*Epidemiol. Infect.* (2014), **142**, 1–11. © Cambridge University Press 2013
doi:10.1017/S0950268813002367

# REVIEW ARTICLE
# The increasing application of multiplex nucleic acid detection tests to the diagnosis of syndromic infections

J. GRAY[1,2] AND L. J. COUPLAND[2]*

[1] *Norwich Medical School, University of East Anglia, NRP Innovation Centre, Norwich Research Park, Norwich, Norfolk, UK*
[2] *Specialist Virology Centre, Department of Microbiology, Norfolk and Norwich University Hospitals NHS Foundation Trust, NRP Innovation Centre, Norwich Research Park, Norwich, Norfolk, UK*

*Received 28 March 2013; Final revision 30 August 2013; Accepted 30 August 2013; first published online 7 October 2013*

## SUMMARY

On 14 January 2013, the US Food and Drug Administration (FDA) announced permission for a multiplex nucleic acid test, the xTAG® Gastrointestinal Pathogen Panel (GPP) (Luminex Corporation, USA), which simultaneously detects 11 common viral, bacterial and parasitic causes of infectious gastroenteritis, to be marketed in the USA. This announcement reflects the current move towards the development and commercialization of detection technologies based on nucleic acid amplification techniques for diagnosis of syndromic infections. We discuss the limitations and advantages of nucleic acid amplification techniques and the recent advances in Conformité Européene – *in-vitro* diagnostic (CE-IVD)-approved multiplex real-time PCR kits for the simultaneous detection of multiple targets within the clinical diagnostics market.

**Key words**: Clinical microbiology, molecular biology, virus infection, virology.

## Introduction

On 14 January 2013, the US Food and Drug Administration (FDA) announced permission for a multiplex nucleic acid test, the xTAG® Gastrointestinal Pathogen Panel (GPP) (Luminex Corporation, USA), which simultaneously detects 11 common viral, bacterial and parasitic causes of infectious gastroenteritis, to be marketed in the USA. This announcement reflects the current move towards the development and commercialization of detection technologies for the diagnosis of infectious syndromes whether, enteric [1–6], respiratory [7–13], sexually transmitted [14], affecting the central nervous system [15] or causing sepsis [16, 17]. Commercially available tests for the detection of specific pathogens associated with urinary tract infection, neonatal infection or infection in the immunocompromised host have also been developed by commercial diagnostic companies including Fast-track Diagnostics (Junglinster, Luxembourg), AusDiagnostics Pty Ltd (Sydney, Australia) and Seegene (Seoul, Korea).

The superior sensitivity, specificity, and reproducibility of nucleic acid amplification techniques combined with the ability to identify a broader range of human pathogens in a rapid format has driven the move from classical diagnostic microbiological techniques including microscopy, microbial culture, antigen detection and serology within the diagnostic setting [18–23]. Moreover, the ability to reduce the myriad of techniques utilized within the routine diagnostic setting such as culture, including selective media and enrichment, biochemical identification, microscopy including immunofluorescence, antigen detection by

* Author for correspondence: Dr L. J. Coupland, Department of Microbiology, Norfolk and Norwich University Hospitals NHS Foundation Trust, NRP Innovation Centre, Norwich Research Park, Colney Lane, Norwich, Norfolk NR4 7GJ, UK.
(Email: lindsay.coupland@nnuh.nhs.uk)

https://doi.org/10.1017/S0950268813002367 Published online by Cambridge University Press

enzyme immunoassay or particle agglutination as well as the delayed serological diagnosis of infectious disease, can streamline sample throughput. Commercial molecular diagnostic technologies encompassing nucleic acid extraction, liquid handling, molecular amplification and identification, which are amenable to partial or complete automation and high throughput, have become increasingly available [24]. However, it is important to understand the limitations of any new technology as well as the clinical relevance of the results obtained. Microbial culture and microscopy can be catch-all methods for detection of a broad spectrum of pathogens including new or unanticipated agents with culture identifying and isolating viable organisms for further study [25, 26]. Nucleic acid amplification technologies, which are limited to existing knowledge of a microorganism's genome, are highly specific and do not discriminate between viable and dead organisms with resultant detection of microbes that are present at non-pathogenic levels. Thus, care is required when designing a molecular amplification assay and interpreting its results [27]. Furthermore, it is common for microbial genomes to contain unexpected mutations, which may render a molecular assay ineffective [27], whereas microbial culture, antigen detection and serology are less likely to be influenced by mutations unless they result in phenotypic changes.

Detection technologies for the diagnosis of syndromic infection range from a number of grouped monoplex real-time polymerase chain reaction (PCR) and real-time reverse-transcription (RT)–PCR assays allowing for the detection of individual pathogens to multiplex real-time PCR and RT–PCR assays with the capacity to detect up to six analytes simultaneously using the same thermal cycling profile. However, the low multiplexing capability of real-time PCR instrumentation represents a major drawback of the technology within the clinical setting [28], which is limited to four or five fluorescent channels detecting fluorescent dyes at different wavelengths or using melting-point analysis to differentiate among PCR products. Limitations are associated with the number of detectors available or the need for discrimination in detectable wavelengths to prevent cross-talk.

## The increasing availability of commercial multiplex nucleic acid detection tests for the diagnosis of infectious syndromes

Recent advances within the clinical diagnostics market has seen an increase in the availability of Conformité Européene – *in-vitro* diagnostic (CE-IVD)-approved, multiplex real-time PCR and RT–PCR kits for the simultaneous detection of multiple targets in the same reaction vessel using commonly available real-time PCR platforms especially for respiratory viruses (Table 1). However, a shift towards the diagnosis of infectious syndromes has driven commercial diagnostic companies to develop multiparametric molecular diagnostic tests within other disease categories. Indeed, Fast-track Diagnostics (Luxembourg) offer optimized multiplexed real-time PCR and RT–PCR primer and probe mixes based on wide-ranging infection syndromes using the same thermal cycling profile. AusDiagnostics Pty Ltd (Australia) also offer highly multiplexed panels using multiplex tandem PCR, which employs two sequential steps. Step 1 is a short (15 cycles) multiplexed pre-amplification reaction using primers homologous to all targets in the panel so competition between primers is avoided allowing low concentrations of targets to be detected, even when multiple pathogens are present. Reverse transcriptase is included in the step 1 reaction for panels including RNA targets. A prior cDNA synthesis reaction is not necessary. In step 2, the product from step 1 is diluted into individual wells for real-time PCR reactions using primers 'nested inside' the primers used for step 1. The Easy-Plex™ liquid handling robot (AusDiagnostics, Pty Ltd) automates this process. The step 2 PCR reaction is performed in the Rotor-Gene 6000 real-time analyser. DNA amplification is measured by the increase in fluorescence when Eva-Green™ dye (Biotium Inc., USA) is incorporated into the DNA being formed in the specific amplification reaction.

