DNA similarity by reassociation and therefore are classified as distinct species, but share 99% to 100% 16S rRNA gene sequence identity. For example, *Mycobacterium chelonae* and *Mycobacterium abscessus* have more than 99% 16S rRNA gene sequence identity, but their DNA similarity by reassociation is only 35% [52]. In such circumstance sequencing of essential genes such as the heat shock proteins (HSP; HSP60, HSP65, groEL, groER, etc.), have been shown to be useful [53,54]. The heat shock response is an important homeostatic response that enables the microbial cells survive the conditions of stress. This phenomenon is observed in all microorganisms from bacteria, fungi, to parasites [55]. A polymorphism in HSP 70 of *Leishmania spp*s has been used for the differentiation of neotropical *Leishmania* species, as well as *Trypanosoma cruzi* [56].

## Antibiotic resistance gene

Antibiotic resistance among microorganisms has become a worldwide issue. A review published by Fluit *et al.* has highlighted on the increasing numbers of multidrug resistant bacteria and its molecular detection [4]. Molecular detection by Xpert MTB/RIF automated system (GeneXpert, Cepheid) has found a wide scale acceptance for the detection of rifampicin resistant tuberculosis, which is on the rise in developing countries [57]. Also the detection of NDM-

1 and its variant has been carried out by conventional PCR against the NDM-1 gene with members of the *enterobacteriaceae* family [58]. For detection of *H pylori* a slow growing ulcerative pathogen resistance markers amplification strategies are used [59]. In parasitology it has found applications for detection of chloroquine resistant *Plasmodium falciparum* by using nested PCR followed by mutation-specific, restriction-endonuclease digestion for detection of mutations in *pfcr*t and *pfmdr* gene for treatment regimens [60].

## Molecular standardised diagnostic protocols

In this section we review some of the standardised protocols employed in a diagnostic molecular microbiology laboratory at the genus and species levels for various clinically important pathogens including bacteria, fungi, parasites and viruses. We also include the sample acceptance and rejection criteria as cited in the literature.

## Bacteriology

Most medically important classes of pathogens belong to the class of bacteria and their molecular detection is the need of the hour for rapid confirmatory diagnosis [61]. Molecular standardised protocols have been cited in Table 1. Whipple's disease is a rare but fatal infection caused by *Tropheryma whipplei* the diagnosis of which until recently only relied on histopathology and electron microscopy, often from post mortem material. PCR now allows the diagnosis of neuro-Whipple's disease and endocarditis by the detection of *T. whipplei* from non-invasive specimens [62]. Molecular diagnosis can also help to diagnose uncultivable pathogens like that of cat scratch disease *Bartonella henselae*, Q fever due to *Coxiella burnetti*, and male urethritis caused by *Mycoplasma genitalium* [63].

## Virology

The field of virology has already adopted diagnosis by molecular methods and Table 2 sites the medically important viruses encountered in clinical labs. Genotypic mutation analysis of the virus which correlates with phenotypic resistance has been designed for retroviruses like HIV in treatment naïve and for viral rebound in patients already on treatment, to establish if resistance has developed which requires a change in treatment [64,65].

## Mycology and parasitology

The molecular diagnosis of parasites and fungi has been a revolution as they are difficult to diagnose at the species level [24]. Although still in a developing phase in parasitology, the lack of culturing techniques has necessitated diagnosis by molecular techniques. The medically encountered parasites and fungi are elaborated in Table 3. In mycology the most common testing employed is for molecular detection of *Pneumocystis jiroveci* (*Pneumocystis carinii*) an opportunistic fungus causing severe pneumonia in HIV-infected patients, which has made the microscopic and silver staining of tissue specimens a thing of the past [66].

## Future applications

The introduction of PCR technology by Nobel Prize winning scientist Dr Kary Mullis in 1983 has revolutionised the field of medical diagnostics including microbiology. A number of automated extraction/detection systems are available in the market which will become essential in molecular labs in the future. This system, such as QiaSymphony (Qiagen) offers automated DNA extraction, PCR setup, including reagent preparation, dilution series and sample pipetting.

