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Application of Multiplex Molecular Assays in the Diagnosis of Acute Respiratory Infectious Diseases

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Abstract: Acute respiratory tract infections (ARTIs) are the most common diseases worldwide and an important cause of morbidity and mortality. Upper and lower respiratory tract infections are caused by a variety of bacterial, viral, and fungal pathogens. Clinical diagnosis of respiratory tract infections is challenging because of indistinguishable symptoms. Laboratory diagnosis is performed by serology, culture, electron microscopy, and immunological antigen detection assays. These conventional diagnostic procedures are time consuming, lack sensitivity, require special laboratory setup, and well-trained staff. In the clinical laboratories, multiplex molecular nucleic acid amplification tests (NAATs) are continuously replacing conventional diagnostic methods. This review summarizes and discusses the availability, clinical use, advantages and disadvantages of multiplex molecular methods in the detection and identification of ARTI pathogens. The multiplex molecular assays can simultaneously detect 20 or more bacterial and viral pathogens and have the advantage of increased sensitivity, specificity, and rapid turn-around time. These assays are helpful in syndromic based testing in high risk patient population, particularly those who are immunocompromised, hospitalized and/or seen in the emergency department. Limitations of multiplex molecular assays include inability to detect all the possible pathogens that can be present, not being able to differentiate between asymptomatic carriers and true infections, and sometimes results are difficult to interpret. Furthermore, additional testing may be required as these assays do not provide any information regarding antimicrobial susceptibility profile. Rapid and accurate diagnosis of respiratory tract infections leads to better treatment decisions, reduction in the further diagnostic procedures, length of hospital stay, better infection control measures, and associated healthcare costs.

Keywords: Respiratory Pathogens, Respiratory Infections, Molecular Diagnostics, Multiplex Assays, Nucleic Acid Amplification Tests

1. Introduction

Respiratory tract infections (RTIs) are the most common, and severe of the infections reported worldwide in terms of the physician's office visit and hospitalization [1]. Acute infections, especially of the lower respiratory tract are the leading cause of morbidity and mortality in young children, elderly and immunocompromised patients [2, 3]. Acute respiratory tract infections (ARTIs) are also the most common infectious diseases in the developed world including the United States. ARTIs include the upper respiratory tract and lower respiratory tract infections and are caused by a variety of bacterial and viral pathogens. The most common symptoms are flu, runny nose, sneezing, cough, congestion, pharyngitis, otitis media, acute bronchiolitis, and pneumonia. The important causative agents of ARTIs are Influenza Virus, Parainfluenza Virus, Respiratory Syncytial Virus (RSV), Human Enterovirus/Rhinoviruses, Adenovirus, Coronavirus, Bocavirus and Metapneumovirus [4, 5]. A variety of bacteria, including true pathogens like *Bordetella spp*., and opportunistic pathogens like *Haemophilus spp*., *Moraxella catarrhalis*, *Streptococcus pyogenes*, *S. pneumoniae* and *Staphylococcus aureus* cause upper (URT) and lower respiratory tract (LRT) infections. Gram negative enteric organisms can cause serious opportunistic infections. Environmental agents e.g., *Acinetobacter spp*., *Legionella spp*., *Pseudomonas* spp., can cause ARTI, whereas *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* are important causative agents of atypical pneumonia [6]. Mixed viral and bacterial infections are very common, and in these

cases coinfections with two or more viruses and bacterial pathogen can occur [6, 7].

Rapid accurate diagnosis and identification of pathogens causing respiratory infections are essential in making appropriate treatment decisions and in the implementation of infection control measures. The clinical differential diagnosis of respiratory infections is difficult, as most of the bacterial and viral pathogens produce similar and nonspecific signs and symptoms. Differentiation between bacterial and viral infections are important, as rapid detection and identification of viral pathogens causing respiratory infections significantly reduce the need for additional laboratory testing and use of antibiotic treatment. Furthermore, identification of viral infections also significantly decreases the unnecessary and prolonged hospitalization and associated management cost [8, 9]. In certain situations, rapid detection and identification of viral pathogens help guide specific antiviral treatment and appropriate isolation precautions [8, 9].

In the last decade, there has been a significant increase in the availability of commercial molecular assays in the point-of-care (POC) and laboratory diagnosis of respiratory tract infections. Recent developments in the field of molecular diagnostics and the availability of commercial nucleic acid amplification tests (NAATs) have changed the way we used to perform laboratory diagnosis of respiratory infections. This review summarizes currently available Food and Drug Administration (FDA) approved (Table 1) and some commonly available European In-Vitro Diagnostic Devices (CE-IVD) marked (Table 2) multiplex molecular assays for the diagnosis of respiratory infectious diseases. Advantages and disadvantages, technical challenges, and problems in ordering, billing, and interpretation of the results are also discussed.

Table 1. *Comparative Summary of Commercial FDA Approved Respiratory Pathogen Multiplex Molecular Assays.*

