Advances in laboratory assays for detecting human metapneumovirus

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Abstract: Human metapneumovirus (HMPV) is one of the major causes of acute respiratory tract infection (ARI) and shows high morbidity and mortality, particularly in children and immunocompromised patients. Various methods for detecting HMPV have been developed and applied in clinical laboratories. When reviewing the literature, we found that polymerase chain reaction (PCR)-based assays have been most frequently and consistently used to detect HMPV. The most commonly used method was multiplex reverse transcriptase-PCR (RT-PCR; 57.4%), followed by real-time RT-PCR (38.3%). Multiplex RT-PCR became the more popular method in 2011–2019 (69.7%), in contrast to 2001–2009 (28.6%). The advent of multiplex PCR in detecting broader viral pathogens in one run and coinfected viruses influenced the change in user preference. Further, newly developed microarray technologies and ionization mass spectrometry were introduced in 2011–2019. Viral culture (including shell vial assays) and fluorescent immunoassays (with or without culture) were once the mainstays. However, the percentage of studies employing culture and fluorescent immunoassays decreased from 21.4% in 2001–2010 to 15.2% in 2011–2019. Meanwhile, the use of PCR-based methods of HMPV detection increased from 78.6% in 2001–2010 to 84.8% in 2011–2019. The increase in PCR-based methods might have occurred because PCR methods demonstrated better diagnostic performance, shorter hands-on and run times, less hazards to laboratory personnel, and more reliable results than traditional methods. When using these assays, it is important to acquire a comprehensive understanding of the principles, advantages, disadvantages, and precautions for data interpretation. In the future, the combination of nanotechnology and advanced genetic platforms such as next-generation sequencing will benefit patients with HMPV infection by facilitating efficient therapeutic intervention. Analytical and clinical validation are required before using new techniques in clinical laboratories.

Keywords: Metapneumovirus; multiplex polymerase chain reaction (multiplex PCR); respiratory tract infection (ARI); reverse transcriptase-polymerase chain reaction (RT-PCR); virus cultivation

Introduction

Human metapneumovirus (HMPV) is one of the main viral etiological agents of acute respiratory tract infection (ARI), which is a major cause of morbidity and mortality, especially in southeast Asia (1). HMPV was first discovered by genetic analysis of nasopharyngeal aspirate samples taken from 28 hospitalized children in the Netherlands. These samples, taken from children with ARIs during the past 20 years, had symptoms ranging from mild respiratory problems to

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severe cough, bronchiolitis, and pneumonia. Some of them were hospitalized and needed mechanical ventilation (2). Since then, HMPV has been detected in approximately 6.39% of patients hospitalized with ARI (3). The prevalence of HMPV varied from 0% (4) to 36.4% (5), showing high heterogeneity in previous studies. HMPV is commonly found in children, particularly those less than 2 years old (6). HMPV infection has also affected immunocompromised patients such as elderly adults (7) and hematopoietic stem cell-transplant recipients (8). The HMPV outbreaks showed an overall fatality rate of 11% (9).

HMPV is an enveloped virus that is comprised of a negative-sense, single-stranded RNA genome. The HMPV genome harbors 8 genes that encode for 9 proteins. The order of these genes in the genome is *N-P-M-F-M2-SH-G-L*. The encoded proteins include the nucleoprotein (N protein), phosphoprotein (P protein), matrix protein (M protein), fusion glycoprotein (F protein), putative transcription factor (M2-1 protein), RNA synthesis regulatory factor (M2-2 protein), small hydrophobic glycoprotein (SH protein), attachment glycoprotein (G protein), and viral polymerase (L protein) (10,11). The *F*, *M*, and *L* genes as well as the *N* gene (the most conserved region), have been targeted to detect HMPV by reverse transcriptase-polymerase chain reaction (RT-PCR) (12,13). In the past few decades, several methods have been developed and established for detecting HMPV (6). Traditional diagnostic techniques (such as virus culture) and immunoassays have been used for pathogen detection. Recently, the advent of newly developed assays utilizing melting-curve analysis, and nucleic acid-amplification technologies has enabled clinical laboratories to detect HMPV rapidly and precisely. When adopting these assays, the comprehensive understanding of the principles, advantages, disadvantages, and precautions for data interpretation should be well-informed. Articles focusing on laboratory methods for HMPV detection have seldomly been published. Therefore, we reviewed the laboratory assays used for HMPV in the real-world laboratories based on published articles.

RT-PCR

RT-PCR is commonly used to detect HMPV (*Table 1*). This technique involves the reverse transcription of RNA into complementary DNA (cDNA) and the amplification of specific DNA targets using PCR. Simultaneous performance of RT-PCR and real-time PCR, which enables combined nucleic acid amplification and detection in a single step, is routinely used to detect viral RNA (14).