**Table 1.** Standardised molecular protocols in diagnostic bacteriology.

Bacteria	Acceptable Specimen	Unacceptable Specimen	Limitations/Comments	Primer Sequence/Probe and Method References
<i>Bordetella pertussis</i>	Nasopharyngeal aspirates, nasal swabs or throat swabs. Sputum, endotracheal tube aspirates, and BAL fluids are also occasionally processed	The potential for false <i>B. pertussis</i> detection due to <i>B. Holmsei</i> infection must also be kept in mind	-	[72-75]
<i>Bordetella parapertussis</i>	Nasopharyngeal aspirates, nasal swabs or throat swabs. Sputum, endotracheal tube aspirates, and BAL fluids are also occasionally processed	-	-	[72-75]
<i>Brucellosis</i>	Bacterial isolates, Blood (EDTA), Serum, Buffy coat, fresh tissue (liver, spleen, thyroid, bone, synovial tissue and fluid)	-	Assays were validated by testing historical culture confirmed <i>B. suis</i> and <i>B. melitensis</i> patient isolates as well as cattle derived <i>B. abortus</i> isolates	[76,77]
<i>Burkholderia cepacia</i>	Designed for the identification of suspected <i>B. cepacia</i> complex and <i>B. gladioli</i> cultures and has not yet been validated for the detection of these organisms in clinical samples.	-	Species specific assays are not currently available for all of the <i>B. cepacia</i> complex species (e.g. <i>Burkholderia dolosa</i> , <i>Burkholderia pyrrocinia</i> )	[78-81]
<i>Chlamydia</i>	Acceptable specimens are urine, urethral swab, endocervical swab, dry swab (including throat, rectal and vaginal), tampon - cells collected in liquid based, cytology medium PreservCyt™ (Hologic, Bedford, MA, USA) and SurePath™ (BD, Franklin Lakes, NJ, USA)	Not validated for other specimens	Most commercial assays, however, do not differentiate between LGV and other serovars of <i>C. trachomatis</i>	[82,83]
<i>Chlamydophila</i>	Respiratory material, including throat swabs, nose swabs, nasopharyngeal aspirates, bronchoalveolar lavage, whole blood (EDTA), urine and CSF, occasionally environmental/bird specimens	Wound swabs, drainage fluid, faeces	No international controls are available for <i>C. psittaci</i> due to restrictions on transport of potential bioterrorism agents	[84-86]
<i>Coxiella burnetii</i>	Blood, serum, bone marrow, biopsies (Including those imbedded in paraffin) and cerebrospinal fluid.	-	-	[63]
<b>Diarrheagenic Escherichia coli Pathotypes (DEP) Including Enterohaemorrhagic (EHEC)/Shiga-toxin E. coli (STEC)</b>	Infectious colonies isolated after processing.	-	-	[87-90]
<i>Haemophilus ducreyi</i>	Impression smears or press slides, swabs, and biopsies only.	-	-	[91-93]
<i>Klebsiella granulomatis</i> .	-----do-----	-	Only <i>K. granulomatis</i> carries the restriction site specific to this organism	[92]
<i>Haemophilus influenzae</i> – Capsulated and Non-typeable	<i>H. influenza</i> cultures only	Not been validated for the detection of <i>H. influenza</i> in clinical samples	-	[94-96]
<b>Mycobacterium tuberculosis Complex</b>	Respiratory tract	Non-respiratory origin, the sensitivity and specificity of the assay has been estimated to be 69.8% and 95.6%, respectively.	Potential for environmental mycobacteria to contaminate samples	[94-96]
<b>Mycobacteria Other Than Mycobacterium tuberculosis</b>	Fresh tissue, cerebrospinal fluid, respiratory specimens, and body fluids, including uncoagulated blood in EDTA tubes	Formalin fixed and paraffin embedded tissue samples, clotted blood, serum, swabs, urine and faeces	-	[97,98]
<i>Mycoplasma genitalium</i>	Urine and genital swabs	-	<i>M. genitalium</i> DNA may be detected in asymptomatic individuals	[99,100]
<i>Mycoplasma pneumoniae</i>	Sputum and Bronchoalveolar lavage (BAL) specimens, nasopharyngeal and throat swabs, nasopharyngeal aspirates, tracheal aspirates and pleural fluid specimens	Not been validated for any other specimen than those listed.	<i>M. pneumoniae</i> may be detected for some time after acute infection following the resolution of clinical symptoms	[101-103]



