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Emerging respiratory tract infections 4

Rapid point of care diagnostic tests for viral and bacterial respiratory tract infections—needs, advances, and future prospects

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Respiratory tract infections rank second as causes of adult and paediatric morbidity and mortality worldwide. Respiratory tract infections are caused by many different bacteria (including mycobacteria) and viruses, and rapid detection of pathogens in individual cases is crucial in achieving the best clinical management, public health surveillance, and control outcomes. Further challenges in improving management outcomes for respiratory tract infections exist: rapid identification of drug resistant pathogens; more widespread surveillance of infections, locally and internationally; and global responses to infections with pandemic potential. Developments in genome amplification have led to the discovery of several new respiratory pathogens, and sensitive PCR methods for the diagnostic work-up of these are available. Advances in technology have allowed for development of single and multiplexed PCR techniques that provide rapid detection of respiratory viruses in clinical specimens. Microarray-based multiplexing and nucleic-acid-based deep-sequencing methods allow simultaneous detection of pathogen nucleic acid and multiple antibiotic resistance, providing further hope in revolutionising rapid point of care respiratory tract infection diagnostics.

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This is the fourth in a **Series** of five papers on emerging respiratory tract infections

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Introduction

Respiratory tract infections are caused by many viral and bacterial pathogens[1] and are the second most common cause of morbidity and mortality worldwide.[2–4] Lower respiratory tract infections come second in the global burden of disease rankings after ischaemic heart disease.[1,4] Surveillance reports[5] from Europe show a substantial rise in the number of infections caused by antimicrobial resistant bacteria. Community acquired pneumonia,[6] hospital-acquired pneumonia, and ventilator associated pneumonia [7] all continue to present clinically significant diagnostic and management challenges. Additionally, the worldwide spread of multidrug resistant tuberculosis[8] and emergence of multidrug resistant Gram-negative bacteria,[9,10] for which few effective therapy options exist, are a major concern. Respiratory tract infections are also the most common infections in an ever increasing number of immunocompromised people[11] in whom a broader differential diagnosis of opportunistic microorganisms presents further diagnostic challenges.[12] Successful treatment outcomes for respiratory tract infections presenting in all types of health-care settings will only be achieved with rapid, sensitive, and specific identification of pathogens and antibiotic resistance profiles to allow effective evidence-based antimicrobial therapy and pathogen-specific infection control measures.[13]

The presence of microbial nucleic acids in respiratory tract samples has been exploited for amplification of targets to identify microbes and antibiotic resistance. In this review, we describe the available diagnostic tests for viral and bacterial causes of respiratory tract infections

Key messages

- Millions of adults and children worldwide continue to die of treatable respiratory tract infections caused by a wide range of microbial pathogens.
- The emergence of multi-antibiotic resistant bacteria and novel respiratory viruses with pandemic potential is of global concern.
- Optimum clinical management outcomes can be achieved only through rapid accurate diagnosis of the microbial cause of respiratory tract infections and initiation of appropriate antibiotic therapy.
- The presence of microbial nucleic acids in respiratory tract samples has been exploited for amplification of microbe species and antibiotic resistance specific genetic targets
- Molecular diagnostic platforms allow for rapid diagnostic tests to be modelled on automated platforms using nucleic acid amplification techniques (NAAT). The clinical dilemma surrounding the use of high sensitivity and specificity NAATs is that identification of pathogen nucleic acid from a respiratory tract sample may not necessarily attribute causation.
- Few validated NAAT tests that screen for respiratory tract infections caused by specific viral or bacterial groups are being used by diagnostic laboratories to diagnose selected pathogens, usually in combination with more traditional methods.
- Laboratories in most developing countries use traditional age-old methods for diagnosis of respiratory tract infections except for the Cepheid GeneXpert MTB/RIF assay, which is being rolled out worldwide for rapid diagnosis of tuberculosis and rifampicin resistance.
- Microarray-based multiplexing and nucleic-acid-based deep-sequencing methods for the simultaneous detection of pathogen nucleic acid and multiple antibiotic resistance provide further hope for revolutionising rapid point-of-care tuberculosis diagnostics, and they have been invaluable in identifying new viral and bacterial pathogens.
- Despite advances, a great need for rapid, point-of-care pathogen-specific, sensitive, and affordable diagnostics remains for the advancement of clinical management, infection control, and improved public health response to emerging pathogens.

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and developments in technologies that offer the potential for improving the quality, speed, and tractability of near point-of-care rapid diagnostic tests.

Clinical and public health diagnostics

When patients with respiratory tract infections present at any point of care, diagnostic tests should be available to simultaneously differentiate bacterial (including tuberculosis), viral, and other microbial causes to achieve the best possible treatment outcomes. At present, patients presenting with acute respiratory tract infections are started on empiric antimicrobial treatment for presumed acute bacterial infection rather than therapy directed at the causal organism.[14] The major drawback in the clinical management of respiratory tract infections worldwide nowadays is the absence of standardised, rapid, accurate, specific point-of-care diagnostic tests able to screen for major pathogen groups, to enable identification of the causative organisms, and to ascertain antimicrobial susceptibilities.[13] Present advances in molecular technologies offer a unique opportunity to address this unmet need.[15]

New lethal viruses and bacteria causing respiratory tract infections, several with epidemic potential, have emerged in the past 10 years, threatening global health security and attracting widespread media and political attention. These include the severe acute respiratory syndrome coronavirus (SARS-CoV [2003]),[16] swine-origin influenza A (H1N1pdm2009),[17] Middle East respiratory syndrome coronavirus (MERS-CoV [2012]),[18] multi drug resistant and extensively-drug resistant tuberculosis,[19] pan-drug resistant Gram-positive and Gram-negative bacteria,[7,9,10] antiviral resistant cytomegalovirus strains in immunocompromised patients,[20] and azole-resistant fungi.[21] Other newly emergent respiratory pathogen threats that merit close monitoring for expanding epidemic potential include avian influenza A H7N9,[22] influenza A swine H3N2v and H1N1v variant,[23] human adenovirus 14p1,[24] and rhinovirus group C,[25] each of which have caused localised outbreaks of great concern.

