Review

Currently used nucleic acid amplification tests for the detection of viruses and atypicals in acute respiratory infections

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Abstract

For the detection of respiratory viruses conventional culture techniques are still considered as the gold standard. However, results are mostly available too late to have an impact on patient management. The latest developments include appropriate DNA- and RNA-based amplification techniques (both NASBA and PCR) for the detection of an extended number of agents responsible for LRTI. Real time amplification, the latest technical progress, produces, within a considerable shorter time, results with a lower risk of false positives. As results can be obtained within the same day, patient management with appropriate therapy or reduction of unnecessary antibiotic therapy in LRTI will be possible. A number of technical aspects of these amplification assays, and their advantages are discussed.

The availability and use of these new diagnostic tools in virology has contributed to a better understanding of the role of respiratory viruses in LRTI. The increasing importance of the viral agents, *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* in ARI is illustrated. A great proportion of ARI are caused by viruses, but their relative importance depends on the spectrum of agents covered by the diagnostic techniques and on the populations studied, the geographical location and the season. The discovery of new viruses is ongoing; examples are the hMPV and the increasing number of coronaviruses. Indications for the use of these rapid techniques in different clinical situations are discussed. Depending on the possibilities, the laboratory could optimize its diagnostic strategy by applying a combination of immunofluorescence for the detection of RSV an IFL, and a combination of real-time amplification tests for other respiratory viruses and the atypical agents. When implementing a strategy, a compromise between sensitivity, clinical utility, turn around time and cost will have to be found.
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1. Introduction

At present there is still a great deficit in the etiologic diagnosis of community-acquired lower respiratory tract infections (LRTI); in most studies more than 50% of cases remain without an etiologic diagnosis resulting in unnecessary or inappropriate antibiotic prescribing.

A wide variety of diagnostic procedures and techniques are applied for the detection of the etiologic pathogens of community-acquired LRTI. Traditional diagnostic culture methods above all lack sensitivity, are not feasable in many contexts, and focus only on a few of the large number of aetiologic agents. During recent years a considerable number of previously unknown respiratory agents were discovered whose in vitro culture is very slow or even unrealized: the human metapneumoviruses, the novel coronaviruses NL63, HKU1, human bocavirus and the new polyomaviruses.

For the so called "atypical" bacterial causes *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Legionella pneumophila*, traditional diagnostic methods are also too insensitve and too slow, producing a result only after several days.

Therefore alternative diagnostic procedures were developed: antigen detection by latex agglutination or immunofluorescence (DIF), ELISA, immunochromatography and nucleic acid amplification techniques (NAATs), particularly PCR and NASBA (nucleic acid sequence based amplification).

Over the past two decades, NAATs are revolutionizing the diagnostic procedures for the management of patients with RTI, resulting from a combination of improved sensitivity and specificity, a potential for automatization and the production of very rapid results. NAATs have already become the gold standard in some diagnostic fields but only a few assays have been approved by the US Food and Drug Administration and fewer still have entered the daily routine diagnosis and management of patients. This can be ascribed to the rapid evolution of the technology, the cost of this technology and the large number of etiological agents, bacterial as well as viral, responsible for community-acquired LRTI.

This overview will therefore provide a look at the general principles, advantages, diagnostic value, and limitations of the most currently used amplification techniques for the etiological diagnosis of respiratory tract infections as they evolve from research to daily practice.

2. Amplification techniques

2.1. Conventional nucleic acid amplification methods

2.1.1. Single target NAATs (Table 1)

The traditional NAATs involve three steps: the sample preparation including extraction of the nucleic acid (NA), the NA amplification and the detection-identification of the amplicons produced. PCR was the first and is still the most common and most frequently applied nucleic acid-based assay. This is mainly because this procedure was widely known and published before the other alternative amplification methods.

Detection of PCR amplicons originally relied on electrophoresis in the presence of ethidium bromide to visualize the resulting bands during UV irradiation and comparison with a reference product. Identification of the amplicons is, however, more specific after hybridization with a labeled probe. This is time consuming and requires multiple PCR product handling steps with the risk of spreading of amplicons throughout the laboratory, resulting in laboratory contamination and false positive results in subsequent assays. Alternatively amplicons may be captured onto a solid phase and detected by an enzyme immunoassay that is more convenient for the examination of clinical samples in batches.