Seegene (Korea) utilize Dual-Priming Oligonucleotides (DPO™) in the Seeplex® multiplex PCR and Anyplex™ multiplex real-time PCR product ranges in combination with detection by automatic capilliary electrophoresis or melting-curve analysis utilizing Tagging Oligonucleotide Cleavage and Extension (TOCE™) technology, respectively, which allow high multiplexing by enabling 'one channel, many targets'. These methods are highly appropriate for the identification and differentiation of viral and bacterial pathogens with very variable genetic characteristics and low availability of primer sites [15].

The benefits that multiplexed nucleic acid detection tests provide to the diagnosis of infection syndromes over conventional techniques within the clinical microbiology setting are increasingly highlighted,

https://doi.org/10.1017/S0950268813002367 Published online by Cambridge University Press

https://doi.org/10.1017/S0950268813002367 Published online by Cambridge University Press



Table 1. *Examples of commercially available multiparametric detection technologies for diagnosis of respiratory infection*

| Test system | FilmArray® Respiratory Panel | Anyplex™ II RV16 | Seeplex® RV15 ACE Detection | eSensor® RVP | xTAG® RVP | xTAG® RVP *FAST* | Respiratory MWS r-gene real-time PCR | RespiFinder® SMART 22 | FTD Respiratory Pathogens 21 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Manufacturer | Biofire Diagnostics, Inc. | Seegene | Seegene | Gen Mark Dx | Luminex Corp. | Luminex Corp. | Argene | PathoFinder B.V. | Fast-track Diagnostics | AusDiagnostics |
| Pathogens detected | <u>Viruses</u>: FA (subtypes H1, H1-2009, H3), FB, RSV, PIV (subtypes 1–4), HMPV, rhinovirus/ enterovirus, ADV, HCOV (NL63/ 229E/ OC43/HKU1). <u>Bacteria</u>: *Bordetella pertussis, Chlamydophila pneumoniae Mycoplasma pneumoniae* | FA, FB, RSV (A/B), PIV (subtypes 1–4), HMPV, rhinovirus (A, B, C) enterovirus, ADV, HCOV (NL63/ 229E/ OC43/ HKU1), HBOV (1–4) | <u>Virus set A</u>: PIV subtypes 1–3, ADV, HCOV NL63, HCOV 229E <u>Virus set B</u>: HCOV 0C43, rhinovirus (A, B, C), FA, RSV (A/B) <u>Virus set B</u>: HCOV 0C43, rhinovirus (A, B, C), FA, RSV (A/B) <u>Virus set C</u>: HBOV (1–4), FB, HMPV, HPIV subtype 4, enterovirus | FA (subtypes H1, H1-2009, H3), FB, RSV (A/B), PIV (subtypes 1–3), HMPV, rhinovirus, ADV (B, C, E) | FA (subtypes H1, H3, H5), FB, RSV (A/B), PIV (subtypes 1–4), HMPV, rhinovirus/ enterovirus, ADV, HCOV (NL63/ 229E/ OC43/ HKU1/SARS) | FA (subtypes H1, H3), FB, RSV, PIV (subtypes 1–4), HMPV, ADV, rhinovirus/ enterovirus, HCOV (NL63/229E/ OC43/ HKU1), HBOV. | <u>Viruses</u>: FA, FB, RSV, PIV (subtypes 1–4), HMPV, rhinovirus/ enterovirus, ADV, and HCOV (NL63/ 229E/OC43/ HKU1), HBOV. <u>Bacteria</u>: *Chlamydophila pneumoniae Mycoplasma pneumoniae* | <u>Panel 1</u>: FA (subtypes H1), FB, RSV (A/ B), HMPV, rhinovirus/ enterovirus, ADV, *Chlamydophila pneumoniae, Mycoplasma pneumoniae*, *Bordetella pertussis* <u>Panel 2</u>: PIV (subtypes 1–4), HBOV, HCOV (NL63/ 229E/OC43/ HKU1) | FA (subtype H1), FB, RSV (A/B), HMPV, PIV (subtypes 1, 2, 3, 4, HMPV (A/B), rhinovirus, enterovirus, ADV, HCOV (NL63/229E/ OC43/ HKU1), HBOV, parechovirus *Mycoplasma pneumoniae*. | <u>Viruses</u>: FA (subtypes H1, H3), FB, RSV, PIV (subtypes 1–3), HMPV, ADV, rhinovirus/ enterovirus, <u>Bacteria</u>: *Bordetella pertussis* |
| Internal control | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Positive control | No | Yes | Yes | No | No | No | Yes | No | Yes | No |
| Time required to result | 65 min*† | 6–7 h | 6–7 h | Within 7 h | Within 8 h | Within 4 h | Within 1·5 h after extraction* | Within 6 h* | Within 6 h | |
| Complexity | Low | Low | High | High | High | High | Low | High | Low | Low |
| CE-IVD labelled | Yes | Yes | Yes | No | Yes | Yes | Yes | Yes | Yes | No |
| Technology | Nested-multiplex PCR | Multiplex real-time PCR | Multiplex PCR system based on DPO technology | RT–PCR and hybridization | Multiplex RT–PCR and hybridization | Multiplex RT–PCR and hybridization | Duplex real-time PCR/real-time RT–PCR | MLPA | Multiplex real-time PCR and real-time RT–PCR | Multiplex tandem PCR |
| Automation | Automated | Semi-automated | Semi-automated | Semi-automated | Semi-automated | Semi-automated | Semi-automated | Semi-automated | Semi-automated | Semi-automated |
| Detection | Endpoint melting curve analysis | Endpoint melting curve analysis | Auto-capillary electrophoresis device | Electrochemical detection | Fluorescent-labelled bead array | Fluorescent-labelled bead array | Multiple fluorophore detection | Endpoint melting curve analysis | Multiple fluorophore detection | Intercalating dye detection |