Whenever a previously unknown potentially lethal microorganism causing respiratory tract infection emerges, clinicians, microbiologists, and public health officials are expected to work synergistically together with national and global health systems to respond to the threat. This response has many components: rapid diagnosis and identification of similar cases; case control studies to determine reservoirs, modes of transmission, and risk factors; collection of individual and case cluster data and reports; ascertainment of transmission patterns; isolation, identification, and characterisation of the specific pathogen, and establishment of Koch's postulates if possible; and development of pathogen-specific diagnostics and genome sequencing to monitor the evolution and transmission patterns. These collaborative activities are essential for the identification of the specific microorganism, guidance of appropriate targeted therapy, monitoring of response to treatment, prediction of prognosis, guidance of infection control measures, and public health surveillance and control recommendations. Rapid, accurate diagnostic laboratory tests are crucial in the public health management of respiratory tract infections caused by new potentially lethal pathogens.

Point-of-care and near-patient testing

The requirements for ideal point-of-care and near-patient testing for respiratory tract infections are similar (table 1) but may differ according to specific needs of the health-care setting. Several diagnostic platforms and tests have great potential to improve management of respiratory tract infections.[26,27] Furthermore, these are becoming increasingly important in response to outbreaks of respiratory tract infections caused by zoonotic pathogens, which jump the species barrier and have epidemic potential.[28–30] Several commercial diagnostic tests and platforms that incorporate the above technologies and promise to substantially reduce turnaround times for diagnosis of a host of microbial infections, including those of the respiratory tract, are on the market or in development (table 2). Typically, these are on automated or semiautomated systems or kits that integrate sample preparation, pathogen detection, and identification of antimicrobial resistance genes, providing an automated read-out of results. These tests and platforms are the most advanced systems requiring the least possible user input throughout the process and are capable of detecting several pathogens simultaneously. Depending on the test, single or multiple pathogens, or antimicrobial resistances may be detected. Such systems can offer not only an improved speed of diagnosis but also increased sensitivity of detection. However, the development of such tests and their successful implementation into clinical practice requires further development.[31–33]

Where the accuracy of results is high with multiplex tests, the desirable characteristics for providing both diagnostic and epidemiological information become convergent, and routine diagnostic laboratories can consider fulfilling a public health role.[34] Molecular multiplex tests need to be transported outside the laboratory as point-of-care tests in busy tertiary care, outpatient clinical settings, or rural areas in developing countries. From this point, the basic requirements of a method may diverge: for field studies, the adopted amplification technology may need to be something more suited to situations where power supply cannot be guaranteed, such as isothermal amplification. For all point-of-care tests, operational simplicity allowing use by non-laboratory trained staff and accurate interpretation of raw signal data are key factors.

Evolution of diagnostics for respiratory tract infections

Before the advent of laboratory tests, the practice of medicine was an art, and making a diagnosis of respiratory infection relied entirely on the taking of

	Technology requirements	Purpose	Desired characteristics	Technological innovation and current stage of development
Viral respiratory infections	Point-of-care (eg, primary care office, outpatient clinics, accident and emergency)	To distinguish viral and bacterial infections and inform antiviral therapy. Infection control and bed management allowing patients with diferent viruses to be separated; outbreak tracing	Rapid <1 h Able to be operated by front-line clinical staff (eg, nurse or family practitioner) Ability to process multiple samples simultaneously Low cost	Multiplexed NAAT based tests for high-throughput test platforms requring minimum user skill and hands-on time (currently available in low-thoughput format) Breath-based tests for key viral pathogens such as influenza (in development) Simple tests on a non-invasive sample able to distinguish viral and bacterial infections (conceptual)
Community acquired pneumonia	Near-patient, rapid response (eg, in larger outpatient clinic or laboratory adjacent to accident and emergency)	To diagnose cause of infection and recommend effective and proportionate antimicrobial therapy, to asses whether patient should be admitted	Rapid <1 h Ability to detect pathogen and distinguish pathogen from colonisers Ability to detect drug resistance Low to medium cost, operation by front-line staff Adaptation for resource limited settings	Multiplex NAAT based tests for a variety of pathogens and resistance determinants requiring minimal user skill and hands-on time (already available but with minimal data regarding performance and clinical utility) Quantitative NAAT-based tests allowing pathogens and colonisers to be distinguished (in concept) Simple tests on a non-invasive sample able to distinguish viral and bacterial infections (conceptual)
Hospital acquired pneumonia and ventilator associated pneumonia	Rapid response (near intensive care unit/in clinical microbiology laboratory with good transport and communication systems)	To diagnose cause of infection and recommend effective and proportionate antimicrobial therapy	Rapid <2 h, round-the-clock service Ability to detect pathogens and distinguish them from colonisers. Ability to detect drug resistance Low to medium cost, operation by trained personnel capable of complex interpretation of results	Rapid, highly multiplexed NAAT based tests and platforms incorporating a wide variety of pahogens and resisance deteminants requiring minimum user skill and hands-on time (currently in development) Quantitative NAAT-based tests allowing pathogens and colonisers to be distinguished (in concept) Next-generation sequencing based diagnostics allowing the identification of rare and unusual pathogens and the rapid generation of antibiotic suscpetibility profiles (in concept)
Tuberculosis	Point of care (eg, doctors office, tuberculosis clinic)	To identify those with acute tuberculosis and needing therapy	Rapid <1 h Reliable detection of drug-resistance Suitable for resource limited setting (eg, requiring minimum operator training, low cost, limited power requirements, room temperature storage)	NAAT based tests for "sample-in answer out" platforms (already available) Hand-held NAAT based tests that can be operated by battery or solar power (in development) Breath–based tests

NAAT= nucleic acid amplification techniques.