Product Name	**BioFire® FilmArray Pneumonia Panel FDA/CE-IVD (M)**	**BioFire® FilmArray Respiratory Panel/ Panel 2 FDA/CE-IVD (M)**	**BioFire® FilmArray Respiratory EZ FDA (W)**	**Verigene® Respiratory Pathogen *Flex* Test FDA/CE-IVD (M)**	**NxTAG® Respiratory Pathogen Panel FDA/CE-IVD (H)**	**eSensor® Respiratory Viral Panel (XT-8) FDA/CE-IVD (H)**	**ePlex® Respiratory Pathogen Panel FDA/CE-IVD (H)**	**QIAstat-Dx® Respiratory Panel FDA/CE-IVD**	**Curetis Unyvero LRT Panel** FDA**
Manufacturer	BioFire, Salt Lake City, UT	BioFire, Salt Lake City, UT	BioFire, Salt Lake City, UT	Luminex, Austin, TX	Luminex, Austin, TX	GenMark, Carlsbad, CA	GenMark, Carlsbad, CA	Qiagen, Germantown, MD	Curetis, Gaithersburg, MD
Specimen	BAL, SPT	NPS	NPS	NPS	NPS	NPS	NPS	NPS	BAL
Turnaround Time	~1 hr	~1 hr	~1 hr	~2 hr	~4 hr	~4 hr	~2 hr	~1 hr	~4 hr
BACTERIA/FUNGI									
Acinetobacter calcoaceticus-baumannii complex	√								√
Bordetella pertussis		√	√	√				√	
Bordetella parapertussis		√*		√ including *B. bronchiseptica, B. holmesii*					
Chlamydia pneumoniae	√	√	√		√		√	√	√
Enterobacter cloacae complex	√								√
Escherichia coli	√								√
Haemophilus influenzae	√								√
Klebsiella aerogenes	√								
Klebsiella oxytoca	√								√
Klebsiella pneumoniae group	√								√ including *K. variicola*
Legionella pneumophila	√								√
Moraxella catarrhalis	√								√
Mycoplasma pneumoniae	√	√	√		√		√	√	√
Pneumocystis jirovecii									√
Proteus spp.	√								√
Pseudomonas aeruginosa	√								√
Serratia marcescens	√								√
Staphylococcus aureus	√								√
Streptococcus agalactiae	√								

Product Name	BioFire® FilmArray Pneumonia Panel FDA/CE-IVD (M)	BioFire® FilmArray Respiratory Panel/ Panel 2 FDA/CE-IVD (M)	BioFire® FilmArray Respiratory EZ FDA (W)	Verigene® Respiratory Pathogen *Flex* Test FDA/CE-IVD (M)	NxTAG® Respiratory Pathogen Panel FDA/CE-IVD (H)	eSensor® Respiratory Viral Panel (XT-8) FDA/CE-IVD (H)	ePlex® Respiratory Pathogen Panel FDA/CE-IVD (H)	QIAstat-Dx® Respiratory Panel FDA/CE-IVD	Curetis Unyvero LRT Panel** FDA
Streptococcus pneumoniae	√								√
Streptococcus pyogenes	√								
VIRUSES									
Adenovirus	√	√	√	√	√	√Including B/E/C	√	√	
Coronavirus	√	√ HKU1, NL63, 229E, OC43	√		√ HKU1, NL63, 229E, OC43		√ HKU1, NL63, 229E, OC43	√ HKU1, NL63, 229E, OC43	
Human Bocavirus					√				
Human Metapneumovirus	√	√	√	√	√	√	√	√A, B	
Human Rhinovirus/Enterovirus	√	√	√	√	√	√	√	√	
Influenza A	√	√ including A/H1, A/H3, A/H1-2009	√ including A/H1, A/H3, A/H1-2009	√ including A/H1, A/H3	√ including A/H1, A/H3	√ including A/H1, A/H3, A/2009 H1N1	√ including A/H1, A/H3, A/2009 H1N1	√ including A/H1, A/H3, A/H1-2009	
Influenza B	√	√	√	√	√	√	√	√	
Parainfluenza Virus	√	√1, 2, 3, 4	√	√1, 2, 3, 4	√1, 2, 3, 4	√1, 2, 3, 4	√1, 2, 3, 4	√1, 2, 3, 4	
Respiratory Syncytial Virus	√	√	√	√A, B	√A, B	√A, B	√A, B	√A, B	
ANTIMICROBIAL RESISTANCE									
Methicillin Resistance: *mec*A/C and MREJ	√								√ *mec*A
Carbapenemases: KPC, NDM, Oxa-48-like, VIM, IMP	√								√ including Oxa 23, Oxa 24, Oxa 58
ESBL: CTX-M	√								√

*Available only on the BioFire Respiratory Panel 2; ** Also include bacterial pathogens *Citrobacter freundii*, *Morganella morganii* and *Stenotrophomonas maltophilia*
Clinical Laboratory Improvement Amendments (CLIA): Waived (W); Moderate Complexity (M); High Complexity (H)
United States Food and Drug Administration (FDA); European CE Marking for In Vitro Diagnostic (CE-IVD)
Bronchoalveolar Lavage (BAL); Nasopharyngeal Swabs (NPS); Sputum (SPT)

Table 2. *Comparative Summary of other Commercial Respiratory Pathogen Multiplex Molecular Assays.*

Product Name	Allplex™ Respiratory Panel Assay CE-IVD	Anyplex™ II RV16 Detection CE-IVD	Seeplex® RV15 ACE Detection CE-IVD	Fast Track Respiratory Pathogens21/33 CE-IVD	PathoFinder RespiFinder® 2Smart CE-IVD	CLART® PneumoVir CLART® PneumoVir 2 CE-IVD	Pneumo CLART® Bacteria CE-IVD
Manufacturer	Seegene, Seoul, South Korea	Seegene, Seoul, South Korea	Seegene, Seoul, South Korea	Fast Track Diagnostics Luxembourg	PathoFinder, The Netherlands	Genomica, Madrid, Spain	Genomica, Madrid, Spain
Specimen	BAL, NPA, NPS	BAL, NPA, NPS	NPS	NPS	NPS	BAL, NPA, NPS	BAL, NPA, SPT
Turnaround Time	~4 hr	~4 hr	~4 hr	~4 hr	~3-4 hr	~4-5 hr	~4-5 hr
BACTERIA/FUNGI							
Bordetella pertussis	√			√	√		√
Bordetella parapertussis	√						√ including *B. bronchiseptica, B. holmesii*
Chlamydia pneumoniae	√			√	√		√
Haemophilus influenza	√			√			√ and others