In NASBA, RNA is amplified by the simultaneous action of three enzymes: a reverse transcriptase which has also polymerase activity, an RNase and an RNA polymerase. The synthesis of cDNA is primed by specially designed oligonucleotide primers one end of which is a target-specific sequence, while the other contains a promoter for the RNA polymerase. The reverse transcriptase synthesizes an RNA–DNA hybrid, the RNase H digests the RNA component and the reverse transcriptase synthesizes double stranded DNA; finally the T7 RNA polymerase produces numerous RNA copies. The amplification product of NASBA is single stranded RNA and can be detected by an enzyme-linked gel assay or electroluminescence (Ieven and Loens, 2006).

One advantage of NASBA compared to PCR is that it is a continuous, isothermal process that does not require a thermocycler. The constant temperature maintained throughout the amplification reaction allows each step of the reaction to proceed as soon as an amplification intermediate becomes available. In addition, RNA is the genomic material of numerous respiratory viruses. The application of an RNA-based amplification technique offers potential advantages com-

Table 1
References for traditional NAAT protocols

Author	Target	Detection procedure
Influenza virus		
Ellis and Zambon (2002)	Review	
Parainfluenza (these viruses are generally part of multiplex reactions)		
Aguilar et al. (2000)	Hexon, 300 bp/139 bp, in urine	A–H
Hibbitts et al. (2003)	HN gene	NASBA, NucliSens Basic kit + ECL detection
RSV		
Paton et al. (1992)	F1 fusion protein, 243 bp	A
Freymuth et al. (1995)	N gene, 278 bp, 1B gene, NS	EIA, EIA
Eugene-Ruellan et al. (1998)	L polymerase gene	A-RFLP
Falsey et al. (2002)	F gene, 411/263 bp	Nested-A
Human metapneumovirus		
van den Hoogen et al. (2001)	Primary description of the virus	
Mackay et al. (2003)	N gene	EIA
Coronaviruses		
Ksiazek et al. (2003)	sars polymerase gene	A
Yam et al. (2003)	RNA polymerase	A
Vabret et al. (2005)	N gene(NL-63)	N-A
Woo et al. (2005)	HKU1	A-sequencing
Adenovirus		
Raty et al. (1999)	Hexon, 308 bp	A + H
Pring-Akerblom and Adrian (1994)	Hexon, 1551 bp; types, 8, 31, 40, 41	A + RFLP
Kidd et al. (1996)	VA region, variable, subgenera	A + RFLP
Morris et al. (1996)	Hexon, 300 bp/243 bp, subgenus C	N-A types
Tiemessen and Nel (1996)	Long-fiber gene, 152 bp, subgenus F	A–H
Avellon et al. (2001)	Hexon, 168 bp, polyvalent	A
Allard et al. (2001)	Hexon, 301 bp/171, typing	N-A–RFLP
Rhinovirus		
Gamma et al. (1989)	5′-Non-coding region	A
Hyypia et al. (1989)	5′-Non-coding region	A + H
Arruda and Hayden (1993)	5′-Non-coding region	A
Johnston et al. (1993)	5′-Non-coding region, 900 bp	A + H
Santti et al. (1997)	5′-Non-coding region, 126/96/533 bp	A
Samuelson et al. (1998)	5′-Non-coding region	NASBA, ECL
Andeweg et al. (1999)	5′-Non-coding region-VP4	N-A–H
Steininger et al. (2001)	5′-Non-coding region, 106/93 bp	N-A
Billaud et al. (2003)	5′-Non-coding region, rhino v	N-A
Loens et al. (2003a)	5′-Non-coding region	NASBA–ECL
Deffernez et al. (2004)	5′-Non-coding region	A
Mycoplasma pneumoniae		
Loens et al. (2003b)	Review	
Daxboeck et al. (2003)	Review	
Chlamydophila pneumoniae		
Dowell et al. (2001)	Review	
Boman and Hammerschlag (2002)	Review	
Legionella pneumophila		
Miller et al. (1993)	ENVIRO AmpKit	H
Jaulhac et al. (1992)	*mip* gene *Legionella pneumophila*	A
Lindsay et al. (1994)	amp *mip* gene	A + H
Matsiota-Bernard et al. (1994)	ENVIRO AmpKit, 5S RNA gene/*mip*	A + H
Jonas et al. (1995)	16S RNA gene, 386 bp	A + H
Van der Zee et al. (2002a,b)	16S RNA gene *Leg.* spp.—*Legionella pneumophila*	A + H