***Table 1*: Clinical needs for rapid point-of-care diagnostics for respiratory tract infections**

medical histories and physical examinations.[35] The discovery of the microscope by Antonie van Leeuwenhoek (1632-1723) was the first step towards the development of laboratory diagnostic tests for respiratory tract infections with microscopic examination of stained sputum coupled with sputum culture on agar[37] followed by liquid-culture methods. Further refinement of bacterial and viral culture methods improved the ability to detect specific pathogens and identify their susceptibility testing against specific antimicrobials, although the time needed for culture growth (24–72 h) did not influence treatment decisions on admission. These diagnostic methods did not change until the late 1980s when major advances in molecular biology, immunology, genomics, and technical engineering led to many new diagnostic tests. Serological tests for detection of microbial antigen or antibody, agglutination tests, complement fixation tests, fluorescent antibody tests, radioimmunoassay, and ELISA have been developed for various pathogens without any great influence on clinical management of respiratory tract infections at points of care.[38] The most relevant development has been the use of nucleic acid amplification techniques (NAAT) for respiratory tract infection diagnostics.[39] The presence of microbial nucleic acids in respiratory tract samples (sputum, nasopharyngeal swabs, tracheal aspirates, and broncho-alveolar lavage) has been exploited for amplification of microbe-specific genetic targets.[26,27] This was initially labour intensive and NAAT technologies have evolved to real time PCR (rtPCR), loop-mediated amplification (LAMP), nucleic acid sequence-based amplification, and strand displacement amplification, the latter three methods avoiding thermocycling.

Diagnostic tests for viral infections

Evolution of viral diagnostics

Before the introduction of NAATs, the mainstay of diagnosis of viral respiratory tract infections was largely based on serology; consisting of a combination of detecting substantial antibody rises with complement-fixation tests, detection of viral antigen by immunofluorescence or colorimetric methods, and virus isolation in cell culture, often with blind passage followed by secondary detection with immunofluorescence or haemadsorption.[40,41] The older generation tests for viruses are still useful today in scenarios in which the time to results is not crucial.[42]

During the past two decades, the sensitivity and specificity of tests to detect viral respiratory pathogens have been improved by developments in genome amplification. Several new respiratory viruses have been

	Desired
Basic	
Sensitivity	Approaching 100%
Specificity	Approaching 100%
Positive predictive value for disease	Approaching 1·0
Negative predictive value for disease	Approaching 1·0
Turnaround time	30 min–2 h
Enhanced	
Control for sample quality	Human single copy gene
Control for reaction inhibition	Heterologous gene
Sample volume	Accepts small volume sample
Multiplex	Ability to multiplex a large number of viral and non-viral pathogens
Typing	Bacterial serotyping, toxin, or viral typing (eg, influenza A or B; haemagglutinin 1 or 3, pneumococcal serotype)
Quantitative	Relative pathogen load to distinguish colonisation from infection
Drug resistance	Resistance to β-lactams, macrolides, fluoroquinolones, antivirals (eg, His275Tyr for oseltamivir)
Automated systems	
Operation	Minimum operator interaction
DNA or RNA extraction	Integrated in automation
Contamination resistant process	Single step, single tube enclosed system
Result analysis	Integrated in automation
Unambiguous interpretation	Positive or negative
Reduce transcription error	LIMS interface
Isothermal	Done at room temperature

LIMS=laboratory information management system.

***Table 2*: Desirable characteristics for respiratory diagnostics**

discovered, and sensitive PCR methods for their diagnostic work-up exist.[26,34,40,43–49] Advances in technology have allowed for development of multiplex PCR tests that use several primer sets running within a single PCR mixture, with short throughput time compared with multiple single-target PCRs. Single and multiplexed PCRs provide rapid detection of respiratory viruses in clinical specimens and are being used in defining the epidemiology of new emerging viruses such as influenza A H1N1pdm2009[17] and MERS-CoV in 2012.[18] Multiplex PCR assays identify several different viruses in a single test.[34] Several multiplex PCR tests are now commercially available, and these are constantly being refined and assessed. Basic laboratory diagnosis of viral causes of respiratory tract infections is being coupled to subtyping,[49] antiviral drug resistance,[50] nucleotide polymorphisms,[51] and, together with viral load assays,[52] provide extensive information for optimum treatment of respiratory tract infections.

Characteristics of viral diagnostic methods

The clinical usefulness of a test was determined by the relative degrees of sensitivity and specificity and the time taken to obtain a result. Monoclonal-antibody direct fluorescent antibody tests usually had adequate specificity for a particular virus, but there was a trade-off between turnaround time and sensitivity. Thus, although some of the colorimetric tests for direct antigen detection had a turnaround time of less than an hour, sensitivity could be around 70%.[51] Further developments in reaction chemistry have enabled the targeted amplification of other viruses in the same reaction, while keeping sensitivity and specificity high and the turnaround time still relevant to clinical need. Available versions of in-house and commercially developed multiplex tests offer potential amplification of up to 20 pathogens from a clinical sample,[26,40,45] although for some of these the turnaround time approaches a whole working day. Several head-to-head comparisons of in-house and commercial tests have been published.[43,44,46–48]

Clinical interpretation of multiplex tests for viruses

The advantage of multiplex tests is that they increase the chance of identifying the microbial causes of respiratory tract infections and can detect more than one pathogen at a single time point when there are co-infections[52] The difficulty lies with interpreting the findings in relation to a patient's clinical status. Detection of a weak signal of one virus may represent a commensal or the tail-end of a previous infection, although it may also show that the infection is recent and evolving. Another possibility is that the weaker signal is a pronounced viral infection in the lower respiratory tract, but the virus is not yet well represented in the upper respiratory tract, where there is a different viral infection present. The issue of which clinical sample will generate the highest diagnostic yield is also dependent on the pathogenesis of the virus.

