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Comparison of two commercial molecular assays for simultaneous detection of respiratory viruses in clinical samples using two automatic electrophoresis detection systems

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A B S T R A C T

Two molecular assays were compared with real-time RT-PCR and viral culture for simultaneous detection of common viruses from respiratory samples: a multiplex ligation-dependant probe amplification (MLPA) and a dual priming oligonucleotide system (DPO). In addition, the positive detections of MLPA and DPO were identified using two different automatic electrophoresis systems. A panel of 168 culture-positive and negative samples was tested by the molecular assays for the presence of influenza A and B virus, respiratory syncytial virus, human metapneumovirus, rhinovirus, coronaviruses, parainfluenza viruses and adenovirus.

One hundred and twenty-nine (77%) samples were positive as detected by at least one method. Sixty-nine (41%) samples were positive by cell culture (excluding human metapneumovirus and coronaviruses), 116 (69%) by RT-PCR, 127 (76%) by MLPA and 100 (60%) by DPO. The MLPA yielded results in one attempt for all samples included while 12 (7.2%) samples had to be repeated by the DPO assay due to inconclusive results. The MLPA assay performed well in combination with either electrophoresis system, while the performance of the DPO assay was influenced by the electrophoresis systems.

Both molecular assays are comparable with real-time RT-PCR, more sensitive than viral culture and can detect dual infections easily. Results can be obtained within 1 day.

1. Introduction

Pneumonia is a serious illness with significant morbidity and mortality rates. A wide variety of bacteria and viruses is held responsible for causing pneumonias, in children as well as in adults, although in about 20–50% of patients the etiology is not established (Johnstone et al., 2008; Michelow et al., 2004). Since signs and symptoms at presentation rarely point to a specific pathogen, it is usual to start empirical therapy aimed at bacterial pathogens. Considering the frequency of viral etiology, antibacterial therapy is often employed inadequately and unnecessarily.

In comparison with conventional detection techniques, multiplex real-time PCR has been shown to be more sensitive and specific, yielding results within 6 h (Bonzel et al., 2008; Templeton et al., 2004) and enabling direct detection of viruses that are difficult to culture (Falsey et al., 2006; Fouchier et al., 2004; Mahony, 2008). Consequently, the results of nucleic acid amplification tests may contribute to timely treatment decisions.

In this study, two commercial molecular assays, both designed for simultaneous detection of the most common viruses from a variety of respiratory samples, were compared with real-time RT-PCR and viral culture: a multiplex ligation-dependant probe amplification (MLPA) and a dual priming oligonucleotide system (DPO). The MLPA technique employs two probes which ligate in the presence of target-specific complementary sequences. The probes consist of a target-specific sequence, common primer sequences and a stretch of nucleotides that allow specific detection based on length differences (Reijans et al., 2008). The DPO technique employs target-specific primers of double the normal oligo length which contain a polydeoxy-inosine linker to gain specificity and sensitivity for the multiplex detection (Drews et al., 2008). In addition, positive detections by the MLPA and DPO assays were identified by two different automatic capillary electrophoresis detection systems.

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Table 1
Detection targets in assays.