Product Name	Allplex™ Respiratory Panel Assay CE-IVD	Anyplex™ II RV16 Detection CE-IVD	Seeplex® RV15 ACE Detection CE-IVD	Fast Track Respiratory Pathogens21/33 CE-IVD	PathoFinder RespiFinder® 2Smart CE-IVD	CLART® PneumoVir CLART® PneumoVir 2 CE-IVD	Pneumo CLART® Bacteria CE-IVD
Klebsiella pneumoniae group				√			
Legionella pneumophila	√			√	√		
Moraxella catarrhalis				√			√
Mycoplasma pneumoniae	√			√	√		√
Pneumocystis jirovecii				√			
Salmonella spp				√			
Staphylococcus aureus				√			√ including *mec*A
Streptococcus pneumoniae	√			√			√
VIRUSES							
Adenovirus	√	√	√	√	√	√	
Coronavirus	√ NL63, 229E, OC43	√ NL63, 229E, OC43	√ NL63, 229E, OC43	√ HKU1, NL63, 229E, OC43	√ NL63, 229E, OC43	√ NL63, 229E, OC43	
Human Bocavirus	√1, 2, 3, 4	√1, 2, 3, 4	√1, 2, 3, 4	√	√	√	
Human Metapneumovirus	√	√	√	√A, B	√	√A, B	
Human Rhinovirus/Enterovirus	√	√	√	√	√	√	
Influenza A	√ including A/H1, A/H3, A/H1-2009	√	√	√ including A/H1-2009	√ including A/H1-2009	√ including A/H3, A/H1-2009	
Influenza B	√	√	√	√	√	√	
Influenza C				√		√	
Parainfluenza Virus	√1, 2, 3, 4	√1, 2, 3, 4	√1, 2, 3, 4	√1, 2, 3, 4	√1, 2, 3, 4	√1, 2, 3, 4	
Respiratory Syncytial Virus	√A, B	√A, B	√A, B	√A, B	√A, B	√A, B	

European CE Marking for *In Vitro* Diagnostic (CE-IVD)
Bronchoalveolar Lavage (BAL); Nasopharyngeal Aspirates (NPA); Nasopharyngeal Swabs (NPS); Sputum (SPT)

2. Traditional Culture, Electron Microscopy and Immunological Techniques

Traditional laboratory diagnosis of ARTIs and respiratory pathogen detection is performed by; 1) Culture methods for bacteria and viruses, 2) Fluorescent antibody staining for pathogenic bacteria and viruses, 3) Electron microscopic examination for viruses, 4) Antigen detection of bacterial and viral pathogens via immunoassays and 5) Serological assays for the detection of antibodies against the pathogenic organisms.

In the case of ARTI, the main challenges faced by the clinicians are to distinguish between common cold viral infections from the respiratory viral infections including influenza, RSV, and bacterial infections. Laboratory diagnosis is needed if bacterial pathogens are involved, especially in the identification of causative pathogens and differentiation of community-acquired pneumonia, atypical pneumonia, secondary bacterial sinusitis, otitis media, and streptococcal pharyngitis [10]. Furthermore, for some of the bacterial isolates, an antibiotic sensitivity profile may be required. Conventional laboratory diagnosis of respiratory infections is complicated and requires separate bacterial and viral laboratory setup with long turn-around time. Additionally, some of the laboratory procedures are technically challenging, very expensive to perform, and require highly trained staff.

Several molecular NAATs and diagnostic methods including multiplex respiratory panels are currently available that can overcome some of the problems with these traditional methods. The major advantages of multiplex molecular assays are the comprehensive coverage of the ARTI pathogens with increased sensitivity and specificity as compared to traditional methods. These methods are easy to perform with rapid turnaround time and have the capability of detection of multiple pathogens including the bacteria, viruses, fungi, and some antibiotic resistant genes simultaneously from a single specimen.

3. Syndromic Multiplex Respiratory Pathogen Testing Algorithm

Multiple factors must be considered by the clinicians before selecting an appropriate multiplex molecular test.

Before considering a multiplex molecular test for the diagnosis of ARTIs, local and/or seasonal epidemiology, travel history, age of the patient, immune status, out-patient or hospitalized, and clinical diagnosis of bacterial or viral infections should be considered. An algorithm for multiplex molecular assays based on the symptoms and patient population can be used (Figure 1).

Commercially available nucleic acid based methods have focused on either the detection of a single pathogen or multiple pathogens in a multiplex assay format. Several molecular assays are available for the detection of a single respiratory pathogen. These assays are mostly focused on the detection of *S. pyogenes*, *Bordetella* spp, atypical bacteria, Influenza, RSV, Adenovirus, and most recently Coronavirus SARS-CoV-2 (COVID-19) infection after the 2019-2020 pandemic. These singleplex molecular NAATs are specially designed to target specific patient population and allow for particular testing that a physician may order. Furthermore, these singleplex assays are also designed to meet medical coding and billing requirements and are not discussed in this article.

According to the symptomatic multiplex NAAT algorithm, the rapid POC antigen detection test can be used if the patients are low-risk, immunocompetent, not seen at the hospital emergency room (ER) or admitted in the hospital and are seen in the Influenza/RSV season. If positive, it confirms Influenza or RSV infection and if negative, further Influenza A/B PCR can be performed on outpatients with corresponding symptoms. Multiplex assays that can detect 12 or more bacterial and viral pathogens can be used if bacterial or viral etiology other than Influenza and RSV is suspected and depending on the patient's immune status and clinical findings. In general, multiplex assays are more suitable for high-risk patient populations including immunocompromised individuals, hospitalized and patients seen and admitted at the hospital ER where rapid screening and diagnosis is required (Figure 1). Multiplex assays are also a good option for screening and detection purposes when the etiology is not very clear.