Table 1 (*cont.*)

| Test system | FilmArray® Respiratory Panel | Anyplex™ II RV16 | Seeplex® RV15 ACE Detection | eSensor® RVP | xTAG® RVP | xTAG® RVP *FAST* | Respiratory MWS r-gene real-time PCR | RespiFinder® SMART 22 | FTD Respiratory Pathogens 21 | |
|---|---|---|---|---|---|---|---|---|---|---|
| Manufacturer | Biofire Diagnostics, Inc. | Seegene | Seegene | Gen Mark Dx | Luminex Corp. | Luminex Corp. | Argene | PathoFinder B.V. | Fast-track Diagnostics | AusDiagnostics |
| Reference | Poritz *et al.* 2011 [12] | Kim *et al.* 2013 [9] | Kim *et al.* 2013 [9] | Pierce & Hodinka, 2012 [11] | Pabbaraju *et al.* 2008 [10] | Dabisch-Ruthe *et al.* 2012 [8] | No reference provided | Dabisch-Ruthe *et al.* 2012 [8] | Sakthivel *et al.* 2012 [13] | Anderson *et al.* 2013 [7] |

ADV, Adenovirus; CE-IVD, Conformité Européene – *in-vitro* diagnostic; DPO, dual-priming oligonucleotides; FA, influenza virus type A; FB, influenza virus type B; HBOV, human bocavirus; HCOV, human coronavirus; HMPV, human metapneumovirus; MLPA, multiplex ligation-dependent probe amplification; MWS, multi-well system; PCR, polymerase chain reaction; PIV, parainfluenza virus; RSV, human respiratory syncytial virus; RT–PCR, reverse transcription–polymerase chain reaction; RVP, respiratory virus panel.

* Time required to obtain result was taken from commercial diagnostic company product literature.

† The FilmArray integrates sample preparation, amplification, detection, and analysis into one simple system that requires 2 min of hands-on time and has a total run time of about 1 h.

especially in relation to gastroenteritis [1–6, 29, 30]. The enteric targets included in multiplex molecular diagnostic tests available on the clinical diagnostics market vary considerably (Table 2). The CE-IVD marked EntericBio® Real-Time Gastro Panel I (Serosep Ltd, Ireland) currently only targets *Salmonella enterica* spp., *Shigella* spp., *Campylobacter* spp., and Shiga toxin-producing *Escherichia coli* (STEC) (Table 2). However, unique to EntericBio Real-Time Gastro Panel I is the ability to perform the test directly from the stool sample thereby removing the usual requirement for the nucleic acid purification, which often presents a bottleneck to the successful implementation of nucleic acid detection within a routine virological setting [31]. The entire master mix required to perform each test is lyophilized into individual reaction wells, which offers an additional improvement to the workflow within the routine diagnostic setting. In combination, the exceptional features of the EntericBio Real-Time Gastro Panel I enable results to be generated within 3 h [4] (Table 2). In contrast, the CE-IVD marked Seeplex® Diarrhea ACE Detection multiplex PCR system based on DPO technology (Seegene, Korea) can detect four viruses and/or 10 bacteria using a virus panel (panel V) and two bacterial panels, bacterial panel 1 (panel B1) and bacterial panel 2 (panel B2) [3] (Table 2). There are several limitations associated with the Seeplex Diarrhea ACE Detection system. First, no option is available within the current system for the detection of human diarrhoeal parasites and although the Seeplex system incorporates quality controls, the internal control is only available for inclusion in each PCR master mix, which does not allow validation of the nucleic acid extraction or reverse transcription processes. Moreover, reverse transcription is performed as a separate step, which in turn increases the duration of the assay [3]. The average turnaround time to process 96 samples using the Seeplex system was 9–10 h or 0·6 h per target in a run of 96 samples compared to 24–48 h [3].

The CE-IVD marked xTAG GPP (Luminex Corporation, USA), and FilmArray® GI Panel (BioFire Diagnostics Inc., USA), which is currently available for research use only, provide the most comprehensive commercial multiplex molecular diagnostic tests available for gastroenteritis diagnosis. The xTAG GPP can simultaneously detect and identify three viruses, nine bacteria and three parasites while the FilmArray GI Panel tests for a panel of five viruses, 14 bacteria and four parasites (Table 2). However,

https://doi.org/10.1017/S0950268813002367 Published online by Cambridge University Press

Table 2. *Examples of commercially available multiparametric detection technologies for diagnosis of infectious gastroenteritis*