Virus	Seeplex RV detection kit		Respifinder		Real-time RT-PCR
	Target gene	Size	Target gene	Size	Target gene
Influenza A	Segment 7	351 bp	Matrixprotein 1	440 bp	Matrixprotein 1
Influenza B	Segment 1	?	Matrixprotein 1	418 bp	Hemaglutinin
RSV A	Fusion protein	273 bp	Major nucleocapsid	271 bp	Major nucleocapsid
RSV B	Fusion protein	391 bp	Major nucleocapsid	247 bp	Major nucleocapsid
Parainfluenza 1	Hemagglutinin-neuramidase	324 bp	Hemagglutinin-neuramidase	298–325 bp cluster	Fusion glycoprotein
Parainfluenza 2	Hemagglutinin-neuramidase	264 bp	Hemagglutinin-neuramidase		Hemagglutinin-neuramidase
Parainfluenza 3	Hemagglutinin-neuramidase	219 bp	Hemagglutinin-neuramidase		Hemagglutinin-neuramidase
Parainfluenza 4	–	–	Major nucleocapsid		Hemagglutinin-neuramidase
Corona 229e	Spike protein	375 bp	Nucleocapsid	163	Nucleocapsid
Corona OC43	Membrane protein gene	231 bp	Nucleocapsid	176	Nucleocapsid
Corona HKU1	Membrane protein gene	231 bp	–	–	–
Corona NL63	Spike protein	375 bp	Nucleocapsid	202	Nucleocapsid
Rhinovirus	5′-Untranslated region	337 bp	5′-Untranslated region	479	5′-Untranslated region
hMPV	Fusion protein	469 bp	Nucleocapsid	364	Nucleocapsid
Adenovirus	E2B DNA polymerase	534 bp	Hexon	350	Hexon

2. Materials and methods

2.1. Samples and strains

Prior to the validation using patient samples, a pilot study was carried out using dilutions of virus strains to confirm all specific detections included in the molecular assays (except human corona HKU1 virus). The viral targets included in the molecular assays are listed in Table 1.

The validation panel consisted of viral cell culture-positive and culture-negative respiratory samples. Eighty-seven samples with positive culture detections for one of the viral targets (or positive RT-PCR results for coronavirus or hMPV) were submitted and 86 culture-negative samples were matched based on sample type and age of the patients (range 1 week to 90 years old, median age 8 months). The samples were collected from several different years (2000–2008) to ensure some strain variability. This panel of respiratory samples, consisting of bronchioalveolar lavages ($n = 20$), sputa ($n = 7$) nasopharyngeal lavages ($n = 60$), nasopharyngeal ($n = 40$) and throat ($n = 46$) swabs, was stored at −80 °C prior to nucleic acid extraction. The samples were submitted to the molecular assays without knowledge of the cell culture results that had been obtained prior to storage at −80 °C.

2.2. Viral culture

Respiratory samples were cultured at 33 °C using four different cell lines on coverslips: HEL (human embryonic lung fibroblast), Vero (African green monkey), Hep-2 (larynxcarcinoma) and LLC-MK2 (rhesus monkey kidney) using minimum essential medium (MEM) with 2 mM L-glutamine and Earle's BSS adjusted to 0.85 g/L sodium bicarbonate, 2% fetal bovine serum and addition of penicillin (100,000 U/L), streptomycin (100,000 μg/L) and amphotericin B (0.5 mg/L). All cultures were examined daily for cytopathic effect (CPE). In addition, all cultures were examined on days 2 and 7 using immunofluorescent (IF) antibodies against common respiratory pathogens including RSV, adenovirus, influenza virus A and B, and parainfluenza virus 1, 2 and 3 (D3 DFA Respiratory Virus Screening/ID kit, ITK Diagnostics BV, Uithoorn, The Netherlands).

2.3. Sensitivity testing

Dilution series of cultured RSV and influenza virus B were submitted to MLPA, DPO and real-time RT-PCR and a $TCID_{50}$ experiment in Hep-2 cells for RSV and LLC-MK2 cells for influenza virus to determine the 50% infective dose for cell culture. Each dilution was prepared in maintenance culture medium and 100 μL of these dilutions was used in all culture wells as well as in nucleic acid extraction. Eight dilution series per virus were used. Positivity was checked using IF and CPE after 3 and 7 days.

2.4. Nucleic acid extraction

Extraction of total nucleic acids was performed with the Nuclisens EasyMAG (BioMérieux, Boxtel, The Netherlands). The protocol used was Specific A with "on board" lysis and 50 μL of silica mix. A mix of 100 μL sample with 100 μL saline (0.9%) was used in the extraction. The MLPA protocol required an internal control which was added after lysis to the extraction mixture.