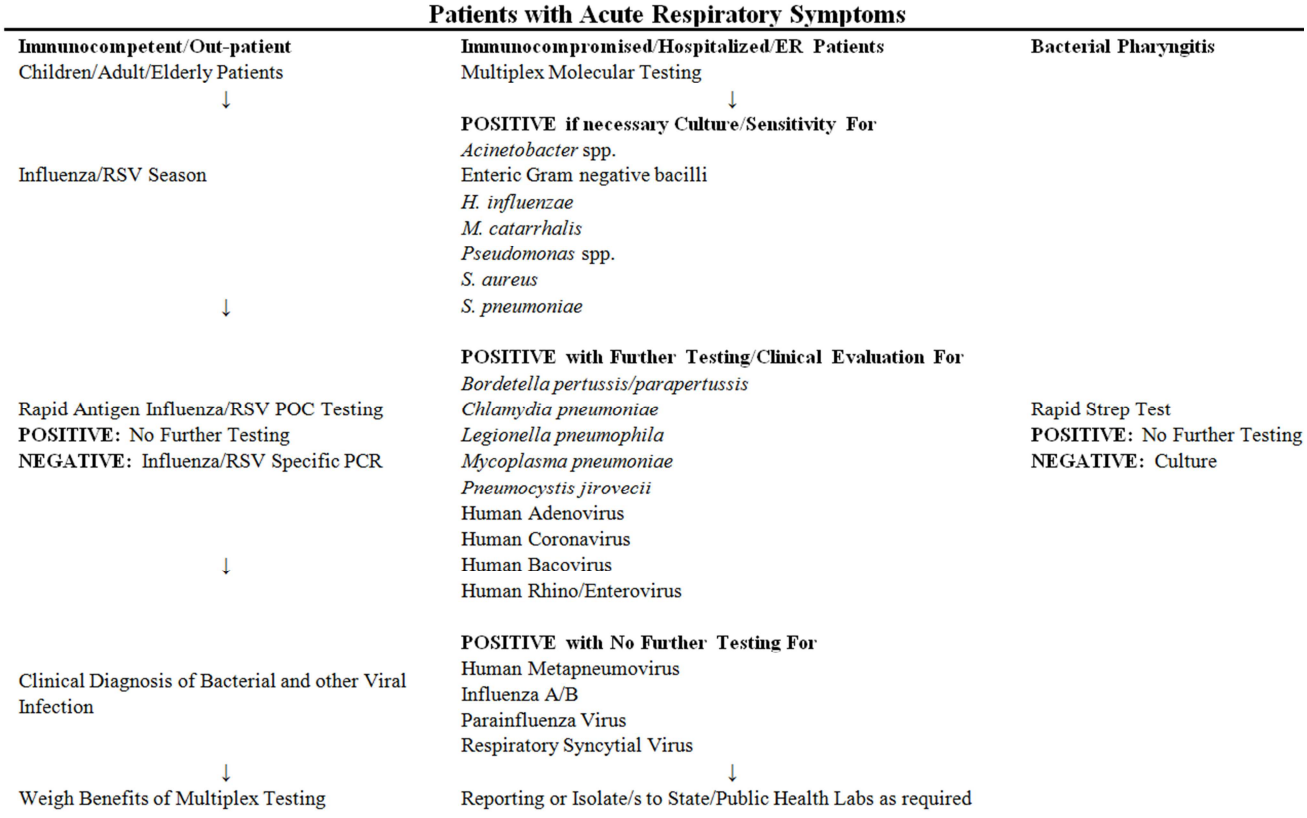

Figure 1. *Multiplex NAAT Algorithm for Acute Respiratory Pathogens.*

4. Specimens for Multiplex Molecular Respiratory Pathogen Testing

For molecular testing, most of the manufacturers recommend nasopharyngeal swabs (NPS) for patients with upper respiratory tract infections (URTI). For patients with lower respiratory tract infection (LRTI), bronchoalveolar lavage (BAL), endotracheal aspirate (ETA), and sputum (SPT) specimens are preferred. Most of the FDA approved assays are intended and are validated for NPS specimens. Clinical microbiology laboratories may also have to validate these assays for the BAL, ETA or SPT specimens if these samples are used [11]. Repeat multiplex molecular testing for respiratory pathogens is common and usually not helpful, as it can leads to confusion with the increased healthcare cost [12]. Testing on both NPS and BAL with 7 days from either specimen is considered as unnecessary repeat testing unless there is change in the patient's clinical presentation since the original testing. For example, NPS specimen was originally

tested for URTI, but later the patient developed lower respiratory tract symptoms for which BAL is required to confirm the pathogen detected in NPS as a causative agent of LRTI. In case of negative NPS, but patient showing symptoms of pneumonia and LRTI, additional bacterial and viral testing may be required (Figure 1). Furthermore, a recent study indicates that repeated multiplex testing for respiratory viruses within short periods of up to 20 days lead to redundant results at the additional costs [13]. Molecular testing does not require the presence of live organisms in the specimen. Therefore, it is recommended that appropriate specimen collection, transport, and storage conditions should be followed if viral culture, or bacterial culture and antibiotic sensitivity are ordered on the same specimen or can be ordered later if found to be positive for a particular pathogen.

5. Commercial Multiplex Molecular Respiratory Pathogen Assays

In the recent years, there has been a remarkable improvement in the diagnosis of respiratory tract infections due to the commercial availability of rapid POC and molecular diagnostic products. There are several Food and Drug Administration (FDA) and European In-Vitro Diagnostic Devices (CE-IVD) approved molecular methods available for the diagnosis of respiratory pathogens. These assays are either pathogen specific and are designed to detect a single pathogen or multiplex assays that can detection several bacterial and viral pathogens as a single test. Multiplex commercial panels can detect most of the common to uncommon pathogens and even some markers of antibiotics resistance. These multiplex assays are either open systems, in which a separate nucleic acid extraction step is required or closed assay systems, in which simultaneous nucleic acid extraction, amplification, and product analysis are performed. For the identification of pathogens, these assays utilize the reverse-transcriptase real-time Polymerase Chain Reaction (PCR) for amplification of genes and pathogen identification by either hybridization to microarray, hybridization to fluorescent probes or by melting curve analysis. In the context of molecular diagnostics, fully integrated systems are commercially available molecular testing platforms that can generate patient results in a single step from the specimen. There is an increased demand for these integrated systems as they are easy to perform and do not require highly trained personnel. These assays are closed systems with less chance of contamination, have reduced hands-on time, and can detect multiple pathogens in a single step.