Nucleic acid extraction and purification are basic steps used for most RT-PCR systems. The sample concentration can be measured to improve the clinical sensitivity. These procedures are automated in numerous laboratories for high-quality and reproducible results. The QIAamp viral RNA Mini Kit from Qiagen (15-17), the MagNaPure Compact system from Roche (18,19), and the NucliSENS easyMAG platform from bioMérieux (20,21) are widely used for automated RNA extraction from respiratory swabs or aspirate specimens. Including control material is necessary to validate the extraction and purification steps. Primers and probes enabling amplification and detection of viral targets have been reported (15,22,23). Primers against conserved regions have been preferred for reliable amplification or for identifying sequence variants. However, these primers can cause decreased amplification efficiency when homologous primers incorporate into products. Several HMPV genes such as *P*, *M*, *F*, or *N* have served as targets for RT-PCR. Among them, the *N* gene is most conserved and has shown high sensitivity and reliability for all four genotypes; thus, it has been widely targeted in RT-PCR assays (12,13,24).

The PCR thermal cycling programs consist of three steps (denaturation, annealing, and elongation) and are slightly varied, according to previous reports for HMPV (15,23). Quantification can be performed by RT-PCR as either a one-step or a two-step reaction (25). One of the main differences between these two procedures is the number of used tubes. For one-step RT-PCR, most processes going from the reverse transcriptase reaction to PCR amplification are conducted in a single tube. However, cDNA synthesis and PCR amplification occur in separate tubes in two-step reactions. The advantage of using a one-step protocol is that it minimizes experimental variation. However, using RNA (which is prone to degradation) as the starting templates for one-step reactions makes it difficult to repeatedly assay the same samples over a period of time. In addition, one-step protocols are reported to be less sensitive than two-step protocols (26). Two-step reaction show high reproducibility and it is possible to perform several different PCR assays after diluting a single cDNA sample (27). Although two-step protocols are vulnerable to DNA contamination, they have been the preferred methods when using DNA-binding dyes such as SYBR Green. Primer dimers can be easily eliminated by manipulating the melting temperatures.

Gel-based detection (28), automated fluorescent capillary

Table 1 Laboratory methods and the main characteristics of studies performed to detect HMPV[†]

First author [year]	Clinical presentation (n)	Patient age (years)	Origin of sample	Sample size	Detection method	Equipment or kit (company)	Study country/area
Loeffelholz [2011]	URI	Children; under 1	Nasopharyngeal	192	Multiplex real-time PCR	FilmArray (Idaho Technology, Inc., USA); Prodesse (Gen-Probe, USA)	USA
Chiu [2017]	Influenza-like illness	All ages	Throat swabs	60	Multiplex real-time PCR; viral culture; IFA	FilmArray (BioFire Diagnostics, USA)	Taiwan
Layman [2013]	URI	Unclear	Nasopharyngeal	61	Multiplex real-time PCR; viral culture; DFA	FilmArray (BioFire Diagnostics, USA)	USA
Poritz [2011]	URI	Children	Nasopharyngeal	328	Multiplex real-time PCR; viral culture; DFA	FilmArray (Idaho Technology, Inc., USA); hMPV monoclonal antibody (Diagnostic Hybrids, Greece)	USA
Rand [2011]	Unclear	All ages	Nasopharyngeal; throat; bronchoalveolar lavage; endotracheal; autopsy	200	Multiplex real-time PCR; multiplex RT-PCR; viral culture; DFA	FilmArray (Idaho Technology, Inc., USA); xTAG RVP (Luminex Corporation, Canada)	USA
Babady [2012]	URI and LRI	Children; 0 to 21	Nasopharyngeal; bronchial; throat; sputum	303	Multiplex real-time PCR; multiplex RT-PCR; viral culture; DFA	FilmArray (Idaho Technology, Inc., USA); xTAG RVP (Luminex Corporation, Canada)	USA
Pierce [2012]	Unclear	Children; median 2.24	Nasopharyngeal; tracheal; bronchoalveolar lavage; autopsy	215	Multiplex real-time PCR; real-time PCR	FilmArray (Idaho Technology, Inc., USA); 7500 real-time PCR system (Applied Biosystems, USA)	USA
Popowitch [2013]	URI	All ages	Nasopharyngeal	300	Multiplex real-time PCR; multiplex RT-PCR	FilmArray (BioFire Diagnostics, USA); eSensor RP (GenMark Dx, USA); Luminex xTAG RVPv1 (Luminex Molecular Diagnostics, USA); Luminex xTAG RVP (Luminex Molecular Diagnostics, USA)	USA
Annamalay [2016]	LRI	Children; under 2	Nasopharyngeal	158	Tandem multiplex real-time PCR assay	In-house designed primers, and Australian Genome Research Facility	Mozambique

Table 1 (*continued*)

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Table 1 (*continued*)