Nucleic acid extracts were eluted in 110 μL elution buffer and stored at -20 °C before molecular testing.

2.5. MLPA

The MLPA (Respifinder DC TwoStep kit, Pathofinder, Maastricht, The Netherlands) was conducted according to the manufacturers' protocol using an ABI 2720 thermocycler (Applied Biosystems, Foster City, CA, USA). In short: 10 μL nucleic acid extract was added to 15 μL pre-amplification mix. Second, a hybridisation step was carried out with 2 μL of diluted pre-amplification product and 6 μL fresh hybridisation reaction mix. Ligation and final PCR were carried out simultaneously in a third reaction mix consisting of 32 μL mix and 8 μL hybridisation product. PCR products were stored at −20 °C until electrophoresis. The total MLPA protocol including extraction was performed in approximately 8 h.

2.6. DPO

The DPO assay (Seeplex RV12 detection kit, Seegene, Rockville MD, USA) was conducted according to the manufacturers' protocol using an ABI 2720 thermocycler. In short: a reverse transcription step was carried out with 8 μL RT reaction mix after an initial denaturation step with 4 μL reaction mix 1 and 8 μL nucleic acid extract. The following PCR was carried out in two reaction mixes consisting of either 17 μL of RV mix A or B and 3 μL cDNA. After PCR, the product was stored at −20 °C until electrophoresis. The total DPO protocol including extraction was completed in approximately 7 h.

2.7. Real-time RT-PCR

All samples included in the validation panel were submitted to two-step real-time RT-PCR. RT-PCR was carried out in sim-

Table 2
Previously unpublished oligos for RT-PCR.

Target virus	Name	Sequence 5′–3′
Parainfluenza 1 virus	GR5504	CGGCATTGAAACTAGGGATCA
Parainfluenza 1 virus	GR5505	GGAGCTGAATGCTGTTGCTAATT
Parainfluenza 1 virus	GR5506	6FAM-TCACCCAACACTACTC-MGBNFQ
Parainfluenza 2 virus	GR5507	TGAAAACCATTTACCTAAGTGATGGA
Parainfluenza 2 virus	GR5508	TCCCGGTATAGCAGTGACTGAAC
Parainfluenza 2 virus	GR5509	6FAM-TCAATCGCAAAAGC-MGBNFQ
Parainfluenza 3 virus	GR5510	GAACATCCAATAAATGAGAATGTAATCTG
Parainfluenza 3 virus	GR5511	CCTATCTGAAAACCATGGACTATGAG
Parainfluenza 3 virus	GR5512	6FAM-CCCGGGAAAACACA-MGBNFQ
Parainfluenza 4A virus	GR5513	CCATCAAAAGTAAGTCTCAGGAGTTTAA
Parainfluenza 4A virus	GR5514	TGGGTCTTGCTAATGAGTCAAGTG
Parainfluenza 4B virus	GR5515	GCACTGGCGATGTCTCAAAA
Parainfluenza 4B virus	GR5516	GGTCTTGCTAACGGATCAAGTGT
Parainfluenza 4 virus	GR5516	6FAM-TTGTTGATCAAGACAATACA-MGBNFQ
Rhinovirus	GR5001	GCCTGCGTGGCTGCC
Rhinovirus	GR5002	CCTGCGTGGCGGCC
Rhinovirus	GR5003	ACGGACACCCAAAGTAGTTGGT
Rhinovirus	GR5004	ACGGACACCCAAAGTAGTCGGT
Rhinovirus	GR5005	6FAM-TCCGGCCCCTGAATGCGGCTAA-TAMRA

plex reactions, using the ABI 7500 system (Applied Biosystems) with standard amplification protocol and reagents (ABI Universal mastermix). The oligonucleotides used for RT-PCR were published previously (human coronavirus NL63: Fouchier et al., 2004; human coronavirus OC43 and 229E: van Elden et al., 2004; influenza A and B virus and RSV A and B: Templeton et al., 2004; hMPV: adapted from Maertzdorf et al., 2004; adenovirus: Heim et al., 2003). The oligonucleotides for the RT-PCR detection of parainfluenza virus 1–4 and rhinovirus were developed by the Laboratory for Infectious Diseases, Groningen, and are listed in Table 2.