In the evaluation of newly developed molecular assays, the terms sensitivity and specificity are important parameters established after comparison with a reference gold standard method. The sensitivity of a newly developed test is its ability to correctly identify those with the disease (true positive), whereas test specificity is its ability to correctly identify those without the disease (true negative). When a newly developed molecular assay is compared with a non-reference method, the numerical calculations are called as positive percent agreement (PPA) and negative percent agreement (NPA) instead of sensitivity and specificity.

5.1. Real-Time Polymerase Chain Reaction (PCR) Assays:

5.1.1. PathoFinder RespiFinder® 2Smart (RF-22)

The RespiFinder 2Smart (PathoFinder, The Netherlands) can detect 22 (4 bacteria and 18 viral) respiratory pathogens from NPS specimens (Table 2). The procedure requires nucleic acid extraction by manual or by automatic specimen processing systems, e.g., NucliSENS easyMAG (bioMérieux, Durham, NC) or QIAsymphony (QIAGEN, Germantown, MD). This assay is validated on LightCycler 480 (Roche, Indianapolis, IN, USA) and Rotor-Gene (QIAGEN, Germantown, MD) real-time PCR equipment. After real-time amplification, target genes are identified by melting curve analysis. Several studies have been performed on the use of this assay [14, 15]. This assay showed increased sensitivity and specificity as compared to culture method for viruses and immunochromatography assay for RSV [16]. A comparative performance evaluation of the NxTAG-RP panel with PathoFinder RF-22 assay showed that concordant results were obtained in 263 (93.3%) cases consisting of concordant positives in 167 (59.2%) and concordant negatives in 96 (34%) of the patient population [17]. Similarly, the analytical sensitivity of three multiplex PCR assays, xTAG RV-Fast, PathoFinder RF-19 and RF-22 assays as compared to monoplex real-time PCR with quantified standardized control material showed concordance [18].

5.1.2. Fast Track Diagnostics Respiratory Pathogens 21/33 (FTD-RP21 or 33)

The Fast Track Diagnostics Respiratory Pathogens panels (Fast Track Diagnostics, Luxembourg) 21 or 33 are a five to eight tube respective multiplex assay designed to detect a variety of bacterial pathogens from NPS samples (Table 2). The procedure requires nucleic acid extraction by manual or by automatic specimen processing system and real-time quantitative PCR amplification of target genes using TaqMan probe technology on a variety of real-time PCR equipment. A comparison of xTAG RV-Fast panel, PathoFinder RF-22, CLART PneumoVir and FTD-RF-33 kits showed these assays overall sensitivities to be 72.63%, 88.42%, 63.86%, 77.89% respectively, and PPA to be 92%, 92.31%, 80.30%, 94.87% respectively [19]. A large epidemiological study in which 38,000 samples were tested for respiratory viruses by FTD-RP 21 showed its usefulness in the improvement and optimization of diagnostic procedures, as well as control and prevention of the respiratory infections [20].

5.1.3. Allplex™ Respiratory Panel Assay (Allplex RP)

The Allplex Respiratory Panel Assay (Seegene, Seoul, South Korea) is a multiplex assay that can detect 26 (7 bacteria and 16 viral) respiratory pathogens from NPS, ETA

and BAL specimens (Table 2). The procedure requires nucleic acid extraction by manual or by an automatic specimen processing system and real-time quantitative PCR amplification using CFX96 (Bio-Rad, Hercules, CA) and other comparable equipment. Target gene detection and quantification is performed by Seegene's Multiple Detection Temperature (MuDT) technology, which reports the individual cycle threshold (Ct) value of each pathogen and allows simultaneous detection and quantification of multiple targets in a single fluorescence channel, without melting curve analysis after amplification. Allplex also offers several separate viral and bacterial assays in this technology format. Comparative studies of Allplex RP with FTD-RP 21 assay for the diagnosis of pediatric respiratory viral infections showed high concordance between the two methods for positive and negative samples [21, 22]. Comparison with reference method showed Allplex RP to have sensitivity and specificity to be 98% and 100% respectively as compared to FTD-RP 21 assay which showed sensitivity and specificity to be 100% [21].

5.1.4. Anyplex™ II RV16 Detection (Anyplex II RV16)

The Anyplex II RV16 Detection (Seegene, Seoul, South Korea) simultaneously can detect 16 of the most prevalent respiratory viruses from NPS, ETA, and BAL specimens. The procedure requires nucleic acid extraction by manual or by an automatic specimen processing system and real-time quantitative PCR amplification using several real-time PCR equipment. Comparison of Anyplex II RV16 with NxTAG-RP panel showed high positive and negative agreement for main viruses that cause acute respiratory infections in children [23], and with FilmArray RP panel [24].

5.1.5. Seeplex® RV15 ACE Detection

The Seeplex RV15 ACE Detection (Seegene, Seoul, South Korea) is a multiplex PCR assay for the detection and identification of 15 most common respiratory viruses from NPS and BAL specimens (Table 2). Related to this product, is Seeplex PneumoBacter ACE Detection, which can detect 6 pneumonia bacteria from NPS, ETA, BAL and SPT specimens. The procedure requires nucleic acid extraction by manual or by an automatic specimen processing system and real-time quantitative PCR amplification using dual priming oligonucleotides as PCR primers on various real-time PCR equipment. A comparative evaluation of the Seeplex RV15 and real‐time PCR for respiratory virus detection produced comparable results [25]. Another comparative evaluation of Anyplex II RV16 with the xTAG RV panel and Seeplex RV15 for detection of respiratory viruses excluding Human Bocavirus results showed the overall sensitivities to be 95.2%, 93.3%, and 87.2% respectively. While the specificities of all three methods ranged from 98.6% to 100% [26]. An evaluation of the AdvanSure RV real-time RT-PCR (AdvanSure, LG Life Sciences, Korea) as compared with culture and Seeplex RV15 for the simultaneous detection of respiratory viruses showed the overall sensitivities of culture, RV15 and AdvanSure to be 74.5%, 89.8%, 95.1% respectively, and specificities to be 100, 98.9, 99.5%, respectively [27].