The rapid development of multiplex tests may have outstripped their clinical need, and rather than provide clinicians with useful information, clinical interpretation and decision making might have become more complex. A demonstration of how challenging interpretation of virus identifications from multiplex PCRs can be is shown by a prospective study of neonates in an intensive care unit.[53] Over 1 year, although some morbidity outcomes were associated with respiratory virus infections, infections were also commonly detected in the absence of clinical illness.

Diagnostic tests for bacterial respiratory tract infections

Despite advances in technology, the gold standard diagnostic technique for respiratory tract infections of bacterial cause is traditional culture, followed by identification and antimicrobial susceptibility testing by various manual or automated methods.[27] Individual diagnostic laboratories may use molecular methods (developed either in-house or externally) for part of the detection process. Such supplementary methods are often aimed at organisms that are difficult or take a long time to culture, such as *Bordetella pertussis, Legionella pneumophila*, *Mycoplasma pneumoniae*, and *Chlamydophila pneumoniae*.[31,54] However, the standard process of culture and susceptibility testing generally takes 2–3 days, with at

least 1 day for culture and a second day for antimicrobial susceptibility testing. Meanwhile, many patients are empirically treated with antibiotics.[27,55] Such treatment will often be ineffective, inappropriate, or both. Ineffective antimicrobials are frequently administered to treat infections caused by resistant organisms or not bacterial at all. In the case of severe infections or those in immunocompromised patients, this ineffective treatment can lead to increased morbidity and mortality.[56] As a result, clinicians often empirically prescribe last resort, broad-spectrum antimicrobials such as carbapenems to treat infections caused by susceptible bacteria, subjecting patients to possible unnecessary side-effects and driving the emergence and spread of antimicrobial resistance. Hence rapid point-of-care and near-patient technology is greatly needed to increase the speed and accuracy of diagnosis, informing the clinicians' choice of appropriate and proportionate anti-infective therapy.[32,56–58]

Existing bacterial diagnostics technology

The laboratory diagnosis of the specific bacterial cause of respiratory tract infections is notoriously difficult. Up to 30% of gold-standard culture tests do not identify a cause because of the existence of unknown pathogens and poor accuracy and sensitivity.[59] Rapid, molecular diagnostic assays based on detection of nucleic acid offer a potential solution to this problem.[60] Accurate and comprehensive detection of antimicrobial resistance with these techniques is fraught with difficulty owing to the multitude of antimicrobial resistance determinants in existence and limited capability of multiplexing for PCR-based technology.[58,61]

New tests for lower respiratory tract bacterial infections

Despite apparent development activity in this area, very few platforms and tests are on the market, and few clinical evaluations of such tests have been published (table 3).[62–65] The only available comprehensive product is the Curetis Unyvero P50 pneumonia cartridge, which can detect 17 bacterial and fungal pathogens and 22 antibiotic resistance markers from respiratory samples in a single run,[63] accomplishing this feat in roughly 4 h. The composition of the panel is general and includes bacteria relevant to both community (eg, *Streptococcus pneumoniae, Haemophilus influenzae,* and atypical bacteria) and hospital acquired pneumonia (*Staphylococcus aureus, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa*) and some resistance determinants relevant to these. Resistance determinants detected include those encoding β-lactam resistance (*mecA, bla*$_{CTX-M}$, *bla*$_{DHA}$, *bla*$_{EBC}$, *bla*$_{OXA-51}$, and *bla*$_{KPC}$), macrolide resistance (*ermB,* and *mefA*), fluoroquinolone resistance mutations (*gyrA83, gyrA87,* and *parC*), and class 1 integron markers (*int1,* and *sul1*). Independent laboratory and clinical evaluation data of this test are not available, but manufacturer sponsored studies suggest variable sensitivity and specificity. Although overall test sensitivity was 80·9% and specificity 99·0%, for individual targets, the sensitivities varied substantially (50–100%) as did the specificities (72·3–100%).[66]

No licensed GeneXpert test for bacterial lower respiratory tract infection exist, although a study[65,70] has reported the use of this platform to detect *S aureus* in respiratory samples. The study examined 135 endotracheal aspirates from suspected ventilator associated pneumonia showing the presence of Gram-positive cocci by microscopy and compared the results with those from both qualitative and quantitative traditional culture. Although the researchers reported good specificity compared with qualitative culture (89·7%), microscopy performed poorly compared with quantitative culture (72·2%).[62,67] At present, most laboratories report quantitative results and generally define counts of 10^4–10^5 colony-forming units/mL as significant infection, and lower counts presumed to show colonisation and contamination.[37] A Cochrane review found no difference in outcome for patients when comparing

	Time to result	Type of technology	Targets	Sensitivity	Specificity
Cepheid Xpert MRSA/SA SSTI[62]	1 h	Automated sample preparation of respiratory specimen, real-time PCR and detection using molecular beacon technology	MSSA and MRSA	99·0% compared with quantitative culture of endotracheal aspirates	72·2% compared with quantitative culture of endotracheal aspirates
Curetis Unyvero Pneumonia P50 Test[63]	4 h	Multiplex endpoint PCR and amplicon detection by hybridisation to oligo probes spotted on membrane arrays direct from respiratory samples	Detection of 17 bacterial and fungal pathogens in addition to 22 antibiotic resistance genes	80·9% overall; target specific values 50–100%	99·0% overall, target specific values 72·3–100%
Biofire Filmarray Respiratory Panel[64,65]	1 h	Pouch format comprising nucleic acid extraction, and nested PCR from nasopharyngeal swabs	20 targets including respiratory viruses, *Bordetella pertussis, Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*	84–100%	98–100%

MSSA=methicillin-sensitive *Staphylococcus aureus*. MRSA=methicillin-resistant *S aureus*. SSTI=skin and soft tissue infection.