A, agarose gel electrophoresis; N, nested; EIA, enzyme immunoassay; H, hybridization; RFLP: restriction fragment length polymorphism; ECL, electrochemo-luminescence.

pared to a DNA-based amplification technique: no additional reverse transcriptase step is required, thus saving time and reducing the risk of contamination. The specificity of the reactions might, however, be lower because the enzymes used are not thermostable, so that the reaction temperature may not exceed 42 °C without compromising the reaction. However, the specificity rate is increased by additional hybridization with target-specific probes.

Table 2
References for multiplex NAAT protocols

Author	N° targets[a]	Organisms detected
Myint et al. (1994)	2	COR 229E, OC 43
Ramirez et al. (1996)	3	*Leg. pn*, *M. pn*, *C. pn*
Valassina et al. (1997)	2	IFLA, RSV
Osiowy (1998)	5	RSV, PFL 1, 2, 3, 4, ADE
Fan et al. (1998)	6	IFLA, B, RSV, PFL 1, 2, 3
Echevarria et al. (1998)	3	PFL 1, 2, 3
Pitkaranta et al. (1998)	3	RHI, RSV, COR
Grondahl et al. (1999)	9	IFLA, B, RSV, PFL 1, 3, ADE, EV, *M. pn*, *C. pn*
Corne et al. (1999)	3	PFL 1, 2, 3
Tong et al. (1999)	3	*M. pn*, *C. pn*, *Chlamydia psittaci*
Xu and Erdman (2001)	3	ADE, 3, 7, 21
Aguilar et al. (2000)	4	PFL 1, 2, 3, 4
Poddar (2002)	4	IFLA, B, typing H1N1, H3N2, H5N1
Coiras et al. (2003)	6	IFLA, B, C, RSVA, RSVB, ADE (48 serotypes)
Coiras et al. (2004)	14	PFL 1, 2, 3, 4, COR 229E, COR OC43, EV, ADE, IFLA, B, C, RSVA, B, ADE in two panels
Bellau-Pujol et al. (2005)	12	IFLA, B, C, PFL 1, 2, 3, 4, RSV, hMPV, COR 229E, COR OC43, RHI
Adachi et al. (2004)	3	COR OC43, COR 229E, COR SARS
Syrmis et al. (2004)	7	IFLA, B, PFL 1, 2, 3, RSV, ADE
Coiras et al. (2005)	14	Coiras (2004) with reverse line blot assay
Li et al. (2007)	6	NGEN (IFL A and B, PFL 1, 2, 3, 4, RSV)
Li et al. (2007)	12	Resplex II (IFL A and B, PFL 1, 2, 3, 4, RSVA, B, hMPN, RHI, ENT, SARS-COR)
Mahony et al. (2007)	20	IFL A, B (typing H1, H3, H5 including H5N1), PFL 1, 2, 3, 4, RSV A, B, ADE, hMPV, RHI, SARS-COR, ENT, COR OC43, COR 229E, COR NL63 and HKU1
Lee et al. (2007)	18	IFL A, B, PFL 1, 2, 3, 4[a], 4[b], RSV A, B, ADE B, C, E, RHI, ENT, hMPV, COR OC43, COR 229E, COR NL63

IFLA: influenza A; RSV: respiratory syncytial virus; ADE: adenovirus; PFL: parainfluenzavirus; COR: coronavirus; EV: enterovirus; RHI: rhinovirus; *Leg. pn*: *Legionella pneumophila*; *M. pn*: *Mycoplasma pneumoniae*; *C. pn*: *Chlamydophila pneumoniae*.

[a] Number of species detected.

To improve the reproducibility of the in-house developed NASBA standardized reagents, the 'NucliSens Basic kit' (bioMérieux) is now commercially available (Fox et al., 2002). It contains the necessary reagents for NA release and inactivation of RNases and DNases, silica extraction of nucleic acids, the NASBA reagents and the reagents for chemoluminescent detection, including the generic ECL probe. The primers and the target specific capture probe are to be synthesized for each target.