5.2. End point Polymerase Chain Reaction (PCR) and Array Based Assays

5.2.1. NxTAG® Respiratory Pathogen Panel (NxTAG-RP)

The NxTAG Respiratory Pathogen Panel (Luminex, Austin, TX) is a multiplex assay that allows the simultaneous detection of 20 bacterial and viral pathogens (Table 1). The assay requires separate nucleic acid extraction step followed by an integrated multiplex PCR amplification in a 96 well plate format, bead hybridization and analysis by using MAGPIX Instrument and SYNCT Software [28]. Clinical evaluation of the NxTAG-RP panel demonstrated an overall sensitivity and specificity to be ≥93% for all respiratory targets except Human Coronavirus OC43 (HCoV-OC43) and HCoV-HKU1 [29]. Also, in this study NxTAG-RP demonstrated 98.8% concordance with the FilmArray-RP in the detection of positive results [29]. A comparative study of NxTAG-RP with PathoFinder RF-22 showed an overall 93.3% concordance between both assay results [17]. Similarly, a study of use of NxTAG-RP and Anyplex II RV16 for multiplex detection of respiratory pathogens in hospitalized children showed the PPA in the range of 83.3 to 100%, while the NPA was more than 99% for all targets except for enterovirus/rhinovirus (EV/RV; 94.4%) [23]. NxTAG-RP is an improved version of xTAG Respiratory Viral Panel Fast (xTAG RV-Fast). Both the assays showed increased detection rate, but was found to be low with the laboratory developed real-time PCR results [30, 31]. Also, NxTAG assay identified *M. pneumoniae* in 32 of 44 (72.7%) PCR-positive samples [31].

5.2.2. CLART® PneumoVir/PneumoVir 2 and Pneumo Bacteria

The CLART PneumoVir/PneumoVir 2 assays allow detection of 21 viruses and Pneumo bacteria, 11 most common bacterial pathogens from NPS, ETA and BAL specimens (Table 2). The procedure requires nucleic acid extraction by manual or by automatic GENOMICA's autoclart plus specimen handling system and amplification by Mastercycler Nexus Thermocycler (Eppendorf, Hauppauge, NY) in an end-point multiplex reverse transcriptase PCR amplification. The amplified products in a 96 well plate format are visualized by GENOMICA's CAR array reader. A comparison of CLART PneumoVir, xTAG RV-Fast, FTD-RP33 and PathoFinder RF-22 showed this assay to have relatively less sensitivity (63.86%, 72.63%, 77.89% 88.42) and PPA (80.30%, 92.00%, 94.87%, 92.31%) respectively as compared to other assays [19]. In the two other studies, PneumoVir has been used to determine its significance in the clinical and epidemiological studies [32, 33].

5.3. Integrated Nucleic Acid Amplification Assays

5.3.1. BioFire FilmArray® Panels

The BioFire FilmArray (BioFire, Salt Lake City, UT) offers comprehensive coverage of respiratory pathogens in the form of Pneumonia Panel, Respiratory Panel/Panel 2, and

Respiratory EZ Panel. BioFire FilmArray panels are a fully integrated system and each test includes nucleic acid extraction, followed by nested reverse-transcriptase PCR and detection of amplified products and identification of the pathogens by melting curve analysis.

The BioFire FilmArray Pneumonia Panel (FilmArray PP) offers the comprehensive detection of 33 clinically relevant targets from BAL, ETA and SPT samples. The FilmArray PP panel can identify 15 bacterial pathogens as semi-quantitative results, which may help determine whether an organism is a colonizer or a true pathogen, 3 atypical bacterial pathogens, 8 viruses, and 7 antibiotic resistance genes as qualitative results (Table 1). A study of the evaluation of FilmArray PP with the culture method demonstrated the overall sensitivity and specificity of this assay to be 98.5% and 76.5%, respectively. FilmArray PP also detected antimicrobial resistance genes in 17 out of 18 specimens (94.4%) that were resistant by antimicrobial susceptibility assays [34]. Semi-quantitative analysis of the bacterial nucleic acid amounts by FilmArray PP revealed that 88.2% of the identified bacteria with $\geq 10^6$ copies/mL also gave culture-positive results [34].

The BioFire FilmArray Respiratory Panel/Panel 2 (FilmArray RP2) detects 21 pathogens (3 bacteria and 17 viruses) directly from NPS samples in a single multiplex reaction (Table 1). BioFire respiratory panel version 2.1 released under the FDA Emergency Use Authorization (EUA) can also detect SARS-CoV-2. Multiple studies have evaluated this assay on the NPS samples for URTI [35], and ETA, SPT specimens for LRTI [36-38]. Performance evaluation of the FilmArray RP2 and FilmArray PP on the LRTI specimens showed PPA to be 87% for viral targets and 100% for atypical bacterial targets. The NPA was 100% for both viral and atypical bacterial targets [38]. This study also reported that FilmArray PP identifies more typical bacterial pathogens in adult LRTI specimens than the FilmArray RP2 while retaining comparable performance for viral targets [38]. Evaluation of the FilmArray RP and the eSensor RV panel on LRTI specimens showed an overall agreement between the two methods to be 89.5% [36]. The lower limit of detection of both assays for all targets in LRTI specimens was comparable to that for NPS specimens [36].