2.8. Virus detection and identification

All reactions were analysed using two microcapillary electrophoresis systems: the Agilent 2100 (Agilent Technologies, Santa Clara, CA, USA) and the Experion (Bio-rad Laboratories, Hercules CA, USA), as prescribed by the respective manufacturers. Input of both systems was 1 μL amplicon. The results were examined by two different technicians to exclude interpretation differences. The identifications obtained in the Agilent 2100 were used in the calculations.

2.9. Calculations and definition of true positive

Defined as true positives were samples that yielded positive viral detections by more than one method (culture, DPO, MLPA or real-time RT-PCR). Samples that remained negative in all methods were considered true negative. Positive results obtained by only one method were considered unconfirmed or false-positive. All calculations were performed using the Microsoft Excel version 2002.

3. Results

3.1. Analytical sensitivity and specificity

The $TCID_{50}$ cut-off of the RSV culture was determined at dilution $10^{6.5}$ in both IF and CPE, the $TCID_{50}$ of influenza virus type B was $10^{5.8}$ in IF and $10^{4.5}$ in CPE. The highest dilution that yielded a specific reaction of RSV in both the MLPA and the DPO was 10^5. The highest dilution that yielded a specific reaction of influenza virus in the MLPA assay was 10^7 and in the DPO assay 10^3. The results of the simplex real-time RT-PCR assays were identical to those of the MLPA. The actual input in the molecular assays is 10-fold lower than in culture because of the extraction protocol. These results show that the analytical sensitivity for the RSV detection is slightly greater for culture compared to the molecular methods. The greatest sensitivity for influenza virus was obtained by the MLPA and RT-PCR and the lowest by the DPO assay.

The specific detections included in the two molecular assays were validated using nucleic acid extracts from cultured virus strains. All virus strains were identified successfully and specificity was confirmed.

3.2. Results clinical samples

Of the total collection of 173 samples that were used in the validation panel, five samples were excluded. One sample was inhibited repeatedly in the MLPA and of four samples an insufficient volume was left to perform all tests. A total of 168 samples were included in the comparison: 129 (77%) samples were positive for one or two viral targets, including unconfirmed (false) detections. Sixty-nine (41%) samples were cell culture-positive, 116 (69%) samples were RT-PCR positive, 127 (76%) samples yielded positive MLPA results and 100 (60%) samples were found positive by the DPO. In Table 3, the performance per method and detection rate are summarised. In Table 4, the virus specific detections have been summarised per method. Both tables show the number of detections which exceeds the amount of positive samples due to mixed infections.

3.3. Detection system

The MLPA results obtained with both detection systems were identical. All virus detections, except for the inhibited samples, could be identified in a single attempt (with no interpretation differences between technicians). One sample was inhibited repeatedly in the MLPA and therefore excluded. For the DPO, the Agilent 2100 performed better than the Experion: The Agilent showed a clearer difference between background signals and specific signals. The Agilent enabled clear interpretation for 167 (of 168) samples in one attempt while the Experion yielded clear interpretation for only 157 (of 168) samples. Of the repeated electrophoresis results for six samples (3.6%: one with the Agilent, five with the Experion), the two technicians disagreed on the interpretation and therefore the complete DPO protocol was repeated. For six other samples the Experion run hampered and for these only the electrophoresis run was repeated. Additionally, six (3.6%) of

Table 3
Performance per technique (combined with Agilent detection). Between square brackets [+] are additional possible positives (unconfirmed or false-positive). A total of 168 samples were included in the comparison.