***Table 3*: Rapid molecular platforms and tests available for the diagnosis of bacterial respiratory tract infections**

quantitative and qualitative culture-based diagnostic methods for ventilator associated pneumonia.[71]

Tests for upper respiratory tract infections

Other tests aimed mainly for the detection of upper respiratory tract infection include the Biomerieux Biofire Filmarray Respiratory Panel.[64,65,68,69] This system integrates sample preparation, amplification and detection with results in roughly 1 h and has minimum hands-on time, making the system suitable for point of care. It uses an upper respiratory tract sample (nasopharyngeal swab) to detect up to 20 viral and bacterial pathogens. Of these, bacteria are limited to *B pertussis, M pneumoniae* and *C pneumoniae*. So far, only limited details of performance and testing are available (table 3).[62–65]

Development of sequencing-based diagnostics for respiratory tract infections

Conventional whole genome sequencing (WGS) requires prior knowledge of the pathogen whereas next generation sequencing (NGS) methods can sequence all genomic material present in a sample.[70] NGS has the ability to sequence many microbial genomes and deliver and interpret the resultant sequence information in near real-time. Thus, NGS provides an unbiased approach for detection of any pathogen present in a clinical sample, its antibiotic resistance genes, and for new pathogen discovery. NGS methods provide sensitivity and multiplexing capabilities, and offer many potential advantages to diagnostic microbiology laboratories for rapid detection of drug resistance and timely identification of nosocomial transmission of a range of bacterial and viral pathogens.[71–73] Therefore, conventional methods are poor for detection of low-level drug resistance mutations, which contribute to phenotypic antimicrobial and antiviral resistance. The need for amplicons limits the length of sequence and thus the usefulness of Sanger methods[74] for pathogen genotyping in outbreaks.

NGS with sputum samples for respiratory tract infections diagnostics

NGS methods can be harnessed for sequencing multiple different pathogens in a single sample or multiple samples in the same run. Barcoding technology,[75] which labels each sample with a unique identifier, can be used to simultaneously sequence multiple samples from patients infected with the same pathogen. Several developments are needed before use of NGS becomes more widespread, such as improving the sensitivity of pathogen sequencing directly from clinical material and development of tractable software for practical use.[72] Methods that can obviate the necessity for prior culture or PCR amplification for enriching target pathogens are needed. A European funded consortium, PATHSEEK, is investigating high multiplicity multiplexing and multiplexing of many different pathogens in the same reaction,[76] using NGS methods with bespoke software for sequencing of whole pathogen genome, including influenza and tuberculosis, directly from clinical material.[73] Alternative approaches retain the unbiased nature of NGS, opting instead for unselective deep sequencing of RNA transcripts isolated from clinical material, thereby capturing RNA and DNA pathogens and discovering new agents. The advent of nanotechnologies such as nanopore sequencing and mobile devices promising rapid turnaround times, small footprints, and decreased costs brings us closer to the possibility that near-patient pathogen genome sequencing and data interpretation will be available within the near future.

NGS for identification of antimicrobial resistance

By contrast with Sanger methods,[74] NGS is able to generate more sequence-data per run, detecting multiple resistance mutations simultaneously, even when these occur in non-contiguous genes. NGS methods[70] can sequence longer regions in a single assay, including whole pathogen genomes, which are particularly powerful for phylogenetic analyses to identify pathogen transmission and for outbreak-monitoring. Although many different NGS methodologies are now available, the principles—namely, unbiased sequencing of populations (libraries) of amplified DNA-template molecules is common to most pathogens. Advances of next generation methods include PacBio and Nanopore, which can sequence from single molecules to provide read lengths of thousands of bases long and throughput with higher overall error rates. NGS methods can be harnessed for sequencing multiple different pathogens in a single sample or multiple samples in the same run.[70] The challenge for the identification of antimicrobial resistance, particularly in complex multi drug resistant organisms, will be to rapidly assemble and analyse the generated data. This will require the construction of robust databases and data analysis algorithms[77] that can rapidly equate a genome with a likely antimicrobial resistance profile.

Development of diagnostic tests for novel viral respiratory tract infections

MERS is a newly described human disease predominantly affecting the respiratory tract. It was first reported from Saudi Arabia in September, 2012, after identification of MERS-CoV (a novel betacoronavirus) from a patient in Jeddah who died from a severe respiratory illness.[18] Subsequently, several community and hospital-based studies defined the epidemiology, transmission dynamics, and spectrum of clinical presentations from the mild to severe, including the relationship of rapidly fulminant disease with comorbid medical disorders.[78–82] A molecular rtPCR diagnostic test for detecting MERS-CoV was rapidly developed and approved by WHO soon after the first case of MERS-CoV infection was reported, and point-of-care tests are being developed.[83–85]

Several studies[29,86–107] have focused on development and assessment of serological tests (table 4) for the screening of human beings and potential animal reservoirs. These