The BioFire Respiratory EZ (BioFire R-EZ) Panel is Clinical Laboratory Improvement Act (CLIA) waived multiplex assay available in the United States to detect 14 respiratory pathogens (3 bacteria and 11 viruses). The BioFire Respiratory 2.1-EZ (RP2.1- EZ) panel released under the FDA-EUA can also detect SARS-CoV-2. This assay is primarily designed for the physician's office and out-patient clinics to test the patient in a POC setting using the syndromic approach as used for Influenza and RSV testing (Figure 1). Validation and performance characteristics of this assay are performed by the manufacturer and there is limited independent evaluation on this assay.

5.3.2. VERIGENE® Respiratory Pathogens Flex (Verigene RP Flex) Nucleic Acid Test

The Verigene Respiratory Pathogens Flex Nucleic Acid Test (Luminex, Austin, TX) can detect up to 16 pathogens (3 bacteria and 13 viruses) from NPS samples (Table 1). This assay is a cartridge based closed sample to the result system. The Verigene RP Flex assay does not require a separate nucleic acid extraction step and the entire procedure is performed by the Verigene sample processor and reader. Even though Verigene RP Flex is a multiplex assay, it offers the advantage of selective testing of one or more of the target pathogens in the patient sample. This flexibility is helpful in certain situations when the physician wants to order specific pathogen testing only, and can also be helpful in resolving billing issues when unnecessary testing is not required. Validation and performance characteristics of this assay are performed by the manufacturer and there is limited independent evaluation on this assay.

5.3.3. ePlex® Respiratory Pathogen (ePlex RP) and Viral (XT-8) (ePlex RV) Panels

The ePlex Respiratory Pathogen (GenMark, Carlsbad, CA) can detect 19 pathogens (2 bacteria and 17 viruses) and XT-8 Viral Panel can detect 14 virus types and no bacterial from the NPS samples (Table 1). These panels are also cartridges base closed sample to result system, does not require nucleic acid extraction step and detection of pathogens are based on electrochemical microfluidics detection technology [39]. A comparison of ePlex RP with laboratory-developed real-time PCR assays for the detection of respiratory pathogens showed an agreement of 97.4% with 464 pathogens found in the clinical specimens [40], and significant decrease in time to result, enabling a reduction in isolation days in half of the patients [41]. Another multicenter evaluation of the ePlex RP for the detection of bacterial and viral pathogens from the NPS specimens and comparison with BioFire RP showed an overall agreement between the ePlex RP and BioFire RP results to be >95% for all targets [42].

5.3.4. QIAstat-Dx Respiratory (QIAstat RP) Panel

The QIAstat-Dx Respiratory Panel (Qiagen, Germantown, MD) can detect 20 pathogens (3 bacteria and 18 viruses) (Table 1) from NPS specimens. During the 2019-2020 SARS-CoV-2 outbreak, under the FDA Emergency Use Authorization (EUA) QIAstat-Dx Respiratory SARS-CoV-2 Panel was introduced that can detect 21 respiratory targets, including SARS-CoV-2. Another version as QIAstat RP 2 is marketed in Europe and other countries in which *C. pneumoniae* is replaced with *L. pneumoniae* and Human Metapneumovirus A/B is added. This test is a cartridge based closed sample-to the result system, which does not require a nucleic acid extraction step, and pathogen detection is performed by multiplex real-time PCR using QIAstat-Dx analyzer. A multicenter evaluation of the QIAstat RP using 445 samples against FilmArray RP, and discrepancy testing by Allplex RP demonstrated that QIAstat RP's PPA to be 98.0% and NPA to be 99.8% [43].

5.3.5. Curetis Unyvero LRT Panel

The Unyvero LRT (Curetis, Gaithersburg, MD) is a recently FDA approved assay that can detect 29 respiratory

pathogens to aid clinicians in the diagnosis of LRTI and earlier targeted antibiotic treatment decisions for critically ill hospitalized pneumonia patients. The Unyvero LRT panel comprises of 19 bacterial pathogens and 10 antibiotic resistance markers with the broad antibiotic resistance genes coverage. This test is a cartridge based closed sample to result system, which does not require a nucleic acid extraction step, and pathogen detection is performed by multiplex real-time PCR using the Unyvero system. Validation and performance characteristics of this assay are performed by the manufacturer and there is limited independent evaluation on this assay. The manufacturer's validation and overall performance of the Unyvero LRT panel for microorganism detection showed the sensitivity and specificity of this assay to be 92.5% and 97.4% respectively. Antibiotic resistance markers detection shows the sensitivity and specificity of this assay to be 93.0% and 98.8% respectively. Another version as Unyvero Hospitalized Pneumonia (HPN) Panel is also available in Europe and other countries.

6. Advantages, Disadvantages and Challenges in the Interpretation of Results

The main advantages of molecular testing are in improved workflow, faster turn-around time with high sensitivity and specificity as compared to traditional methods. Additionally, the multiplex molecular assay eliminates the need of running multiple specialty labs with highly trained staff in specialty areas, as multiplexing allows simultaneous detection of the majority of respiratory pathogens. Multiplex assays can be particularly helpful for severely ill patients and in certain patient populations where rapid diagnosis, treatment, and management decisions are required. Multiplex molecular assays can be helpful from the therapeutic point of view to avoid inappropriate and unnecessary antimicrobial treatment [44]. One of the areas in which molecular testing is very helpful is the epidemiological studies in which broad, highly sensitive, and specific testing is required to identify the particular organism involved in the outbreaks and in taking appropriate isolation procedures and infection control measures.