First author [year]	Clinical presentation (n)	Patient age (years)	Origin of sample	Sample size	Detection method	Equipment or kit (company)	Study country/area
Annamalay [2016]	Respiratory symptoms	Children; under 10	Nasopharyngeal	277	Tandem multiplex real-time PCR assay	In-house designed primers, and Australian Genome Research Facility	Mozambique
Cohen [2015]	Severe acute respiratory illness	All ages	Nasopharyngeal; throat	963	Multiplex real-time, RT-PCR	In-house	South Africa
Cohen [2015]	Severe acute respiratory illness	All ages over 5	Nasopharyngeal; throat	7,056	Multiplex real-time, RT-PCR	In-house	South Africa
Cohen [2015]	LRI	Children; under 5	Nasopharyngeal	8,393	Multiplex real-time, RT-PCR	In-house	South Africa
Cohen [2015]	Severe acute respiratory illness	All ages	Respiratory specimen	1,376	Multiplex real-time, RT-PCR	In-house	South Africa
Cohen [2016]	LRI	Children; under 6 months	Nasopharyngeal	3,537	Multiplex real-time, RT-PCR	In-house	South Africa
Feikin [2012]	Acute respiratory illness	All ages over 5	Naso- and oropharyngeal	1,039	Quantitative real-time RT-PCR	In-house using AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems, USA)	Kenya
Madhi [2006]	LRI	Children; under 5	Nasopharyngeal	2,715	Nested RT-PCR	In-house	South Africa
Madhi [2007]	LRI	Children; under 5	Nasopharyngeal	2,715	Nested RT-PCR	In-house	South Africa
Moyes [2017]	Severe acute respiratory illness	Adults	Naso- and oropharyngeal	329	Multiplex real-time, RT-PCR	In-house	South Africa
Moyes [2013]	Severe acute respiratory illness	Children; under 5	Nasopharyngeal	1,157	Multiplex real-time, RT-PCR	In-house	South Africa
Nunes [2014]	LRI	Children; under 2	Nasopharyngeal	1,460	Multiplex real-time PCR	In-house using oligo-dT primers (Invitrogen, Life Technologies, USA) and ABI 7500 RT-PCR system (Applied Biosystems, Life Technologies, USA)	South Africa
Peterson [2016]	Severe acute respiratory illness	Children; 0 to 14	Nasopharyngeal	1,505	Multiplex real-time, RT-PCR	In-house	Malawi
Venter [2011]	Acute respiratory infection	Children; under 5	Nasopharyngeal	610	Multiplex real-time, RT-PCR	In-house	South Africa
Zash [2016]	Pneumonia	Children; under 5	Nasopharyngeal	85	Real-time, RT-PCR	Pro hMPV Real Time Assay (Prodesse, Gen-Probe, USA)	Botswana

Table 1 (*continued*)

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First author [year]	Clinical presentation (n)	Patient age (years)	Origin of sample	Sample size	Detection method	Equipment or kit (company)	Study country/area
Brittain-Long [2010]	Acute respiratory infection	Adults	Nasopharyngeal; throat	309	Multiplex real-time, RT-PCR	In-house	Sweden
Jennings [2008]	Pneumonia	Adults	Nasopharyngeal	100	Conventional PCR; viral culture; DFA	In-house	New Zealand
Lieberman [2010]	Pneumonia and LRI	Adults	Naso- and oropharyngeal	834	Real-time, RT-PCR	Multiplex TaqMan Hydrolysis probe-based real-time PCR (Integrated DNA Technology, USA)	Israel
Self [2016]	Pneumonia	All ages	Naso- and oropharyngeal	1,783	Real-time, RT-PCR	In-house	USA
Shih [2015]	Acute respiratory infection	Adults	Nasopharyngeal; throat	267	PCR/electrospray ionization mass spectrometry	PLEX-ID Respiratory Virus assay (Abbott Laboratories, USA)	Taiwan
Tokman [2014]	Pneumonia	All ages	Nasopharyngeal	140	RT-PCR microarray	CLART PneumoVir (Genomica, Spain)	Turkey
Zhan [2014]	Pneumonia; Influenza-like illness	Adults	Throat swabs	251	Real-time, RT-PCR	TaqMan real-time RT-PCR (Guangzhou HuYanSuo Medical Technology Co., China)	China
Jain [2015]	Pneumonia	Adults	Naso- and oropharyngeal	2,320	PCR	In-house (CDC-developed methods)	USA
Dare [2007]	Pneumonia; influenza-like illness	All ages	Nasopharyngeal	1,890	Multiplex real-time, RT-PCR	In-house	Thailand
Falsey [2003]	Acute respiratory infection	All ages	Nasopharyngeal	1,201	Real-time, RT-PCR; viral culture; Enzyme immunoassay	In-house	USA
Falsey [2006]	Respiratory illness	Adults	Nasopharyngeal	304	Multiplex real-time, RT-PCR	In-house	USA
Akhras [2010]	Acute respiratory infection	Children; under 18	Nasopharyngeal	256	Real-time, RT-PCR; viral culture; DFA	NucliSENS real-time analyte specific reagent assay performed on the EasyQ instrument (bioMérieux, USA)	USA

Table 1 (*continued*)

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Table 1 (*continued*)