	Details	Human studies	Animal studies
Virus			
ELISA	MERS-CoV infected Vero cells used as crude lysate in ELISA[89]	NA	MERS-CoV in dromedary camels, Saudi Arabia[89]
WB	MERS-CoV infected Vero cells used as crude lysate in Western blot analysis [89]	NA	MERS-CoV in dromedary camels, Saudi Arabia[89]
IFA	MERS-CoV infected Vero cells fixed to glass slides[90]	Serology Essen patient;[88] case contact study Essen patient;[90] clinical feature of MERS patient Munich;[93] slaughterhouse serostudy Saudi Arabia;[91] stillbirth during MERS infection, Jordan;[94] study on cross reactivity of SARS patient sera[92]	Serostudy on dromedary camels from UAE;[97] serostudy hedgehogs[95]
PRNT	Plaque assay based virus neutralisation test[90]	Case contact study Essen patient;[90] clinical feature of MERS patient;[93] slaughterhouse serostudy Saudi Arabia;[91] camel and human infection in Saudi Arabia[99]	First serostudy on dromedary camels, Oman and Spain;[98] Camel and human infection in Saudi Arabia[99]
MicroNT	Cytopathogenic-based virus neutralisation test[98]	Stillbirth during MERS infection, Jordan;[94] study on cross reactivity of SARS patient sera[92]	First serostudy on dromedary camels, Oman and Spain;[98] serostudy on dromedary camels from UAE[97] serostudy on livestock in Egypt and Saudi Arabia;[96,102] serostudy on livestock in Jordan[104]
Spike protein pseudotyped viruses			
MicroNT	Reporterviruses carrying the spike protein of MERS-CoV	Serostudy eastern Saudi Arabia[101]	Serostudy on livestock in Egypt;[102] serostudy on livestock in Saudi Arabia[96]
Spike protein			
rELISA	Recombinant spike protein expressed by Venezuelan equine encephalitis replicons[100]	Serology MERS patient NA1[100]	NA
rWB	Recombinant spike protein expressed by Vero cells (denatured protein)[86] or by Venezuelan equine encephalitis replicons[100]	Serology Essen patient;[88] serology MERS patient NA1[100]	NA
rIFA	Vero cells expressing recombinant MERS-CoV full-length spike protein[88]	Serology Essen patient;[88] slaughterhouse serostudy Saudi Arabia;[91] clinical feature of MERS patient;[93] camel and human infection in Saudi Arabia[95]	Serostudy on dromedary camels from UAE;[97] camel and human infection in Saudi Arabia[95]
Differential rIFA	Vero cells expressing recombinant full-length spike proteins of all known human pathogenic CoV[90]	Case contact study Essen patient[90]; slaughterhouse serostudy Saudi Arabia[95]	NA
Spike S1 subunit			
rELISA	Spike S1 subunit expressed as described[105]	Camel and human infection in Saudi Arabia[95]	Camel and human infection in Saudi Arabia[99]
Protein microarray	Glass chips carrying S1 subunit protein spots of MERS-CoV, hCoV-OC43 and SARS-CoV[98]	NA	First serostudy on dromedary camels, Oman and Spain;[98] first identification of dromedary camels carrying MERS-CoV in Qatar;[97] serostudy on livestock in Jordan;[104] serostudy on dromedary camels from UAE[29]
Nucleocapsid			
rELISA	HCoV-HKU1-nucleocapsid as substitute for MERS-CoV N,different N proteins expressed by Venezuelan equine encephalitis replicons[94,100]	Stillbirth during MERS infection, Jordan;[94] serology MERS patient NA1[100]	NA
rWB	MERS-CoV nucleocapsid expressed in Vero cells or by Venezuelan equine encephalitis replicons[100]	Serology Essen patient;[88] serology MERS patient NA1[100]	NA
LIPS	Immunoprecipitation with MERS-CoV nucleocapsid protein[89]	NA	MERS-CoV in dromedary camels, Saudi Arabia[89]
rIFA	MERS-CoV nucleocapsid expressed in Vero cells[86]	Serology Essen patient[88]	NA
Differential rIFA	MERS-CoV and other human pathogenic CoV nucleocapsid expressed in Vero cells[90]	Case contact study Essen patient[90]	NA

MERS-CoV=Middle-East respiratory syndrome coronavirus. SARS-CoV=severe acute respiratory syndrome coronavirus. WB=western blot. rWB=recombinant western blot. IFA=immunofluorescence antibody assay. rIFA=recombinant immunofluorescence assay. PRNT=plaque reduction neutralisation test. HCoV= human coronavirus. HKU1=Hong kong University 1. NA=not applicable.

***Table 4*: Serological tests using virus, spike protein, and nucleocapsid antigens for Middle East respiratory syndrome coronavirus**

include immunofluorescence assays with Vero cells expressing recombinant N or S proteins of MERS-CoV, conventional immunofluorescence assays with virus-infected cells, and western blot analysis of lysates from cells expressing recombinant N or S protein 2. A cell-free protein microarray was developed that uses the correctly folded and glycosylated S1 fragment of the MERS-CoV S protein as an antigen. MERS-CoV and MERS-CoV-S protein-pseudotyped viruses were used in neutralisation assays. Comparison of conventional virus neutralisation test with the S pseudotyped lentivirus-based neutralisation test on 1343 human serum samples collected from healthy donors in Egypt and Hong Kong as controls were negative in both neutralisation test formats.[102] Large-scale serological

and case-controlled studies of the population in affected countries are urgently needed to further examine spread, prevalence, and transmission of MERS-CoV.

WGS has been useful for studying viral transmission and evolution.[79–81] Several studies,[87–89] based on nucleic acid detection assays, found closely related coronaviruses in different species of bat in Africa, Saudi Arabia, and north America. Comparison of PCR, with serological methods on livestock animals from MERS-CoV, showed that dromedary camels harbour MERS-CoV neutralising antibodies,[98] and this finding was verified by studies of camels on farms where human MERS rtPCR-confirmed cases occurred.[86] A report[107] showed identical MERS-CoV sequences obtained from a patient who died of laboratory-confirmed MERS and those obtained from a dromedary camel with rhinorrhoea that the patient had contact with.

Development of rapid diagnostic tests for pulmonary tuberculosis

An estimated 3 million of the world's 8·8 million cases of pulmonary tuberculosis are not diagnosed and thus are still untreated, continuing to spread the disease in the community. In 2012, of an estimated 450 000 cases of multidrug resistant tuberculosis worldwide, 80% were undiagnosed.[108] Patients with pulmonary tuberculosis present with respiratory symptoms and receive repeated courses of antibiotics before being screened for tuberculosis. The continued use of century-old sputum microscopy and the time required for traditional culture-based diagnosis of *Mycobacterium tuberculosis*, coupled with the large global health burden and associated mortality of tuberculosis, led to focused global efforts on new rapid and more sensitive tuberculosis diagnostics (table 5). The past 5 years have seen an unprecedented activity in development of a range of new diagnostic tests based on culture, molecular, and non-molecular methods by scores of small-to-medium sized enterprises.[109–138] A major concern is that not all marketed tests have been assessed rigorously for diagnostic accuracy, robustness under operational conditions in the field, cost-effectiveness, and practical usefulness.