However, there are several disadvantages and challenges in the use of multiplex molecular assays. The first one is the initial equipment and setup cost, which can be quite high and comparative evaluation needs to be performed before selection and replacing the traditional methods. The multiplex assays can detect pathogens that may or may not be prevalent in a setting and local epidemiology as well as institutional needs should be considered before testing and results should be interpreted carefully. There is limited information available on the actual benefits of the multiplex assays and their role in the everyday clinical practice as well how these multiplex assays have improved the patient care, outcomes and impacts on healthcare system [45]. Furthermore, the current practices do not address the issue of their clinical use and interpretation of the results. The results of multiplex assays can be confusing as physicians may not be familiar with the role of all organisms/strains and antibiotic resistance genes. The use of multiplex molecular assays can have billing issues as these assays can detect and report what physicians have not ordered, and these tests may be considered as medically unnecessary. In order to resolve these billing issues, some of the assays offer separate bacterial and viral panels, making them flexible in situations where specific testing may have been requested on the basis of clinical symptoms by a physician.

The interpretation of the multiplex molecular assays in case of viral infections is difficult as some of the viruses can be present in healthy individuals or in a transient/carrier state and there can be prolonged shedding after asymptomatic/recent infection [46, 47]. Rhinoviruses are responsible for more than one-half of the URTI, can cause self-limiting common cold to severe pneumonia in the elderly and immunocompromised patients, as well as exacerbations of chronic obstructive pulmonary disease and asthma [48]. However, this virus can be identified in about 10-35% of the healthy population [49, 50]. Human Adenovirus account for about 7-8% of viral respiratory infections in children under the age of 5 years with the symptoms of fever, runny nose, sneezing, coughing, congestion, bronchitis and pneumonia [51, 52] and in the adult patient population [53]. However, this virus can be detected in asymptomatic children [54, 55] and in patients with prolonged intermitted shedding of the virus [56]. Human Bocavirus is found worldwide and can cause severe acute respiratory tract infection in children with rhinitis, cough, dyspnea, wheezing, fever, and diarrhea. The role of Human Bocavirus as the single causative agent for respiratory tract infections remains unclear as detection in children is frequently in combination with other viruses or bacteria in a co-infection state [57, 58]. Furthermore, Human Bocavirus can remain in transient state after recovery and can be isolated from healthy individuals [59]. In the last two decades, three novel coronaviruses have emerged in the humans that can cause ARTI with symptoms of fever, sore throat, cough, chills, and muscle pain leading to more severe acute respiratory distress syndrome. These include, the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and most recently Coronavirus Disease 2019 (COVID-19) virus infections [60, 61]. Studies indicate asymptomatic or transient infection in all of the strains of Coronavirus [62, 63]. While on other hand some of the respiratory viruses e.g., Influenza, RSV, Human Metapneumovirus and Parainfluenza Virus are relatively common in patients ARTI symptoms and are rarely isolated form asymptomatic patients [45]. In certain of these viral infections, quantitative measurement of the viral load may be helpful in determining the true disease state [45].

The interpretation of the multiplex molecular assays in case of some of the atypical bacterial infections are also very difficult. Particularly in case of *C. pneumoniae*, *L.*

pneumophila, and *M. pneumoniae*, where these pathogens can be present in healthy individuals or in transient/carrier state [64, 65]. Even though one study indicates that multiplex assays can be helpful in the diagnosis of underlying *M. pneumoniae* infections [66]. Multiplex molecular assay results in case of *Pneumocystis jirovecii* pneumonia need to interpret carefully. This fungal infection commonly affects immunocompromised individuals with severe life-threatening infections. Healthy asymptomatic individuals with normal immune system can have lung colonization and can transfer this infection to immunocompromised patients. Immunocompromised patients with altered host immunity with underlying disease states, cancer, the HIV, transplant recipients, or those on immunosuppressive therapies and medications are more susceptible to develop *P. jirovecii* pneumonia [67].

In general, multiplex molecular testing increases detection of potential pathogens as compared to traditional methods [68]. This increase in the detection rate is because of the increased sensitivity and specificity of the assays and the fact that it can detect a variety of bacterial, viral, and fungal pathogens as a single test without the involvement of multiple laboratories. These assays can detect pathogens that the physician does not suspect in their clinical setting or on the basis of the clinical findings. There can be true viral-viral coinfections, or bacterial-viral coinfection or one or both may be just colonizer or transient flora, making interpretation of result more difficult and in the determination of the true etiological pathogen [69].

Multiplex molecular assays provide identification of bacterial pathogens, but do not address the issue of antibiotic sensitivity profile of these pathogens. Some of the molecular assays can detect Methicillin-Resistant *Staphylococcus aureus* (MRSA) staphylococcal cassette chromosome *mec* element (SCC*mec*) and right extremity junction (MREJ) region, Carbapenemase and Extended-Spectrum Beta-Lactamases (ESBLs) genes that can be helpful in the treatment decisions. However, in certain situations, and if there is a need of antibiotic susceptibility profile, traditional identification and susceptibility testing for Minimum Inhibitory Concentration (MIC) or procedures may be required.

7. Conclusions

Multiplex molecular assays have been increasingly developed in the recent years and a number of commercial kits are routinely available for the diagnostic microbiology laboratories, which can detect 12 to 33 different pathogens. Molecular methods are easy to perform and offer significantly faster turn-around times and with increased sensitivity and specificity for many respiratory pathogens. Multiplex molecular panels can be appropriately utilized in a syndromic diagnostic approach for high risk immunocompromised, hospitalized, and ER patients. Cost-effectiveness, impact on patient outcome, antimicrobial use, length of hospital stay, and associated healthcare cost need to be determined before implementing multiplex molecular assays. Results of the multiplex NAAT need to be interpreted carefully. Additional testing may be required based on the pathogen involved in the infection, which may include isolation of organisms for antimicrobial susceptibility testing and submission of isolates to the state public health laboratories for further epidemiological studies.

Disclaimer and Statement of Conflict of Interests

The author does not have any possible conflicts of interest. This article reviews commonly available commercial products on which independent studies have been performed and it does not necessarily include all the available products. Furthermore, this article overviews mostly independent studies and does not reflect or endorse manufacturers' data and views.

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