<i>Neisseria gonorrhoeae</i>	Urine, urethral swab, endocervical swab, dry swab (including throat, rectal and vaginal)	Not been validated for any other specimen than those listed	PHLN guidelines for the use and interpretation of nucleic acid detection tests for <i>Neisseria gonorrhoeae</i>	[104-107]
<i>Neisseria meningitidis</i>	EDTA whole blood, CSF and aspirates from joints. Heparinised or citrated samples can be tested	Plasma and serum samples show a decreased recovery of <i>N. meningitidis</i> DNA	The <i>ctrA</i> PCR assay is used as a diagnostic test for meningococcal disease	[108,109]
<i>Pseudomonas aeruginosa</i>	<i>P. aeruginosa</i> isolates only	Used in sputum samples, but has not been validated	-	[110-113]
<i>Rickettsia</i>	Blood, biopsies, and cerebrospinal fluid	Clotted blood, serum, swabs and urine	-	[114]
<i>Streptococcus pneumoniae</i>	CSF and other sterile fluids.	Not appropriate to test respiratory specimens	<i>ply</i> gene is not specific for pneumococci and been detected in <i>Streptococcus mitis</i> and <i>Streptococcus oralis</i> isolates	[115]
<i>Treponema pallidum</i>	Swabs from skin lesions only	Not validated for other specimens	The gold standard for the detection of <i>T. pallidum</i> is probably rabbit inoculation	[116-118]

**Table 2.** Standardised molecular protocols in diagnostic virology.

Virus	Acceptable Specimen	Unacceptable Specimen	Limitations/Comments	Primer Sequence/Probe and Protocol References
<i>Adenovirus</i>	Clinical specimens (throat or nasal swabs or faecal samples)	Not been validated for any other specimen than those listed	Most common technique is to passage Adenovirus in A549 cells before genetic analysis	[119-121]
<i>Cytomegalovirus (CMV)</i>	New born Screening card, fresh placenta, fresh umbilical cord, amniotic fluid, urine, serum, plasma, buffy coats, formalin-fixed, paraffin-embedded tissues	Not been validated for any other specimen than those listed	Generally two different CMV PCR are performed on each sample to minimise false positivity	[122-126]
<i>Dengue Virus</i>	Serum	Not been validated for any other specimen than those listed	'Not detected' should not be considered as absence of virus.	[127-131]
<i>Enterovirus</i>	Faeces, CSF, nasopharyngeal aspirates, cell culture supernatant, throat, vesicle and rectal swabs	Blood and tissue samples	The specimen should be kept at 4 degree C	[132-135]
<i>Epstein Barr Virus</i>	CSF, Blood, Biopsies	Not been validated for any other specimen than those listed	Stored at -20 degree C	[136,137]
<i>Flavi virus</i>	CSF, CNS tissues and whole blood	Not been validated for any other specimen than those listed	West Nile virus assay also detects Kunjin virus.	[137]
<i>Hepatitis A Virus</i>	Whole blood, serum, and EDTA tubes	Heparin tubes as it is a potent inhibitor of PCR	Haemolysed samples are causes PCR inhibition	[138]
<i>Hepatitis B Virus</i>	-----do-----	-----do-----	-----do-----	[138]
<i>Hepatitis C Virus</i>	-----do-----	-----do-----	-----do-----	[139]
<i>Hepatitis D Virus</i>	-----do-----	-----do-----	-----do-----	[140]
<i>Hepatitis E Virus</i>	-----do-----	-----do-----	-----do-----	[141]
<i>Herpes Simplex Virus Type 1 and 2</i>	CSF, brain biopsy, FFPE	Not been validated for any other specimen than those listed	PCR detection has become a confirmatory test for HSV encephalitis	[142-147]
<i>Human Bocavirus</i>	Respiratory secretions, nose/throat swabs, stool samples, whole blood	Not been validated for any other specimen than those listed	-	[148]
<i>Human Coronaviruses</i>	Respiratory specimens (Nasopharyngeal aspirates, nose and throat swabs and bronchial samples)	Not been validated for any other specimen than those listed	-	[149-151]
<i>Human Herpes Viruses 6, 7 and 8</i>	EDTA blood, bone marrow and body fluids (amniotic fluid, CSF), biopsies, newborn screening cards	Not been validated for any other specimen than those listed	-	[122,124,125]
<i>Human Papillomavirus</i>	Vaginal swab, tampon, FFPE, cells collected in liquid based cytology medium PreservCyt™ (Hologic, Massachusetts, USA) and SurePath™ (BD, New Jersey, USA)	Not been validated for any other specimen than those listed.	For keratinised tissues, specific methods should be followed	[152,153]
<i>Human Polyomaviruses – JCV and BKV</i>	EDTA blood, CSF, body fluids, urine, biopsies	Not been validated for any other specimen than those listed	JCV and BKV may occur at the same time in same patients	[93,154-158]
<i>Human Polyomaviruses – KIV and WUV</i>	Nasopharyngeal aspirates, bronchial washes and other respiratory secretions, nose/throat swabs, cerebrospinal fluid, urine, whole blood, and stool samples	Not been validated for any other specimen than those listed	-	[159-161]
<i>Human Rhinoviruses</i>	Nasopharyngeal aspirates, nose and throat swabs, nasal or bronchial washings, broncho alveolar lavage or middle ear fluids	Not been validated for any other specimen than those listed	The assay may miss some viral strains	[162]
<i>Influenza Virus A H5N1 ("Avian Influenza")</i>	Throat swab, nasal swab or nasal wash	Not been validated for any other specimen than those listed	Prior testing with current strains is mandatory	[130]