NAATs are available for all respiratory agents. Whenever possible, consensus primers able to detect all viruses from a family or genus must be used and their ability to amplify all viruses with the same efficiency must be carefully evaluated, particularly for entero- and rhino-viruses (Loens et al., 2003a). By the judicious choice of primers a high specificity of the NAATs can be ascertained.

References to protocols for traditional NAATs are presented in Tables 1–4. Tables 1 and 3 present either the earliest publication for the diagnosis of a particular agent, or an assay directed at an alternative target or the use of a different method for this agent, or a review. Tables 2 and 4 present protocols for the detection of various combinations of etiologic agents.

In the conventional PCR reactions mentioned in Tables 1 and 2 amplicon detection is done by agarose gel electrophoresis with or without hybridization, occasionally by restriction fragment length polymorphism and in some by an enzyme immunoassay.

For some virus groups several protocols have been proposed. They differ in the target chosen or in the amplicon detection techniques applied with different conveniences. All RSV procedures have a sensitivity of 94.5–97% compared with cell culture and immunofluorescence, but PCR is definitely more sensitive for adults and older persons as a result of the lower virus production in these patients (Landry et al., 2000). Multiple protocols are more frequent for those virusgroups that contain multiple virustypes. Several amplification protocols were developed to cover particular types or groups among the adenoviruses. More than 100 rhinovirus types and their close relationship with enteroviruses constitute a special challenge. The judicious choice of primers and particularly of the hybridization probes should ensure a satisfactory coverage of the rhinovirus types as illustrated by Loens et al. (2003a); by the use of new primers, rhinoviruses were detected five times more frequently in clinical samples than traditional culture techniques and two to

Table 3
References of real-time single target NAAT protocols

Author	Targets	Procedure
Influenza virus		
Habib-Bein et al. (2003)	Matrix IFLA	Smart Cycler
Henrickson (2004)	IFLA nucl. caps. gene	NASBA ECL
Parainfluenza		
These viruses are generally part of multiplex reactions		
RSV		
Whiley et al. (2002)	L gene	Light Cycler
van Elden et al. (2003)	N gene types A, B	TaqMan
Mentel et al. (2003)	F gene	TaqMan
Gueudin et al. (2003)	N gene	Light Cycler
Borg et al. (2003)	F gene	TaqMan
Kuypers et al. (2004)	Matrix protein gene	TaqMan
O'Shea and Cane (2004)	N gene	AmpliTaq
Perkins et al. (2005)	F, N genes types A, B	AB Prism 7900HT
Human metapneumovirus		
Cote et al. (2003)	N gene	Light Cycler
Mackay et al. (2003)	N gene	Light Cycler
Maertzdorf et al. (2004)	N gene	ABI Prism 7000
Coronavirus		
Drosten et al. (2003)	sars BNI fragment	Light Cycler/BioSystems 7000 SDS
Poon et al. (2003)	sars RNA polymerase	Light Cycler
van Elden et al. (2004)	(229E, OC43) N gene sars polymerase gene seminested	TaqMan ABI Prism 7700
Fouchier et al. (2004)	(NL-63) N gene	TaqMan
Mahony et al. (2004)	sars, nuclocapsid gene	Light Cycler
van der Hoek et al. (2004)	NL-63	i Cycler
Emery et al. (2004)	sars, polymerase, two regions of nucleocapsid	TaqMan, i Cycler
Hourfar et al. (2004)	sars Roche replicase "Aztus" (genome fragment)	Roche assays and Aztus
Nitsche et al. (2004)	sars, three different sequences	BioSystems DS 7700, 7000, Light Cycler
Booth et al. (2005)	sars, P, N, M genes	i Cycler
Woo et al. (2005)	HKU1 N gene	Light Cycler
Chui et al. (2005)	Comparison of nine primer seq.	
Adenovirus		
Poddar (1999)	Hexon gene	TaqMan
Houng et al. (2002)	Hexon gene	TaqMan
Heim et al. (2003)	Hexon gene	Light Cycler
Rhinovirus		
Nijhuis et al. (2002)	5′-Non-coding region	N-A
Dagher et al. (2004)	5′-NCR	Light Cycler
Kares et al. (2004)	5′-NCR	Light Cycler
Bocavirus		
Lu et al. (2006)	NS1, NP genes	Taqman
Mycoplasma pneumoniae		
Ursi et al. (2003)	P1	Light Cycler
Templeton et al. (2003a)	P1	i Cycler
Loens et al. (2003b)	Review	
Chlamydophila pneumoniae		
Mygind et al. (2001)	pmp 4 gene	Light Cycler
Kuoppa et al. (2002)	MOMP gene	ABI Prism 7700
Tondella et al. (2002)	Two regions MOMP gene	AB Prisma 7700
Ciervo et al. (2003)	Pst fragment	Light Cycler
Apfalter et al. (2003)	MOMP	TaqMan
Hardick et al. (2004)	16S rRNA gene	TaqMan
Loens et al. (2006a)	16S rRNA gene	NASBA
Legionella		
Ballard et al. (2000)	mip gene *L. pneumophila*	Light Cycler
Wellinghausen et al. (2001)	16S rRNA gene *Leg.* spp.	Light Cycler
Rantakokko-Jalava and Jalava (2001)	16S rRNA gene *Leg.* spp.	Light Cycler