Characteristic	MLPA	DPO	RT-PCR	Viral culture	True positives	All positives
Positive detections of viral target	140 [+13]	115 [+3]	137	69 [+2]	142	160
Mixed infect	22 [+4]	15 [+3]	21	1	24	31
Inhibited samples in first run	3	6				
Inhibited after repeat (excluded)	1	0				

the samples were inhibited using the DPO and three (1.8%) using the MLPA. The calculated amplicon sizes differed slightly between the Agilent and the Experion electrophoresis systems. The amplicon sizes were calculated based on the markers included with the kit reagents. Because of the greater performance of the Agilent, all virus detections of the MLPA and DPO, as mentioned in the results, were based on the combination with the Agilent electrophoresis.

3.4. Discrepancy analysis

All 168 samples were tested by the simplex real-time RT-PCRs to confirm the results from the comparison. Of 18 positive viral detections that were found only by one method (those unconfirmed or false detections are listed in brackets in Table 4), a specific RT-PCR was repeated. Seven of 18 detections could be confirmed. These included six MLPA detections (three rhinovirus, two coronavirus and one adenovirus detection) and one DPO detection (a rhinovirus detection). All seven had high threshold cycle (Ct) values: ranging between Ct 36.65 and Ct 38.40.

3.5. Dual infections

Of 31 mixed infections found initially, 24 were detected by more than one method. MLPA detected 26 dual infections of which 22 were confirmed, RT-PCR detected 21 dual infections, viral cell culture detected one dual infection and DPO detected 18 dual infections of which 15 were confirmed (Table 3). Of the 62 virus detections involved in mixed infections, the distribution of viruses is shown in Table 4.

4. Discussion

The performance of two commercial molecular detection assays, the MLPA and the DPO, was compared using a panel of respiratory samples. Both molecular assays yielded comparable sensitivities to that of RT-PCR and significantly greater sensitivities in respiratory specimens than viral culture (excluding hMPV and coronaviruses for which cell culture was not undertaken), which is in accordance with earlier publications (Kim et al., 2009; Reijans et al., 2008; Yoo et al., 2007). The MLPA assay appeared to have a greater sensitivity for all viral targets compared to that of the DPO and similar sensitivity compared to that of real-time RT-PCR. The overall specificity of the DPO assay appeared greater compared to that of MLPA. However, the sensitivity of the MLPA decreases the specificity due to a number of false-positives. It might well be that these so-called false-positive detections were in fact accurate detections as shown by the seven additional detections that were confirmed by RT-PCR during discrepancy testing. The low viral load in these samples was confirmed by the high Ct values that were obtained with the repeated extraction and RT-PCRs.

Comparison of the analytical sensitivity with the sensitivity obtained in the respiratory samples for influenza virus type B showed similar differences between the MLPA and the DPO assays. The differences in sensitivity for RSV were less obvious, but the greater detection rate of the molecular assays can be explained by the greater potential to detect co-infections. These two viruses were chosen for the $TCID_{50}$ sensitivity comparison because of the difference in sensitivity observed in the validation panel. The sensitivity of the DPO assay might be enhanced by lowering the amount of internal control (IC), because the strength of the IC signal compared to the specific target signals might be due to competitive amplification.

The type-specific viral detections per molecular assay (Table 1) could all be differentiated except for parainfluenza virus types 1–4, which are detected as a cluster by the MLPA and coronavirus OC43/HKU1 and 229E/NL63 which are not further differentiated by the DPO assay. In the sample panel used here coronavirus HKU1 was not present because all OC43/HKU1 DPO detections were identified as OC43 by the MLPA. At least one parainfluenza 4 was detected by the MLPA and identified as such by RT-PCR.