First author [year]	Clinical presentation (n)	Patient age (years)	Origin of sample	Sample size	Detection method	Equipment or kit (company)	Study country/area
Ali [2010]	Acute respiratory infection	Children; under 5	Nasopharyngeal; throat	728	Multiplex real-time, RT-PCR	In-house, Smart Cycler II (Cepheid, USA) using QuantiTect Probe RT-PCR chemistry (Qiagen, Jordan)	Jordan
Chano [2005]	Acute respiratory infection	Children; under 18	Nasopharyngeal; throat; bronchoalveolar lavage; endotracheal	1,132	RT-PCR	In-house, QIAamp UltraSens Virus Kit coupled with QIAvac purification module 6S, and One-Step RT-PCR Kit (Qiagen, Canada)	Canada
Chen [2010]	LRI	Children; under 18	Nasopharyngeal	6,296	Real-time, RT-PCR	In-house, QIAamp Viral RNA Mini Kit (Qiagen, Germany), PrimeScript RT Reagent Kit (TaKaRa, China), China Premix Ex Taq Kit (TaKaRa), and LightCycler instrument (Roche Diagnostics, USA)	China
Cuevas [2003]	LRI	Children; under 3	Nasopharyngeal	111	RT-PCR	In-house	Brazil
Hombrouck [2012]	Influenza-like illness	Children; under 5	Nasopharyngeal; throat	139	Real-time, RT-PCR	In-house, QIAamp Viral RNA Kit (Qiagen, Germany), real-time RT-PCRs on a Stratagene Mx3000P™ using the SuperScript III Platinum One-Step qRT-PCR System (Invitrogen, USA) or the Brilliant QRT-PCR Core Reagent Kit (Stratagene, Netherlands)	Belgium
Moreno-Valencia [2015]	Acute respiratory infection	Children; under 12	Nasopharyngeal	432	Multiplex RT-PCR	In-house, High-throughput gene expression analysis using 48.48 dynamic array integrated fluidics chips (BioMark platform, USA)	Mexico
Schuster [2015]	Acute respiratory infection	Children; under 2	Nasopharyngeal; throat	3,175	Quantitative RT-PCR	In-house, Step One Plus using the AgPath-ID RT-PCR Kit (Applied Biosystems, USA)	Jordan

Table 1 (*continued*)

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First author [year]	Clinical presentation (n)	Patient age (years)	Origin of sample	Sample size	Detection method	Equipment or kit (company)	Study country/area
von Linstow [2004]	Acute respiratory infection	Children; under 2	Nasopharyngeal	374	Real-time, RT-PCR	In-house, MagNA Pure LC Total Nucleic Acid Isolation Kit, and Light-Cycler instrument (Roche Diagnostics, Germany)	Denmark
Yan [2017]	LRI	Children; under 15	Nasopharyngeal	387	Real-time, RT-PCR	In-house, One Step RT-PCT Kit (Applied Biosystems, USA)	China
Zimmerman [2014]	URI	Children; under 2	Naso- and oropharyngeal	662	Multiplex RT-PCR	eSensor RVP multiplex PCR assay (GenMark Diagnostics, USA)	USA
Tempia [2017]	Influenza like- and severe acute respiratory illness	All ages	Naso- and oropharyngeal	13,335	Multiplex real-time, RT-PCR	In-house	South Africa

†, the included studies were based on meta-analyses recently published in the 3 years. DFA, direct immunofluorescence assay; FilmArray, BioFire FilmArray Respiratory Panel multiplex PCR assay; HMPV, human metapneumovirus; IFA, indirect immunofluorescence assay; LRI, lower respiratory infection; PCR, polymerase chain reaction; Prodesse, Prodesse ProFlu+, ProFAST+, ProParaflu+, Pro hMPV+, and ProAdeno+ real-time PCR assays; RP, respiratory panel; RT, reverse transcriptase; URI, upper respiratory infection.

electrophoresis (29), or hybridization to target specific probes (30) have been used to detect the amplified products. Nested PCR was adopted in several laboratories because of its enhanced sensitivity and specificity (31,32). However, problems with amplicon contamination have occurred even in experienced laboratories (33). As a preferred method in clinical laboratories, real-time PCR assays utilize target-specific probes to detect the amplified products. Among various types of fluorescent probes used for real-time PCR, TaqMan and molecular beacon probes are most widely used. The fluorescent signals generated by TaqMan and molecular beacon probes depend on Förster resonance energy transfer (FRET)-based coupling of the dye molecule and a quencher moiety to the oligonucleotide substrate (34). During PCR amplification, TaqMan probes hybridize to the target sequences and cleave the fluorescent probe. Decoupling of the quencher molecule and the fluorescent probe increases the fluorescence intensity in direct proportion to the number of probes that are cleaved (35). TaqMan probes were frequently used to detect HMPV in previous studies (36-39). Molecular beacon probes also utilize FRET detection. However, they remain intact and rebind to a new target during each PCR cycle. When molecular beacon probes hybridize to a target, the fluorescent dye and the quencher are separated and emit fluorescent light upon excitation (40). Both TaqMan and molecular beacon probes are expensive and time-consuming to synthesize and require separate probes for each RNA target. HMPV detection was previously achieved using the NucliSENS EasyQ instrument (bioMérieux) and molecular beacon probes (3,41). The appropriateness of selecting the real-time amplification format for HMPV detection depends on the number of targets to be tested, laboratory throughput, level of expertise available, and acceptable hands-on or run time. The spectral overlap of fluorophores should be considered, especially for fluorescent real-time PCR assays. Checking the specificity of probe hybridization is recommended due to its usefulness and relative convenience.