Table 3 (*Continued*)

Author	Targets	Procedure
Hayden et al. (2001)	5S rDNA *Leg.* genus mip *Leg.* spp.	Light Cycler
Wilson et al. (2003)	mip gene *L. pneumophila*	Light Cycler
Loens et al. (2006d)	16 Rrna gene	NASBA

For abbreviations see Table 2.

three times more than in studies using previously described primers.

Studies comparing two different molecular amplification techniques applied to a considerable number of clinical specimens are rare. Face-to-face comparison of two amplification protocols was done for sars-coronavirus by Yam et al. (2003).

Both NASBA and reverse transcriptase PCR have their advantages.

In a recent study Loens et al. (2006b) compared NASBA and PCR on more than 500 nasopharyngeal aspirates collected from children presenting with acute respiratory tract infections at the University Hospital Antwerp. Both NASBA and reverse transcriptase PCR produced comparable results and were significantly more sensitive than virus culture.

Protocols for *Legionella* spp. were developed for the detection of single types in clinical specimens or of mutiple species in environmental water.

An overview of the literature on the use of NAATs to detect *M. pneumoniae* since 1989 is given in the review by Loens et al. (2003b) with a description of the currently available molecular amplification methods. Topics discussed include specimen collection and transport, preparation of nucleic acid from clinical specimens, choice of the target sequence, and detection of the amplicons. Methods to recognize and prevent false positive and false negative results, the results of NAATs in comparison with results obtained by conventional diagnostic tests, and clinical applications are also reviewed.

In their review on the diagnosis of *C. pneumoniae* Kumar and Hammerschlag (2007) conclude that studies of NAATs on respiratory specimens revealed significant variations of test performance from laboratory to laboratory await validation and standardization.

2.1.2. Multiplex NAATs (Table 2)

In a multiplex PCR several independent amplifications are carried out simultaneously in one tube with a mixture of primers.

Multiplex NAATs were developed to detect 2, 6, up to 14 microorganisms simultaneously, in some instances in two or more separate assays (Table 2). There are indications that increasing the number of targets in one reaction results in loss of sensitivity (Tong et al., 1999; Vernet, 2004). Tong