| Test system | FilmArray® GI Panel | xTAG® Gastrointestinal Pathogen Panel | Seeplex® Diarrhea ACE Detection | FTD Gastroenteritis | EntericBio Panel I® system | RIDA® GENE Gastrointestinal Infections | Gastroenteritis Multiplex | Faecal pathogens |
|---|---|---|---|---|---|---|---|---|
| Manufacturer | Biofire Diagnostics Inc. | Luminex Corporation | Seegene | Fast-track Diagnostics | Serosep Ltd | R-Biopharm | Diagenode | AusDiagnostics |
| Pathogens detected | Viruses: norovirus GI/GII, rotavirus A, adenovirus 40/41, astrovirus and sapovirus. Bacteria: *Salmonella, Vibrio cholerae, Campylobacter , Clostridium difficile* toxin A/B, ETEC LT/ST, *E. coli* O157, STEC *stx1*/*stx2*, EAEC, EPEC, *Shigella*/EIEC, *Yersinia enterocolitica, Aeromonas, Plesiomonas shigelloides.* Parasites: *Giardia lamblia, Cryptosporidium*, and *Entamoeba histolytica Cyclospora cayetanensis* | Viruses: norovirus GI/GII, rotavirus A, adenovirus 40/41, and astrovirus Bacteria: *Salmonella* spp., *Shigella* spp., *Vibrio cholerae, Campylobacter* spp., *Clostridium difficile* toxin A/B, ETEC LT/ST, *E. coli* O157, STEC *stx1*/*stx 2*, and *Yersinia enterocolitica* Parasites: *Giardia lamblia, Cryptosporidium*, and *Entamoeba histolytica* | Panel V: norovirus GI/GII, rotavirus, adenovirus, and astrovirus Panel B1: *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Campylobacter* spp., and *Clostridium difficile* toxin B Bacteria: *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica, Clostridium difficile, Campylobacter coli*/*jejuni, E. coli* O157, EIEC Panel B2: *Clostridium perfringens, Yersinia enterocolitica, Aeromonas* spp., *E. coli* O157:H7, and Verocytotoxin-producing *E. coli* | Viruses: norovirus GI/GII, rotavirus, adenovirus, astrovirus and sapovirus Parasites: *Giardia lamblia, Cryptosporidium*, and *Entamoeba histolytica* | *Salmonella enterica* spp., *Shigella* spp., *Campylobacter jejuni*/*coli*/*lari, and* STEC *stx1*/*stx2* | Viruses: norovirus GI/GII, rotavirus, adenovirus. Bacteria: *Salmonella* spp., *Campylobacter* spp., *Yersinia enterocolitica, Clostridium difficile* toxin A/B, EHEC, STEC, EPEC, EIEC/*Shigella* spp., ETEC LT/ST, EAEC. | Viruses: norovirus GI/GII, rotavirus A, adenovirus 40/41, and astrovirus Bacteria: *Salmonella* enterica, *Campylobacter jejuni* Parasites: *Giardia lamblia, Cryptosporidium parvum, Entamoeba histolytica* and *Dientamoeba fragilis* | Viruses: norovirus GII, rotavirus, adenovirus 40/41 Bacteria: *Salmonella* spp., *Shigella* spp., *Clostridium difficile* toxin B, *Campylobacter coli*/*jejuni*/*doylei* Parasites: *Giardia lamblia, Cryptosporidium parvum, Entamoeba histolytica* Parasites: *Giardia* spp., *Cryptosporidium* spp. |
| Internal control | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| Positive control | No | No | Yes | Yes | Yes | Yes | Yes | No |
| Time required to result | 65 min*† | Within 5 h*‡ | 9–10 h | Within 6 h | Within 3 h | Information not supplied | Information not supplied | Within 3 h |
| Complexity | Low | High | High | Low | Low | Low | Low | Low |
| CE-IVD labelled | No | Yes | Yes | Yes | Yes | Yes | Yes | No |
| Technology | Nested-multiplex PCR | Multiplex RT–PCR and hybridization | Multiplex PCR system based on DPO technology | Multiplex real-time PCR and real-time RT–PCR | Multiplex real-time PCR | Multiplex real-time PCR and real-time RT–PCR | Multiplex real-time PCR and real-time RT–PCR | Multiplex tandem PCR |
| Automation | Automated | Semi-automated | Semi-automated | Semi-automated | Semi-automated | Semi-automated | Semi-automated | Semi-automated |
| Detection | Endpoint melting curve analysis | Fluorescent-labelled bead array | Auto-capillary electrophoresis device | Multiple fluorophore detection | Multiple fluorophore detection | Multiple fluorophore detection | Multiple fluorophore detection | Intercalating dye detection |

https://doi.org/10.1017/S0950268813002367 Published online by Cambridge University Press

Table 2 (*cont.*)

| Test system | FilmArray® GI Panel | xTAG® Gastrointestinal Pathogen Panel | Seeplex® Diarrhea ACE Detection | FTD Gastroenteritis | EntericBio Panel I® system | RIDA® GENE Gastrointestinal Infections | Gastroenteritis Multiplex | Faecal pathogens |
|---|---|---|---|---|---|---|---|---|
| Manufacturer | Biofire Diagnostics Inc. | Luminex Corporation | Seegene | Fast-track Diagnostics | Serosep Ltd | R-Biopharm | Diagenode | AusDiagnostics |
| Reference | No reference provided | Claas *et al.* 2013 [1]; Mengelle *et al.* 2013 [29]; Navidad *et al.* 2013 [30] | Coupland *et al.* 2012 [3] | McAuliffe *et al.* 2013 [5] | Koziel *et al.* 2013 [4] | Coste *et al.* 2013 [2] | Coste *et al.* 2013 [2] | Stark *et al.* 2011 [6] |

DPO, Dual-priming oligonucleotides; EAEC, enteroaggregative *Escherichia coli*; EHEC, enterohaemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC LT/ST, enterotoxigenic *E. coli* LT/ST; PCR, polymerase chain reaction; RT–PCR, reverse transcription–polymerase chain reaction; STEC *stx1/stx2*, Shiga-like toxin-producing *E. coli stx1/stx2*;

* Time required to obtain result was taken from commercial diagnostic company product literature.

† The FilmArray integrates sample preparation, amplification, detection, and analysis into one simple system that requires 2 min of hands-on time and has a total run time of about 1 h.

‡ Time estimate of within 5 h is for a maximum of one extraction (24 samples), unless multiple extractors are available. Does not include pre-treatment.

there are significant differences between these tests. The xTAG GPP procedure incorporates sample pre-treatment, which consists of bead beating in lysis buffer to ensure maximum efficiency during nucleic acid purification, multiplex RT–PCR, bead hybridization and detection using the proprietary universal tag sorting system and data acquisition and analysis. The workflow can be completed within 5 h, which is based on one extraction of 24 samples but does not include the pre-treatment stage. In contrast, the FilmArray system brings sample to results in about 1 h, with minimal hand-on time to process. However, a significant drawback of the FilmArray system is its low throughput, as only a single sample can be processed on the instrument at one time, which limits the overall utility of the test in laboratories with moderate to high numbers of specimens to be tested [32].