Multiplex RT-PCR

In multiplex PCR, multiple primer sets are included within a single PCR tube to produce amplicons specific to different target sequences. The primer pairs should be optimally designed to function at the same annealing temperature. Different amplicons are detected using target-specific probes labeled with different fluorescent dyes (42). This technique enables detection of a broader range

of respiratory viruses in a single test run. Additionally, information pertaining to coinfection can be obtained through multiplex PCR (35,43). A 30% to 50% increase in the detection of respiratory viruses was reported when using multiplex RT-PCR methods compared to conventional methods, such as viral culture or direct fluorescent antibodies (44). Commercial multiplex PCR kits, cleared by the Food and Drug Association (FDA), have become available, although they offer mixtures of pros and cons (45). Among them, the FilmArray system (BioFire Diagnostics, Inc.)—integrating nucleic acid extraction, nested PCR, and data analysis in a disposable pouch—enables HMPV detection. This system is recommended considering its short hands-on and run time, and reagent storage condition (room temperature). Limitations of the FilmArray system (such as the ability to only perform a single test per run) should also be considered when selecting an instrument(s) suitable for a clinical laboratory (45-47).

Introducing multiple primer and probe sets can enable the detection of multiple viral pathogens; however, the potential for reduced amplification efficiency exists. Separating the PCR amplification step from the hybridization and detection steps solve problems associated with diagnostic efficiency and enhances the capacity for detecting a broader array of pathogens. These separate hybridization techniques have become a forerunner of array-based methods. Microarrays comprise collections of microscopic DNA or RNA spots bound to a solid surface, with the potential of analyzing complex amplified PCR products. The microarray substrate can be fabricated from nylon, membrane, glass, silicon, or polystyrene microbeads, based on probe design and hybridization conditions. These microarray-based approaches have proven useful for detecting multiple respiratory viruses, including HMPV. High-throughput gene analysis using a dynamic array of integrated fluidics chips on the BioMark platform (38,48), or the CLART PneumoVir platform from Genomica (49) have been utilized for HMPV detection. Furthermore, liquid-phase, bead-conjugated microarray methods have been applied with commercial Luminex systems for detecting amplified products. When adding new probes to solid-phase microarrays, it is necessary to reformat the procedures and print new arrays, although these steps are not required for suspension microarrays. Therefore, liquid-phase microarray techniques exhibit flexibility in terms of assay design and format, rapid hybridization kinetics, and lower cost (50,51). The Luminex xTAG RVP fast assay is a multiplexed microsphere-based suspension microarray platform that enables the analysis and reporting of 12 respiratory viruses from nasopharyngeal swabs. Several investigators have adopted the commercially available Luminex system for HMPV detection (52-54). The Luminex system showed higher positivity (47.5%) than real-time RT-PCR (42.5%) and immunofluorescence assays (2.7%) (55). However, as previously mentioned, the FilmArray system was reported to have higher sensitivity than the Luminex xTAG RVP fast assay and shorter a run time (1 hour) and hands-on time (2 minutes) (45,52). Additional enzymatic amplification steps for viral RNA prior to hybridization are required for microarray-based methods. Moreover, detection requires labeling of multiple probes or the incorporation of fluorescent dye- or biotin-conjugated nucleotides into cDNA. In addition, the sensitivities of most conventional microarrays have depended on the efficiency of target amplification and probe hybridization. The multiple steps required for these microarrays make them complex, expensive, labor-intensive, and vulnerable to contamination. These array systems are also prone to false-negative results because of the gene mutations, PCR inhibitors, and degradation of RNA products. Further investigations are required to optimize the design of multiple, virus-specific primers and assay systems.

The detection methods used and their main characteristics observed in previous HMPV studies are presented in *Table 1*. The included studies were mainly based on four HMPV meta-analyses recently published in 3 years [2017–2019] (45,56-58). Forty-seven studies are included to reflect methods used in clinical laboratories. The countries/areas included in this study were Belgium (59), Botswana (60), Brazil (61), Canada (22), China (23,39,62), Denmark (38), Israel (37), Jordan (19,63), Kenya (36), Malawi (64), Mexico (48), Mozambique (65,66), New Zealand (67), South Africa (18,31,32,68-76), Sweden (77), Taiwan (17,78), Thailand (15), Turkey (49), and USA (3,7,16,20,21,52-54,79-83). The most frequently used method was multiplex PCR (57.4%), followed by real-time RT-PCR (38.3%) (*Figure 1*). Commercially available products from diverse companies, such as the One-Step RT-PCR Kit (Applied Biosystems or Qiagen), TaqMan real-time PCR kits, NucliSENS EasyQ instrument (bioMérieux), and Pro hMPV Real Time assay (Gen-Probe) were used to detect HMPV. The differences in the assays used to detect HMPV according to previous publications are illustrated in *Figure 2*. Multiplex PCR (69.7%) became the most commonly used method in 2011–2019, in contrast to 2001–2009. The advent of multiplex PCR for detecting multiple
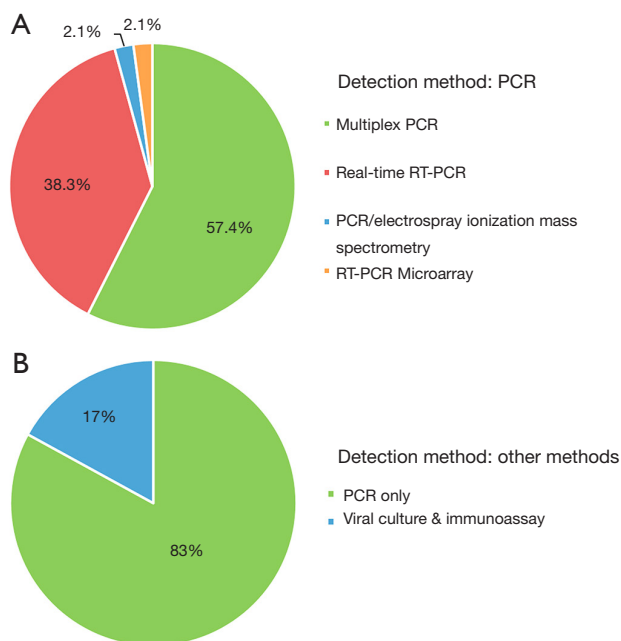
Figure 1 Applied laboratory assays for detecting human metapneumovirus. (A) Pie charts showing the use of PCR-based assays. (B) Pie charts showing the use of viral culture and immunoassays. RT-PCR, reverse transcriptase polymerase chain reaction.