New point-of-care, near-patient innovations in tuberculosis diagnostics have several targets: rapid diagnosis of tuberculosis and identification of rifampicin resistance with Xpert MTB/RIF assay identification of multidrug resistant tuberculosis with the Hain Genotype multidrug resistant Plus System, and routine prospective variable number of tandem repeats-mycobacterial interspersed repetitive units (VNTR-MIRU) typing to allow prioritisation of cases for contact tracing. The Xpert MTB/RIF assay, which uses the Cepheid GeneXpert system, has been a forerunner in rapid molecular point-of-care diagnostics.[115,131–137] The results of sputum analysis are available in 2 h, and operationally within 24 h.[136,137] Numerous assessment studies at several points of care have shown that the assay is sensitive and specific and has increased detection of smear-negative patients with pulmonary tuberculosis (table 4).[115,137] However, this diagnostic improvement does not always lead to better clinical outcomes. In a randomised multicentre trial of clinical outcomes of with Xpert MTB/RIF assay, although a high proportion of patients started treatment on the day of presentation, there was no significant improvement in lowering of tuberculosis related morbidity; the researchers suggested that the lack of benefit was a result of effective empirical management in the control group.[134] Other promising test platforms are being introduced for detection of *M tuberculosis* aligning improved functionality at point of care, increased accuracy of detection, and developing more drug resistance targets.[109–116] Although tuberculosis-centric diagnostic test development is important, it is prudent to realise that it might not fit into the longer term goals of optimum converging delivery of health care for both noncommunicable and other communicable diseases, which is moving away from disease-specific silos.

Although genotypic analysis of drug-resistant strains of *M tuberculosis* is possible, limitations in laboratory methods exist, such as faster and more accurate determination of the antimicrobial resistance phenotype, which need to be overcome. Direct sequencing from sputum requires prior pathogen enrichment by culture or other enrichment methods. Microarray-based multiplexing and nucleic-acid-based deep sequencing methods, for the simultaneous detection of *M tuberculosis* DNA and multiple drug resistance to several first-line and second-line tuberculosis drugs, now provide further hope in revolutionising rapid-point-of-care tuberculosis diagnostics. Next generation benchtop sequencing systems have the potential to allow for *M tuberculosis* sequencing for resistances to all first-line and second-line tuberculosis drugs direct from sputum[76] and could overcome the problem of low bacterial loads in sputum and provide a timescale weeks quicker than culture-based resistance testing. There is also a need for comprehensive mapping of antimicrobial resistance mutations and bespoke software for easy interpretation of resistance assays. WGS approaches linked with quantitative bacteriology will generate comprehensive genotype–phenotype correlations across all the multidrug resistant *M tuberculosis* isolates and provide the opportunity to extract genome data exploitable for both development of point-of-care diagnostic tests coupled to drug resistance screening, and for epidemiological and public health control purposes.

Needs and challenges for the future

Several manufacturers are developing potentially relevant diagnostic technologies that are beginning to enter the market.[130–139] There is a need to improve our understanding of the role of individual microorganisms in respiratory disease and the true relationship between pathogen quantity and disease. A major challenge of implementation of molecular testing technology will be the ability of the test to distinguish between microbial colonisation,

	Status
Volatile organic compounds	
Breathlink, Menssana Research USA[128]	In development, CE marked
Breath analyser, Next Dimension Technologies, USA[111]	In development
Molecular technologies	
Alere Q, Alere, USA[112]	In development
B-SMART, LabCorp, USA[113]	In development
Genedrive MTB/RIF ID, Epistem, UK[114]	CE marked, clinical sample testing in progress
LATE-PCR, Brandeis University, USA[129]	Clinical sample testing in progress
GeneXpert MTB/RIF Cartridge, Cepheid, USA[115,116]	On market, CE marked and FDA cleared, evaluated and endorsed by WHO
GeneXpert XDR Cartridge, Cepheid, USA[117]	In development
TruArray MDR TB, Akkoni, USA[109]	In development
INFINITI MTB-TB Assay, Autogenomics, USA[116]	Available for research use only
Tuberculosis LAMP, Eiken, Japan[118]	On market, CE marked, evaluation by WHO in progress
Genotype MTBDRsl, Hain Lifescience, Germany[119]	On market, CE marked, evaluation by WHO in progress
iCubate Myco Cassette, iCubate, USA[120]	Available for research use only
Mycobacterium Identification Array, Capital Bio, China[121]	On market, not yet assessed by WHO
Truelab/TruenatMTB, Molbio Diagnostics, India[122]	On market, not yet assessed by WHO
Non-molecular methods	
Alere Determine TB-LAM, Alere, USA[123]	On market, not yet assessed by WHO
TB Rapid Screen, Global BioDiagnostics, USA[124]	In development
TBDx, Signature Mapping Medical Sciences, USA[129]	Clinical sample testing in progress
Culture-based rapid tests	
BNP Middlebrook, NanoLogix, USA[125]	In development
MDR-XDR TB Colour Test, FIND, Switzerland/Imperial College, United Kingdom[126]	In development
TREK Sensititre MYCOTB MIC plate, Trek Diagnostic Systems/Thermo Fisher Scientific (USA)[127]	In development, clinical sample testing in progress