There are also an increasing number of multiparametric molecular diagnostic tests available for the diagnosis of meningitis and sexually transmitted disease (STD). The Seeplex® Meningitis ACE Detection (Seegene, Korea) multiplex PCR system based on DPO technology detects 12 common bacterial and viral causes of acute meningitis, i.e. *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *group B streptococcus*, *Listeria monocytogenes*, herpes simplex virus types 1 and 2, cytomegalovirus, Epstein–Barr virus, varicella zoster virus, human herpes virus 6 and human enterovirus [15]. DPO technology is also utilized in the Seeplex® STD6 ACE Detection system, which is designed to simultaneously detect six STD pathogens, i.e. *Trichomonas vaginalis*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma urealyticum*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae* [14]. Similarly, the Anyplex™ II STI-7 Detection system detects the aforementioned STD pathogens plus *Ureaplasma parvum* using DPO and TOCE technology in a single real-time PCR.

## The advantages and challenges of multiplex nucleic acid detection tests

Despite the differences between these multiparametric molecular diagnostic tests all have in common the ability to provide a more comprehensive assessment of the aetiology of disease [1] due to increased diagnostic yield compared to conventional diagnostic tests [33, 34]. These tests can also accelerate the microbial detection/identification phase of the laboratory diagnostic cycle to meet the critical 6- to 24-h

https://doi.org/10.1017/S0950268813002367 Published online by Cambridge University Press

window [35]. The ability to rapidly detect and distinguish multiple potentially infectious pathogens is critical for the accurate diagnosis of seasonal and sporadic outbreaks, emerging pathogens and agents of bioterrorism [12]. Clinical syndromes are seldom specific to a single pathogen, so detection strategies that allow multiple agents to be simultaneously considered [36] can have a significant impact on infectious disease management since multiparametric molecular diagnostic tests can provide a more accurate representation of the true pathogen spectrum in clinical samples [37].

In the absence of rapid tests, infections are managed using empirical antibiotic regimens, which are associated with overuse of broad-spectrum antibiotics, which has major implications for the development of bacterial resistance and emergence of hospital-acquired infections [35]. Inadequate or inappropriate antimicrobial treatment and the delayed administration of appropriate antimicrobial therapy correlate with negative clinical outcomes in patients with bacteraemia and sepsis compared to patients who receive appropriate therapy from the onset [38]. This was reflected in a longer hospital stay, a higher risk of *Clostridium difficile*-associated infection, excess mortality and higher cost of therapy per bacteraemic episode [38–45].

Rapid diagnosis can have a major impact on patient care and outcome, most importantly significant reductions in hospital stay, inappropriate or unnecessary antibiotic use, and associated common adverse reactions including rash, abdominal pain, diarrhoea and vomiting as well as informing decisions regarding infection control measures [46–52]. However, most studies have concentrated on the diagnostic capabilities of multiparametric molecular diagnostic tests [5, 53, 54] while the clinical and economic impact of these tools has received limited attention [33]. Oosterheert *et al*. [33] conducted a randomized controlled trial involving 107 adults with lower respiratory tract infections at two hospitals in The Netherlands. All patients had upper respiratory tract specimens tested for viral and atypical bacterial pathogens by real-time PCR as well as by conventional diagnostic procedures, but only results for patients in the intervention group were reported to the treating physician; results for patients in the control group were unavailable. The implementation of multiparametric detection technologies for diagnosing respiratory infections increased the diagnostic yield compared to conventional diagnostic tests but did not reduce antibiotic use, antibiotic costs, or the duration of hospital stay [33]. Wishaupt *et al*. [55] also found that RT–PCR testing had a high yield of viral diagnoses, but rapid communication did not lead to decreases in hospital admissions, shorter hospital stays, or less antibiotic use for children with acute respiratory infections. In contrast, Brittain-Long *et al*. [56] demonstrated that access to a rapid molecular diagnostic tool for aetiological diagnosis of viral respiratory infection significantly reduced antibiotic prescriptions at the initial visit in a primary-care setting but this effect was no longer evident at follow-up. These studies highlight the difficulties in evaluating the impact of molecular diagnostic tests on patient management, especially in relation to respiratory tract infection since bacterial co-infection is associated with about 40% of viral respiratory tract infections requiring hospitalization [57]. Hence, clinicians are unwilling to alter therapy based on discovery of a viral pathogen [58]. Fortunately, new rapid multiplex molecular diagnostic tools are becoming available, which are designed to detect bacterial respiratory pathogens. These include the Anyplex™ II RB5 Detection system (Seegene, Korea), which detects and differentiates the most common causes of atypical pathogens, *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella pneumophila* and two causative agents of whooping cough, the commonly detected *Bordetella pertussis* as well as the less common but vaccination-ineffective *Bordetella parapertussis.* Fast-track Diagnostics (Luxembourg), Pathofinder B.V. (The Netherlands) and AusDiagnostics Pty Ltd (Australia) also offer multiplex PCR assays for the simultaneous detection and differentiation of relevant respiratory viruses and bacteria associated with respiratory tract infection.

The LightCycler® Septi*Fast* Test MGRADE (Roche Diagnostics, Switzerland), a commercial real-time PCR designed to detect and identify 25 bacterial and fungal species that comprise >90% of the pathogens causing bloodstream infections in critical care [59] epitomizes the difficulties associated with changing the clinical management of infectious disease. The LightCycler Septi*Fast* MGRADE test was the first PCR-based system to be awarded a CE mark for pathogen detection and identification in blood samples and, to date, is the most intensively investigated multiplex real-time PCR assay in the clinical setting of sepsis [59]. It offers demonstrable diagnostic value in terms of enhanced detection of the most common pathogen species in patients with suspected sepsis

https://doi.org/10.1017/S0950268813002367 Published online by Cambridge University Press

and for the timely diagnosis of bloodstream infections, particularly in antibiotic pre-treated patients [60, 61]. The LightCycler Septi*Fast* Test MGRADE system is now part of a clinical diagnostic validation study to determine whether this multiplex molecular diagnostic technology has sufficient clinical diagnostic accuracy, which represents a crucial phase of detailed independent health technology assessment of the first multiplex real-time PCR technique aimed at helping deliver more effective care to critically ill patients internationally [62].