pathogens (including coinfecting viruses) in a single run influenced these changes in user preferences.

Viral culture

The isolation of viruses from culture is considered a gold standard for diagnosing infection (52,64). After inoculating a permissive cell line with an infectious sample, such as a nasopharyngeal swab or aspirate, the specimen is incubated for 7 to 10 days to observe the development of a cytopathic effect (CPE). CPE refers to structural changes in cells caused by viral invasion. Established cell lines, such as Madin-Darby canine kidney cells, A549 cells, mink lung epithelial cell lines, human lung diploid fibroblast cells (MRC-5 and WI-38), HeLa cells, rhesus monkey kidney cells (LLC-MK2), and buffalo green monkey kidney cells are utilized for isolating diverse viruses, including influenza virus and respiratory syncytial virus (52,64,84). Regarding HMPV, various cell lines, such as the Vero (85), HEp-2, Hep G2 (86), 293 (87), and LLC-MK2 (2,7,52) cell lines have been used to isolate the virus. Among them, a human Chang conjunctiva cell line (clone 1-5C4) and a

feline kidney CRFK cell line were suggested as the most suitable cells for HMPV isolation, based on a previous study of 19 different cell lines used to grow HMPV (88). HMPV culture in LLC-MK2 cell lines has been frequently performed in clinical laboratories, based on previous reports (7,52). The tolerance of LLC-MK2 cells to trypsin make it an ideal cell line for HMPV cultivation (85).

Traditional viral culture has been generally replaced by shell vial culture. This technique involves the inoculation of samples onto a cell monolayer in shell vials, followed by centrifugation and further incubation. After 24 to 48 hours, the HMPV antigens are measured with virus-specific antibodies. Shell vial culture enables rapid detection of slow-growing viruses, including HMPV. Moreover, this method is relatively straightforward and more sensitive than traditional viral culture, due to the centrifugation steps (89). In addition, a modified shell vial culture method has been introduced. This technique, utilizing R-Mix or R-Mix Too cells from Diagnostic Hybrids (a mixture of mink lung cells and human adenocarcinoma cells), has shown higher sensitivity than the shell vial culture method with a run time of 1.4 days (80,84).

HMPV grows so slowly that it shows late CPEs varying from cell rounding and detachment from the culture matrix to the formation of small syncytia (i.e., large cytoplasmic masses that contain many nuclei). Therefore, immunoassays such as direct fluorescent assays (DFAs) and enzyme-linked immunosorbent assays (ELISAs) are frequently utilized along with cell culture to detect HMPV antigens (85).