<b>Influenza Virus A H1N1 (2009) ("Human Swine Influenza")</b>	Nasopharyngeal aspirates, bronchial washings, throat swab, nose swab	Not been validated for any other specimen than those listed	-	[113,163,164]
<b>Influenza Type C</b>	Respiratory secretions, bronchial lavage and washings, nose/throat swabs	Not been validated for any other specimen than those listed.	-	[165]
<b>Measles Virus</b>	EDTA blood, Urine, nasopharyngeal aspirates, throat and nasopharyngeal swab	Not been validated for any other specimen than those listed	No cross-reactivity identified between human and viral DNA in the cited protocols	[166,167]
<b>Norovirus</b>	Faeces	Not been validated for any other specimen than those listed		[168-171]
<b>Respiratory Syncytial Virus Types A and B</b>	Respiratory swabs, sputa or respiratory tissues, and paranasal aspirates	Not been validated for any other specimen than those listed	The coat protein gene of MS2 RNA coliphage and A and B (RSVA and RSVB)	[172]
<b>Rotavirus</b>	Faeces	Not been validated for any other specimen than those listed	RNA should be completely free from residual stool inhibitors	[173-177]
<b>Varicella Zoster virus</b>	CSF and CNS tissue	Not been validated for any other specimen than those listed	-	[178-180]

Table 3. Standardised molecular protocols in diagnostic mycology and parasitology.