The extraordinary sensitivity associated with molecular diagnostic tests also brings a new set of challenges that include detection of dead microbes or potential pathogens that simply colonize non-sterile sites [63]. Certainly, quantitative or semi-quantitative molecular methods are utilized to establish a clinically significant result for viral infection as asymptomatic infection is associated with a significantly lower viral load [64, 65] and may help to separate bacterial colonization from disease [63]. It seems that only with this information can clinicians make well-informed decisions, which would promote judicious antibiotic use and permit pathogen-targeted antibacterial therapy [63]. Multiparametric diagnostic tests may also be augmented by the inflammatory biomarker procalcitonin (PCT) since a growing body of evidence supports PCT use to differentiate bacterial from respiratory viral diagnoses, which may improve individualized decision-making regarding antibiotic treatment when multiparametric molecular diagnostic tests cannot exclude the possibility of bacterial superinfection [66].

Multiparametric detection technologies can come with a price in human resources and qualified technical staff [35]. Nevertheless, multiplex nucleic acid detection tests also offer the opportunity to adapt clinical microbiology services within the current austere environment through rationalizing or redistributing labour and costs while maintaining and improving the provision of routine diagnostic services [67]. Common to all multiplex nucleic acid detection tests and recently demonstrated using the Luminex xTAG respiratory virus panel is the ability to increase laboratory efficiency by reducing hands-on time and operational steps while standardizing workflow in comparison to viral direct immunofluorescence assay (DFA) and culture [67]. The cost–benefit studies constructed using multiplex PCR demonstrate savings in the absence of reduced rates of antibiotic usage. Multiplex PCR testing for respiratory viruses achieved using the xTAG respiratory virus panel test was the least costly strategy for the diagnosis of respiratory virus infections compared to standard non-molecular diagnostic methods such as viral culture and DFA [68].

### Conclusions

PCR-based technologies have become standard within the clinical laboratory setting over the last two decades. Experience of the clinical significance of results generated using these powerful molecular diagnostic tools has accumulated during this time and there are many benefits to be gained by utilizing sensitive, specific, and rapid molecular diagnostic techniques. However, the clinical utility of these techniques urgently needs to be determined through well-structured clinical trials comparing new with traditional methods. Diagnosis of syndromic infections represents a new pathway for the diagnosis of infection and newer molecular diagnostic tools, which will streamline workflows in the routine diagnostic setting, must incorporate all relevant pathogens in order to improve patient management.

## DECLARATION OF INTEREST

None.

## REFERENCES

1. **Claas E, *et al.*** Performance of the xTAG® Gastrointestinal Pathogen Panel (GPP), a multiplex molecular assay for simultaneous detection of bacterial, viral and parasitic causes of infectious gastroenteritis. *Journal of Microbiology and Biotechnology* 2013; **23**: 1041–1045.
2. **Coste JF, *et al.*** Microbiological diagnosis of severe diarrhea in kidney transplant recipients by use of multiplex PCR assays. *Journal of Clinical Microbiology* 2013; **51**: 1841–1849.
3. **Coupland LJ, *et al.*** Simultaneous detection of viral and bacterial enteric pathogens using the Seeplex® Diarrhea ACE detection system. *Epidemiology and Infection*. Published online: 5 December 2012. doi:10.1017/S0950268812002622.
4. **Koziel M, *et al.*** Improved detection of bacterial pathogens in patients presenting with gastroenteritis using the EntericBio Real-Time Gastro Panel I® assay. *Journal of Clinical Microbiology* 2013; **51**: 2679–2685.
5. **McAuliffe GN, *et al.*** Systematic application of multiplex PCR enhances the detection of bacteria, parasites, and viruses in stool samples. *Journal of Infection* 2013; **67**: 122–129.

https://doi.org/10.1017/S0950268813002367 Published online by Cambridge University Press