Florescent immunoassay

Among the various florescent immunoassays, DFA testing directly determines the presence of specific antigens with fluorescently labeled antibodies and has been widely employed to detect viruses in clinical laboratories. This method involves the direct staining of respiratory epithelial cells derived from nasopharyngeal swabs or aspirates with fluorescently labeled, virus-specific antibodies (82,90). DFA methods coupled with viral culture or shell vial culture are also widely used for enhanced sensitivity and specificity (80). Data interpretation using immunofluorescence microscopy is necessary. Determination of the correct absorption wavelength is required to excite the fluorophore tag bound to the antibody and detect the released fluorescence, where positive cells are indicated by the presence of the targeted virus. DFAs have been used for several decades due to their simplicity and short turnaround
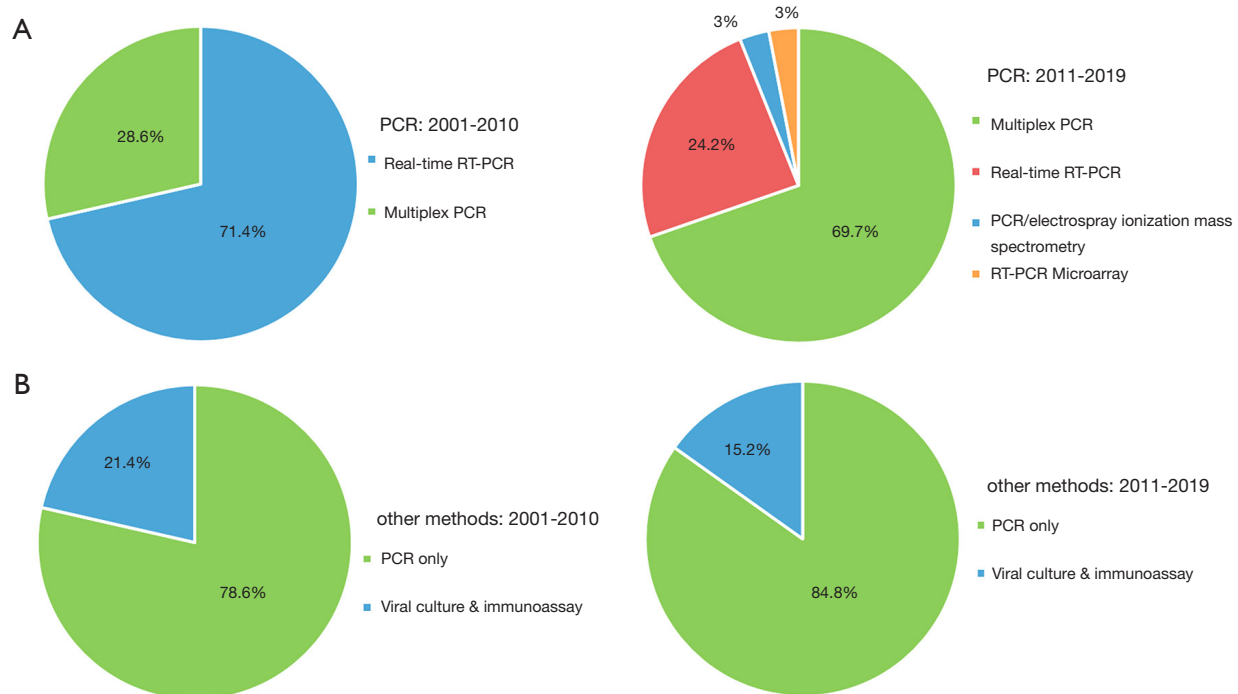
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Figure 2 Changes in the application of laboratory assays for detecting human metapneumovirus over time. (A) Pie charts showing changes in the use of PCR-based assays. (B) Pie charts showing changes in the use of viral culture and immunoassays. RT-PCR, reverse transcriptase polymerase chain reaction.

time. A modified cytospin-enhanced DFA has also been used to enhance the sensitivity in terms of virus detection. Cytospinning decreases inadequate smears and improves cell morphology, resulting in enhanced performance. Data from a previous study showed that 85.4% (41/48) of samples were positive by cytospin-enhanced DFA, compared to TaqMan RT-PCR (91). Among commercially available DFA kits approved by the FDA, the D³ DFA test (Diagnostic Hybrids) has been widely used (44,80). This kit enables the identification of HMPV in less than 25 minutes after sample receipt and shows high sensitivity (95.2%) and specificity (100%) compared to RT-PCR (92). When compared to shell vial culture, DFA showed comparable results (93.3%) based on the number of positive samples determined by RT-PCR (93). In another study, comparing viral culture with DFA showed a 92% sensitivity and a 90% specificity for HMPV (80). However, no recent studies have involved HMPV detection without molecular techniques because of the shorter turnaround times and higher sensitivity of molecular tools.

The assays used and their main characteristics in studies involving viral culture and fluorescent immunoassays are shown in *Table 1*. Among 47 included studies, 8 studies (17.0%) involved viral culture and fluorescent immunoassays, whereas the remaining 39 studies (83.0%) employed only PCR-based methods for HMPV detection (*Figure 1*). LLC-MK2 cells (Diagnostic Hybrids) have been frequently used for HMPV culture in several clinical laboratories (7,52). The usefulness of trypsin-tolerant LLC-MK2 cells for HMPV cultivation has influenced these trends (85). In terms of DFAs, the FDA-approved D³ DFA kit from Diagnostic Hybrids have been widely used (44,80). The short turnaround time (less than 25 minutes) and high performance compared to RT-PCR make it commonly utilized. Changes over time in the use of culture and fluorescent immunoassays for detecting HMPV are presented in *Figure 2*. The combined proportions of culture-based and fluorescent immunoassays decreased from 21.4% in 2001–2010 to 15.2% in 2011–2019. Meanwhile, PCR-based methods showed an increasing trend (78.6% in 2001–2010 versus 84.8% in 2011–2019). These changes might have occurred because PCR methods such as RT-

PCR and multiplex PCR demonstrated better diagnostic performances (45). In a previous study, the sensitivity and specificity of viral culture were reported to be 68% and 99%, respectively, as compared to real-time RT-PCR detection of HMPV (94). In addition, the shorter run- and hands-on times, and more reliable results than traditional methods make PCR techniques preferable tools in many clinical laboratories (45).