Fungi/Parasites	Acceptable Specimen	Unacceptable Specimen	Limitations/Comments	Primer Sequence/Probe and Method References
<b>Aspergillus Species</b>	BAL, EDTA whole blood, serum, fresh and paraffin embedded tissue, vitreous fluid, ascitic fluid and CSF	Sputum and plasma	<i>Penicillium</i> spp. can also be amplified by the assay	[181-184]
<b>Cryptosporidium</b>	Fresh or frozen stool samples	-	PCR has a high sensitivity	[185-196]
<b>Cyclospora</b>	Fresh or frozen stool samples	-	The cited assay has a detection limit of 0.5 oocyst	[189]
<b>Dientamoeba fragilis</b>	Fresh or frozen stool samples	-	The cited assay amplifies 18s rDNA	[197-199]
<b>Entamoeba histolytica</b>	Fresh or frozen stool samples		The cited assay amplifies small subunit (18S) rRNA gene	[200-211]
<b>Giardia</b>	Fresh or frozen stool samples	-	-	[212-216]
<b>Malaria (<i>P. falciparum</i>, <i>P. vivax</i>, <i>P. malariae</i>, <i>P. ovale</i>)</b>	Whole blood samples	-	-	[155,217-219]
<b>Microsporidia</b>	Fresh or frozen stool samples	-	-	[220-227]
<b>Pneumocystis jirovecii</b>	Sputum, induced sputum, bronchial washings, bronchial lavages and lung biopsies	-	The cited assay can be used only as qualitative assay	[228,229]
<b>Trichomonas vaginalis</b>	Genital swabs and urine	-	The cited assay targets the $\beta$ -tubulin and 18S rRNA genes	[230]
<b>Toxoplasma gondii</b>	CSF, CNS tissue, and FFPE	CSF supernatant, plasma, and serum	Negative results should be confirmed with serology	[231-233]

Other automated systems such as the Abbott m1000 system, the ABI PRISM 6100 Nucleic Acid PrepStation, 6700 Automated Nucleic Acid Workstation, and the Corbett Robotics X-tractor Gene perform DNA extraction in a closed system.

In future a diverse array of next generation sequencing (NSG) platforms like Roche 454 will be routinely used as it can sequence ~500 bp approaching that of Sanger sequencing at lower cost [67]. For large bacterial sequences IlluminaHiSeq platform sequence analysers are the most useful, as it delivers ~300 Gb of raw data per eight-lane flow cell in the form of a 100 bp reads and provide rapid sequencing [68]. The future is promising for bench top NGS analysers, like Ion PGM and the IlluminaMiSeq than the larger counterparts [43]. A new platform of NSG released in 2012 by UK startup Oxford Nanopore Technologies has led to landmark improvement by introducing two sequencing platforms (the GridION and MinION) capable of delivering high-throughput, ultra-long sequence reads at low cost. It offers chips that are configured to read 2,000 or 8,000 pores simultaneously and that reads can be up to tens of kilobases in length. Because it reads native DNA, the Oxford Nanopore technology is anticipated to work with fairly crude samples and low DNA concentrations this makes colony

processing a thing of the past [45]. Another future application lies in Micorarrays which are designed to simultaneously monitor whole-genome, host and pathogen gene expression, providing a complete view of disease progression [69]. The most recent generation of microarrays performs sequence analysis often performing WHG in a single experiment. One such array the Gene Chip Customseq Arrays (Affymetrix, CA, US) can sequence up to 300 kb of a genome in 48hrs with minimal amplification of the genomic target thus enabling pathogen detection and identification [70]. The limit of detection in Gene Chip is as low as 10 femtograms for pathogenic DNA much below the detection limits of existing technologies. Thus the flexibility and high-throughput nature of microarrays offers an unprecedented opportunity for infectious disease diagnosis. Also the platform which holds a huge scope is digital PCR (dPCR) which is still in its early stage of development. The sample is diluted and partitioned into hundreds or even millions of separate reaction chambers so that each contains one or no copies of the sequence of interest. By counting the number of 'positive' partitions (in which the sequence is detected) versus 'negative' partitions (in which it is not), scientists can determine exactly how many copies of a DNA molecule were in the original sample [71]. In

one type of platform manufactured by Fluidigm and Life Technologies, reactions are created in within specially designed chips or plates. While in other platforms developed by BioRad and RainDance, reagents are sequestered into individual droplets *i.e.*, Droplet Digital PCR (ddPCR).

Thus the future of diagnostic microbiology holds a tremendous scope offered by molecular diagnostics which has the potential to transform the precision, sensitivity and specificity of pathogen detection in a rapid and cost effective manner.

## Competing interests

None declared.

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