6. **Stark D, *et al.*** Evaluation of multiplex tandem real-time PCR for detection of *Cryptosporidium* spp., *Dientamoeba fragilis*, *Entamoeba histolytica*, and *Giardia intestinalis* in clinical stool samples. *Journal of Clinical Microbiology* 2011; **49**: 257–262.
7. **Anderson TP, *et al.*** Comparison of four multiplex PCR assays for the detection of viral pathogens in respiratory specimens. *Journal of Virological Methods* 2013; **191**: 118–121.
8. **Dabisch-Ruthe M, *et al.*** Comparison of three multiplex PCR assays for the detection of respiratory viral infections: evaluation of xTAG respiratory virus panel fast assay, RespiFinder 19 assay and RespiFinder SMART 22 assay. *BMC Infectious Diseases* 2012; **12**: 163.
9. **Kim HK, *et al.*** Comparison of Anyplex II RV16 with the xTAG respiratory viral panel and Seeplex RV15 for detection of respiratory viruses. *Journal of Clinical Microbiology* 2013; **51**: 1137–1141.
10. **Pabbaraju K, *et al.*** Comparison of the Luminex xTAG respiratory viral panel with in-house nucleic acid amplification tests for diagnosis of respiratory virus infections. *Journal of Clinical Microbiology* 2008; **46**: 3056–3062.
11. **Pierce VM, Hodinka RL.** Comparison of the GenMark Diagnostics eSensor respiratory viral panel to real-time PCR for detection of respiratory viruses in children. *Journal of Clinical Microbiology* 2012; **50**: 3458–3465.
12. **Poritz MA, *et al.*** FilmArray, an automated nested multiplex PCR system for multi-pathogen detection: development and application to respiratory tract infection. *PLoS One* 2011; **6**: e26047.
13. **Sakthivel SK, *et al.*** Comparison of fast-track diagnostics respiratory pathogens multiplex real-time RT-PCR assay with in-house singleplex assays for comprehensive detection of human respiratory viruses. *Journal of Virological Methods* 2012; **185**: 259–266.
14. **Lee SJ, *et al.*** Evaluation of Seeplex® STD6 ACE Detection kit for the diagnosis of six bacterial sexually transmitted infections. *Journal of Infection and Chemotherapy* 2012; **18**: 494–500.
15. **Shin SY, *et al.*** Evaluation of the Seeplex® Meningitis ACE Detection kit for the detection of 12 common bacterial and viral pathogens of acute meningitis. *Annals of Laboratory Medicine* 2012; **32**: 44–49.
16. **Blaschke AJ, *et al.*** Rapid identification of pathogens from positive blood cultures by multiplex polymerase chain reaction using the FilmArray system. *Diagnostic Microbiology and Infectious Disease* 2012; **74**: 349–355.
17. **Josefson P, *et al.*** Evaluation of a commercial multiplex PCR test (SeptiFast) in the etiological diagnosis of community-onset bloodstream infections. *European Journal of Clinical Microbiology and Infectious Diseases* 2011; **30**: 1127–1134.
18. **Amar CF, *et al.*** Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case-control Infectious Intestinal Disease Study (1993–1996). *European Journal of Clinical Microbiology & Infectious Diseases* 2007; **26**: 311–323.
19. **Corless CE, *et al.*** Development and evaluation of a ‘real-time’ RT-PCR for the detection of enterovirus and parechovirus RNA in CSF and throat swab samples. *Journal of Medical Virology* 2002; **67**: 555–562.
20. **Stránská R, *et al.*** Routine use of a highly automated and internally controlled real-time PCR assay for the diagnosis of herpes simplex and varicella-zoster virus infections. Journal of Clinical Virolog*y* 2004; **30**: 39–44.
21. **Templeton KE, *et al.*** Improved diagnosis of the etiology of community-acquired pneumonia with real-time polymerase chain reaction. *Clinical Infectious Diseases* 2005; **41**: 345–351.
22. **Templeton KE, *et al.*** Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. *Journal of Clinical Microbiology* 2004; **42**: 1564–1569.
23. **Weinberg GA, *et al.*** Superiority of reverse-transcription polymerase chain reaction to conventional viral culture in the diagnosis of acute respiratory tract infections in children. *Journal of Infectious Diseases* 2004; **189**: 706–710.
24. **Association for Molecular Pathology (AMP).** FDA-cleared/approved molecular diagnostic tests (http://www.amp.org/FDATable/). Accessed 22 February 2013.
25. **Leland DS, Ginocchio CC.** Role of cell culture for virus detection in the age of technology. *Clinical Microbiology Reviews* 2007; **20**: 49–78.
26. **Ogilvie M.** Molecular techniques should not now replace cell culture in diagnostic virology laboratories. *Reviews in Medical Virology* 2001; **11**: 351–354.
27. **Mackay IM.** Real-time PCR in the microbiology laboratory. *Clinical Microbiology and Infection* 2004; **10**: 190–212.
28. **Reddington K, *et al.*** Advances in multiparametric molecular diagnostics technologies for respiratory tract infections. *Current Opinion in Pulmonary Medicine* 2013; **19**: 298–304.
29. **Mengelle C, *et al.*** Simultaneous detection of gastrointestinal pathogens with a multiplex Luminex-based molecular assay in stool samples from diarrhoeic patients. *Clinical Microbiology and Infection*. Published online: 29 April 2013. doi:10.1111/1469-0691.12255.
30. **Navidad JF, *et al.*** Evaluation of Luminex xTAG® gastrointestinal pathogen analyte specific reagents for high-throughput, simultaneous detection of bacteria, viruses, and parasites of clinical and public health importance. *Journal of Clinical Microbiology* 2013; **51**: 3018–3024.
31. **Niesters HG.** Molecular and diagnostic clinical virology in real time. *Clinical Microbiology and Infection* 2004; **10**: 5–11.
32. **Pierce VM, *et al.*** Comparison of the Idaho Technology FilmArray system to real-time PCR for detection of respiratory pathogens in children. *Journal of Clinical Microbiology* 2012; **50**: 364–3671.
33. **Oosterheert JJ, *et al.*** Impact of rapid detection of viral and atypical bacterial pathogens by real-time polymerase chain reaction for patients with lower respiratory

https://doi.org/10.1017/S0950268813002367 Published online by Cambridge University Press

tract infection. *Clinical Infectious Diseases* 2005; **41**: 1438–1444.