Other methods

Among traditional methods for detecting HMPV, serological assays including virus-neutralization assays (95-97) and enzyme immunoassays (7) were once the main techniques. Virus-neutralization assays are used to detect virus-specific antibodies induced by viral infection. Virus-specific antibodies are produced by the immune system to neutralize viruses. The titer can be measured and the highest serum dilution at which virus infection is blocked is considered as the virus-neutralization titer. When performing virus-neutralization assays, the hazards due to the use of infectious viruses should considered. Several studies have been performed to develop better neutralization assays for HMPV detection (95-97). Plaque-reduction, virus-neutralization assays showed faster turnaround times and better sensitivities than conventional methods requiring around 7 days for completion (95). Micro-neutralization assays for measuring antibody titers (96) and neutralization assay based on recombinant HMPV expressing Renilla luciferase (97) also enabled efficient HMPV detection.

In terms of enzyme immunoassays, enzymes are conjugated to secondary (detection) antibodies, which bind to the primary viral antigen-antibody complex. When the appropriate substrate is added and incubated, the enzyme catalyzes the production of a colored end-product, which can be visualized and quantified. The most frequently used enzymes are alkaline phosphatase, horseradish peroxidase, and β-galactosidase (98). It is necessary to distinguish specific HMPV antigens from nonspecific complexes, for most commercially available systems. These systems are known as ELISA or solid-phase immunosorbent assays. Separation is feasible through binding of the antigen or capturing the antibody on a solid material, such as a microtiter plate or paper strip. Lysates from representative HMPV strains (7) or the carboxy-terminal domain of the N protein of HMPV (99) can be used for antigen preparation. Although ELISA-based tests have been developed with rapid turnaround times (100)

and better diagnostic performance than traditional methods, PCR-based assays offering higher sensitivity have become preferred techniques in clinical laboratories.

In terms of modified PCR-based methods, the PCR-electrospray ionization mass spectrometry (ESI-MS) approach provided by the PLEX-ID system (Abbott Laboratories) was applied to detect HMPV. After RT-PCR, automated post-PCR desalting, ESI-MS signal acquisition, spectral analysis, and data reporting can be performed on a biosensor platform (Ibis Biosciences, USA). Virus identification was based on the MS data and base compositions of the PCR amplicons when compared to those in the molecular database established by the PLEX-ID manufacturer (17,101). Accurate identification based on both MS data and base compositions of PCR amplicons are an advantage of the ESI-MS technique. However, the process involves a longer turnaround time from sample to result (within 6 to 8 hours) and is more laborious than currently available multiplex RT-PCR systems (45). Regarding advanced multiplex RT-PCR methods, microarrays using nanoparticles coupled with silver staining have been adopted and served as tools for detecting viruses. This nanoparticle-based microarray offers better sensitivity than fluorescent dyes commonly utilized in most microarrays. When combining with next-generation sequencing (NGS) technology, this assay can be suitable for screening and identifying a broader range of viruses with shorter run- and hands-on times, due to the simplified procedures (102). This approach has been investigated mainly for influenza viruses and further studies with HMPV are necessary.

NGS has provided a quantum leap in the field of molecular diagnostics. NGS offers significant improvements in sequencing speed and throughput due to automated streamline workflow and reduces the cost of sequencing. Several manufacturer-specific platforms such as the Roche 454 system and the Illumina MiSeq and HiSeq systems (which use different sequencing strategies, reagents, and bioinformatics software) have been developed. Among them, Illumina's NGS platforms (employing a sequencing-by-synthesis approach) have been used to analyze small non-coding RNAs of HMPV (103) and HMPV viral metagenomics (104).

Conclusions and future perspectives

In conclusion, we reviewed applied laboratory methodologies for detecting HMPV, one of the major causes of morbidity

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and mortality derived from ARI. In clinical laboratories, the main methods used for HMPV detection are PCR-based assays including conventional RT-PCR, multiplex RT-PCR, and microarray-based approaches. Viral culture such as shell vial assays and fluorescent immunoassays (combined with or without culture) were once the major methods for detecting HMPV. Comparison of the assays using the wide range of available data showed that PCR-based methods have become the more appreciated methods. Although viral culture and immunoassays have remained in use as reference methods due to their high specificity, PCR-based methods offer shorter run- and hands-on times, higher sensitivity, a broader range of available viral targets, a lower risk of exposure to virus, and economic feasibility, which could have influenced the changes in use. When using these assays, it is important to have a comprehensive understanding of the associated principles, advantages, disadvantages, and precautions for data interpretation.

Sophisticated techniques for detecting HMPV including nanotechnology and biosensors, with micro-sized instruments, ultra-sensitive and specific detectors, faster turnaround times, and lowered costs will be developed in the future. Furthermore, rapid and accurate diagnosis based on the advent of newly developed methods for differentiating subtypes and emerging viral variants resistant to currently used therapies will greatly benefit patients with HMPV infections. In addition, the genetic profiling of putative virulence factors and antiviral resistance markers using molecular tools such as NGS might improve patient prognosis and increase the accuracy in predicting responses to therapeutic intervention. Analytical and clinical validation should be conducted before using each new technique in clinical laboratories.

Acknowledgments

Footnote

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