In two cases, virus was isolated by culture (influenza B virus and parainfluenza 3) but were not detected by the molecular assays. These viruses were found in patients with dual infections, parainfluenza 3 combined with enterovirus in a neonate and influenza B virus and herpes simplex 1 virus in bronchoalveolar fluid in a 74-year-old patient, and were only found by specific IF tests on cover slips. The amount of virus in the original clinical sample was probably very low and duration of storage or handling might have influenced the quality of the sample, although a laboratory contamination might be a plausible explanation as well.

The molecular assays are comparable in several aspects: the multiplex format, target identification based on length differences of the amplicons and the most common respiratory viruses are included. However, the DPO assay is faster and easier to perform than the MLPA, which consists of more hands-on steps. On the other hand, the MLPA has several advantages over the DPO. First, it con-

Table 4
Positive detections per target organism. Between square brackets [+] are additional possible positives (unconfirmed or false-positive). In the last column "detected as mixed infection", all positive detections were considered and therefore the percentages were calculated versus "All positives". A total of 168 samples were included in the comparison.

Target virus	MLPA	DPO	RT-PCR	Viral culture	True positives	All positives	Detected as mixed infection
Influenza A virus	11	11 [+1]	11	11	11	12	1 (8.3%)
Influenza B virus	11	6	11	8 [+1]	11	12	3 (25%)
RSV A/B	19	15	19	12	19	19	11 (58%)
Adenovirus	15 [+3]	13	13	10	15	18	7 (39%)
Parainfluenza 1–4 virus	24 [+2]	22	23	22 [+1]	24	27	7 (26%)
Rhinovirus	36 [+6]	30 [+2]	37	6	38	46	21 (46%)
Coronavirus	10 [+2]	7	10	–	10	12	9 (75%)
hMPV	14	11	13	–	14	14	3 (21%)

tains an internal control added prior to extraction and therefore controls the extraction as well as inhibition, while that of the DPO is added after the extraction. Second, the difference between the performances of the electrophoresis systems was only apparent for the DPO assay for which 11 samples had to be repeated in the Experion run (inhibited samples not taken into account). Overall, peaks were sharper and seemed more pronounced by the Experion compared to those obtained by the Agilent. However, this resulted in false-positive and/or false target identifications due to background signals. As the MLPA showed virtually no background, this problem was not encountered by the MLPA and the results of the two electrophoresis systems were identical for the MLPA assay.

Both assays are unable to produce quantitative results whereas real-time RT-PCR can estimate semi-quantitative results per sample. However, in both assays the relative peak-height provides information on the viral load compared to that of the internal control and between multiple virus detections.

Another drawback of both assays is the fact that sample cups have to be opened after the formation of cDNA. Therefore the risk of cross-contamination exists and a sufficient number of control samples should be included.

The molecular assays revealed a high frequency of double infections (Table 4). Specifically coronaviruses (75%), RSV (58%) and rhinovirus (46%) were frequently detected as a mixed infection. By viral culture only one dual infection was identified. This is probably due to overgrowth of the faster growing virus which is identified as the infecting agent. Following a positive identification, the cultures are usually not examined further and dual infections could thus be missed easily. The viruses that are involved commonly in dual infections, such as coronavirus, cannot be detected easily in culture. Several studies have pointed out that a more severe clinical course is associated with dual infection (Caracciolo et al., 2008; Johnstone et al., 2008; Paranhos-Baccalà et al., 2008). Therefore, the importance of a sensitive test that is able to identify dual infections is emphasized.

Asymptomatic infection with these respiratory tract viruses is described both as uncommon (Falsey et al., 2006; Kumar et al., 2008) as well as common (Peltola et al., 2008; Zalm et al., 2009). The importance of low viral load detections, therefore, is often unclear and has to be correlated with the clinical diagnosis.

In conclusion: The MLPA assay has a greater sensitivity, is easier to interpret and has a greater success rate than the DPO (in particular when the DPO was combined with the Experion). The DPO needs less "hands-on" time than the MLPA. However, both assays are more rapid and sensitive than viral culture and are good alternatives for real-time RT-PCR.

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