34. **van de Pol AC, *et al.*** Diagnostic value of real-time polymerase chain reaction to detect viruses in young children admitted to the paediatric intensive care unit with lower respiratory tract infection. *Critical Care* 2006; **10**: R61.
35. **Bissonnette L, Bergeron MG.** Multiparametric technologies for the diagnosis of syndromic infections. *Clinical Microbiology Newsletter* 2012; **34**: 159–168.
36. **Briese T, *et al.*** Diagnostic system for rapid and sensitive differential detection of pathogens. *Emerging Infectious Diseases* 2005; **11**: 310–313.
37. **Brunstein JD, *et al.*** Evidence from multiplex molecular assays for complex multipathogen interactions in acute respiratory infections. *Journal of Clinical Microbiology* 2008; **46**: 97–102.
38. **Lueangarun S, Leelarasamee A.** Impact of inappropriate empiric antimicrobial therapy on mortality of septic patients with bacteremia: a retrospective study. *Interdisciplinary Perspectives on Infectious Diseases* 2012; **2012**: 765205.
39. **Malhotra-Kumar S, *et al.*** Effect of azithromycin and clarithromycin therapy on pharyngeal carriage of macrolide-resistant streptococci in healthy volunteers: a randomised, double-blind, placebo-controlled study. Lancet 2007; **369**: 482–490.
40. **Bouza E, *et al.*** Bloodstream infections: a trial of the impact of different methods of reporting positive blood culture results. *Journal of Infectious Diseases* 2004; **39**: 1161–1169.
41. **Chen HC, *et al.*** Outcome of inadequate empirical antibiotic therapy in emergency department patients with community-onset bloodstream infections. *Journal of Antimicrobial Chemotherapy* 2012; **68**: 947–953.
42. **Garnacho-Montero J, *et al.*** Mortality and morbidity attributable to inadequate empirical antimicrobial therapy in patients admitted to the ICU with sepsis: a matched cohort study. *Journal of Antimicrobial Chemotherapy* 2008; **61**: 436–441.
43. **Ibrahim EH, *et al.*** The influence of inadequate antimicrobial treatment of bloodstream infections on patient outcomes in the ICU setting. *Chest* 2000; **118**: 146–155.
44. **Retamar P, *et al.*** Impact of inadequate empirical therapy on the mortality of patients with bloodstream infections: a propensity score-based analysis. *Antimicrobial Agents and Chemotherapy* 2012; **56**: 472–478.
45. **Shehab N, *et al.*** Emergency department visits for antibiotic-associated adverse events. *Clinical Infectious Diseases* 2008; **47**: 735–743.
46. **Barenfanger J, Drake C, Kacich G.** Clinical and financial benefits of rapid bacterial identification and antimicrobial susceptibility testing. *Journal of Clinical Microbiology* 1999; **37**: 1415–1418.
47. **Barenfanger J, *et al.*** Clinical and financial benefits of rapid detection of respiratory viruses: an outcomes study. *Journal of Clinical Microbiology* 2000; **38**: 2824–2828.
48. **Byington CL, *et al.*** The effect of rapid respiratory viral diagnostic testing on antibiotic use in a children's hospital. *Archives of Pediatrics and Adolescent Medicine* 2002; **156**: 1230–1234.
49. **Doern GV, *et al.*** Clinical impact of rapid in vitro susceptibility testing and bacterial identification. *Journal of Clinical Microbiology* 1994; **32**: 1757–1762.
50. **Galar A, *et al.*** Clinical and economic evaluation of the impact of rapid microbiological diagnostic testing. *Journal of Infection* 2012; **65**: 302–309.
51. **Henrickson KJ.** Cost-effective use of rapid diagnostic techniques in the treatment and prevention of viral respiratory infections. *Pediatric annals* 2005; **34**: 24–31.
52. **Woo PC, *et al.*** Cost-effectiveness of rapid diagnosis of viral respiratory tract infections in pediatric patients. *Journal of Clinical Microbiology* 1997; **35**: 1579–1581.
53. **Chen YS, *et al.*** Comparison of diagnostic tools with multiplex polymerase chain reaction for pediatric lower respiratory tract infection: a single center study. *Journal of Microbiology, Immunology and Infection*. Published online 29 September 2012. doi:10.1016/j.jmii.2012.07.016.
54. **Tsalik EL, *et al.*** Multiplex PCR to diagnose bloodstream infections in patients admitted from the emergency department with sepsis. *Journal of Clinical Microbiology* 2010; **48**: 26–33.
55. **Wishaupt JO, *et al.*** Clinical impact of RT-PCR for pediatric acute respiratory infections: a controlled clinical trial. *Pediatrics* 2011; **128**: e1113–1120.
56. **Brittain-Long R, *et al.*** Access to a polymerase chain reaction assay method targeting 13 respiratory viruses can reduce antibiotics: a randomised, controlled trial. *BMC Medicine* 2011; **9**: 44.
57. **Falsey AR, *et al.*** Bacterial complications of respiratory tract viral illness: a comprehensive evaluation. *Journal of Infectious Diseases* 2013; **208**: 432–441.
58. **Murdoch DR.** Impact of rapid microbiological testing on the management of lower respiratory tract infection. *Clinical Infectious Diseases* 2005; **41**: 1445–447.
59. **Dark PM, Dean P, Warhurst G.** Bench-to-bedside review: the promise of rapid infection diagnosis during sepsis using polymerase chain reaction-based pathogen detection. *Critical Care* 2009; **13**: 217.
60. **Westh H, *et al.*** Multiplex real-time PCR and blood culture for identification of bloodstream pathogens in patients with suspected sepsis. *Clinical Microbiology and Infection* 2009; **15**: 544–551.
61. **Yanagihara K, *et al.*** Evaluation of pathogen detection from clinical samples by real-time polymerase chain reaction using a sepsis pathogen DNA detection kit. *Critical Care* 2010; **14**: R159.
62. **Dark P, *et al.*** The clinical diagnostic accuracy of rapid detection of healthcare-associated bloodstream infection in intensive care using multipathogen real-time PCR technology. *BMJ Open* 2011; **1**: e000181.
63. **Infectious Diseases Society of America.** An unmet medical need: rapid molecular diagnostics tests for respiratory tract infections. *Clinical Infectious Diseases* 2011; **52**: S384–S395.

https://doi.org/10.1017/S0950268813002367 Published online by Cambridge University Press

64. **Gerna G, *et al*.** Correlation of rhinovirus load in the respiratory tract and clinical symptoms in hospitalized immunocompetent and immunocompromised patients. *Journal of Medical Virology* 2009; **81**: 1498–1507.
65. **Phillips G, *et al*.** Diagnosing norovirus-associated infectious intestinal disease using viral load. *BMC Infectious Diseases* 2009; **9**: 63.
66. **Schuetz P, Raad I, Amin DN.** Using procalcitonin-guided algorithms to improve antimicrobial therapy in ICU patients with respiratory infections and sepsis. *Current Opinion in Critical Care*. Published online 11 July 2013. doi:10.1097/MCC. 0b013e328363bd38.
67. **Dundas NE, *et al*.** A lean laboratory: operational simplicity and cost effectiveness of the Luminex xTAG™ respiratory viral panel. *Journal of Molecular Diagnostics* 2011; **13**: 175–179.
68. **Mahony JB, *et al*.** Cost analysis of multiplex PCR testing for diagnosing respiratory virus infections. *Journal of Clinical Microbiology* 2009; **47**: 2812–2817.

https://doi.org/10.1017/S0950268813002367 Published online by Cambridge University Press