TB=tuberculosis, MTB/RIF=*M tuberculosis*-rifampicin, LAMP=loop-mediated amplification, MDR-XDR= multi-drug resistant and extensively-drug resistant. *Adapted from WHO Global Tuberculosis Report 2013.[108]

Table 5*: Examples of tuberculosis diagnostics in development and assessment

For **Next Dimension Technologies** see http://www.nextdimensiontech.com

For **Cepheid** see http://www.cepheid.com

For **Akkoni** see http://www.akonni.com/

For **Autogenomics** see http://www.autogenomics.com/infiniti_main.php

For **iCubate** see http://icubate.com/index.php/product/myco/

For **Global BioDiagnostics** see http://www.globalbiodiagnostics.com

For **NanoLogix** see http://nanologix.com/

infection, and disease causation. Standard laboratory culture generally incudes a quantitative element, with a usual cut-off being 10^5 CFU/mL. Respiratory tract specimens are invariably contaminated with colonising organisms from the nasopharynx and the increased sensitivity of molecular techniques will detect such colonisers. Additionally, multiple pathogenic species can be present in one specimen. The extent to which these represent genuine co-infections as opposed to a mixture of infection and colonisation needs to be determined. The difficulty in distinguishing between infection and colonisation creates a dilemma as to whether such results should be used to guide treatment. Incorporation of a quantitative element to diagnostics, such as use of quantitative PCR will go some way towards improved interpretation.

At present, the biggest technology gap exists within the diagnosis of lower respiratory tract infections and these are now the focus of consortia partnerships funded by the EU and the Innovative Medicines Initiative such as PATHSEEK,[76] rapid identification of respiratory tract infections (RiD-RTI),[140] and development of rapid point-of-care test platforms for infectious diseases (RAPP-ID).[141]

Search strategy and selection criteria

We searched for articles published in English in PubMed, Embase, Cochrane database, Google scholar, and WHO publications website with the terms "respiratory tract", "lung"', "infections" and combined these with the terms "diagnostics", "diagnostic tests", "diagnostic platforms", "PCR", "serology", "rapid", "molecular", "antibiotic resistance", "sequencing" "point of care", and "development" for the period between March 21, 2000, and June 4, 2014. Substantive reviews identified on the subject have been referenced.

The aim of the RiD-RTI consortium is to develop a rapid sample-in, answer-out nucleic-acid-based platform for the diagnosis of all types of pneumonia (community acquired pneumonia, hospital-acquired pneumonia, and ventilator associated pneumonia) caused by viral and bacterial pathogens[140] while RAPP-ID proposes to use various technologies to develop point-of-care tests for influenza, ventilator associated pneumonia, and community acquired pneumonia.[141] An increased array

of good quality point-of-care products for diagnosis of respiratory tract infections is hoped and expected to be available on the market in the next 5 years.

As further developments in NAAT tests progress, further coanalyses of several viral and bacterial targets will be possible. Fully automated multiplex NAAT tests such as GeneXpert,[67] Nanosphere, and FilmArray[64,69] are only suitable for low throughput scenarios; although for Nanosphere technology, some modest scale-up is possible by the addition of up to 16 processing modules. Batched processing of validated NAAT tests to diagnose some pathogens is usually done in combination with more traditional microbiological methods. In the future, a three-point arrangement for cost-effective rapid diagnosis of respiratory tract infection might be possible. The first point is a low throughput, fully automated NAAT platform, situated as a point-of-care test in primary-care or secondary-care emergency areas, where tests are done by clinical staff, and provide out-of-hours diagnostic information to manage admission of patients and infection control practice. The second stage is a robust in-house or commercial NAAT test of large batch size to provide the main diagnostic laboratory capacity for managing the significant and varied range of targeted respiratory tract infection requests that are generated within a secondary care setting. The third point, which is not yet well established as a diagnostic pathway, is a pathogen-discovery process formed by an initial non-targeted amplification of polyadenylated RNA in a clinical sample, then an array-based selection of potential pathogens. The sequencing of any captured structures might have a much longer turnaround time, but would provide a final opportunity to obtain a diagnosis where no result could be obtained via the first or second point. This three-point diagnostic process would provide the maximum opportunity for obtaining relevant diagnostic information.

The first of the fully automated NAAT platforms are in the early stages of commercialisation, and should these platforms prove successful they will likely be rapidly adopted by health-care systems worldwide. However, the third, so-called pathogen-discovery approach has not yet been developed for clinical use, and it might be some time before the proposed scenario becomes a reality. The use of automation and reliability improvements will facilitate testing out of the laboratory and toward the interface between patients and clinicians at points of care and ideally, in rural areas in developing countries, run on solar power.

Conclusions

Several technological advances are showing great promise, and although substantial progress is being made in the development of new pathogen-specific rapid diagnostic tests, there are issues of interpretation, sensitivity, and specificity that need to be resolved. The clinical dilemma surrounding the use of high sensitivity and specificity NAATs is that identification of pathogen nucleic acid from a respiratory tract sample might not necessarily attribute causation.

In practical management terms, for patients with respiratory tract infection at any point of care, a rapid diagnostic test is needed which, from a single respiratory tract sample, can distinguish bacterial from viral infection, identify any bacteria to the species level, and delineate antibiotic sensitivities. Such a test would enable prompt initiation of pathogen-specific treatment, or enable the prompt modification of empiric antibiotic therapy, and thus improve management and outcomes of patients presenting with respiratory tract infection. For any new test to be widely adopted it should be possible to power with solar energy and use reagents should not require cold chain storage.

Contributors

AZ wrote and coordinated this Series paper, developed the draft outline, contributed his sections and finalised the manuscript. All authors contributed relevant text and tables on their expert sections and contributed to finalising the manuscript.

Declaration of interests

AZ is the principal investigator, and VE and VG are co-principal investigators of the EU FP7 grant, RiD-RTI. JB is the principal investigator of the EU FP7 grant, PATHSEEK. All other authors declare no competing interests.

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