Table 4

References real-time multiplex NAATs

Author	N° targets	Species detected
Fan et al. (1998)	2	RSVA, RSVB
Schweiger et al. (2000)	2	IFLA, IFLB + subtypes
Hindiyeh et al. (2001)	7	Hexaplex
Kehl et al. (2001)	6	Hexaplex
van Elden et al. (2001)	2	IFLA, IFLB
Templeton et al. (2003b)		*L. pn*, *L.* spp.
Poddar (2003)	2	IFL and subtypes
Smith et al. (2003)	2	IFLA, IFLB
Welti et al. (2003)	3	*M. pn*, *C. pn*, *L. pn*
Herpers et al. (2003)		Differentiation *L. pn/L.* spp.
Boivin et al. (2004)	2	IFL, RSV
Stone et al. (2004)	2	IFL and subtypes
Templeton et al. (2004)	7	IFLA, IFLB, RSV, PFL 1, 2, 3, 4
von Linstow et al. (2004)	2	RSV, hMPN
Maltezou et al. (2004)	2	*M. pn*, *L.* spp.
van Elden et al. (2004)	2	COR E229, OC 43
Gruteke et al. (2004)	7	IFL A and B, PFL 1, 3, RHI, RSV, ENT in two panels
Scheltinga et al. (2005)	2	hMPN, RHI
Raggam et al. (2005)	3	*M. pn*, *C. pn*, *L.* spp.
McDonough et al. (2005)	4	*M. pn*, *C. pn*, *L. pn*, *B. pertussis*
Ginevra et al. (2005)	3	*M. pn*, *C. pn*, *L.* spp., commercial
Gunson et al. (2005)	12	IFL A and B, PFL 1, 2, 3, RHI, hMPN, RSVA and B, COR E229, OC 43, NL63 in four triplex reactions
Loens et al.	3	*M. pn*, *C. pn*, *L. pn*
Choi et al. (2006)	12	In four multiplex and one monoreaction

For abbreviations see Table 2.

observed a lower sensitivity of about 1 log for both *M. pneumoniae* and *Chlamydia pneumoniae* compared with their individual PCRs. On the respiratory samples, the sensitivity of the multiplex assay for *M. pneumoniae*, *C. pneumoniae* and *Chlamydia psittaci* were 82% (9/11), 100% (11/11) and 86% (6/7), respectively. Vernet observed that the analytical sensitivity of multiplex RT-PCR detection of six viruses, i.e. influenza A, influenza B, RSV A/B, parainfluenza 1, 2 and 3 is reduced by a factor of 1–2 logs compared to single detections, depending on the virus. Nevertheless, this multiplex assay was able to identify correctly 95% (21/22) infections in respiratory specimens. This decrease in sensitivity is not unexpected since the presence of several pairs of primers may increase the probability of mispairing resulting in non-specific amplification products and the formation of primer-dimers. Furthermore enzymes, primers and salt concentrations as well as temperature cyclings required for each target may be slightly different. One assay, the commercially available Pneumoplex (Prodesse, Milwaukee, USA) targeting seven respiratory viruses was included in a quality control exercise. Although the limit of detection of this assay was reported to be 5 cfu/ml for *M. pneumoniae* and 0.01 $TCID_{50}$/ml for *C. pneumoniae* and 10 copies of recombinant DNA for each organism (Khanna et al., 2005) the test did not perform well in this evaluation (Loens et al., 2006c). The manufacturer was contacted and is aware of the sensitivity problems of the Pneumoplex assay. They intended to improve the sensitivity of the assay. These commercially available multiplex tests such as the Hexaplex test are still technically demanding, requiring 3–4 h hands-on-time (Hindiyeh et al., 2001).

A different approach was applied by Coiras et al. (2005), samples were submitted to two multiplex transcription PCR assays followed by reverse-line blot assays for the detection-identification of 14 groups of viruses. This procedure, however, also takes time.

The latest evolution combines conventional PCR with microarray detection as recently described by Li et al. (2007) who evaluated positively two commercial multiplex panels, NGEN and ResPlex II, detecting 6 and 12 respiratory viruses or virusgroups, respectively by microarray and Luminex liquid chip hybridization and identification, respectively. Sensitivities of these two assays were also lower than those of the monoplex real-time reversed transcriptase PCR assays, most noticeably for RSV and PIV-3. Although these might be improverd by further primer/probe optimization, changes in primer/probe sequences could negatively influence other assays targeted in the multiplexed reaction. Although hands-on-time of these tests are only approximately 60 min, turnaround times are still 6 h for the ResPlex II and 9 h for the NGEN.

Based on the same principle, eight distinct virus groups and 20 different respiratory viruses were amplified in a multiplex reaction by Lee et al. (2007) and by Mahony et al. (2007), respectively. The amplicons were detected by fluid microsphere-based array (Universal Array™, Bioscience) and the Luminex x-MAP system.

These techniques are therefore in competition with and in many cases gradually replaced by real-time multiplex reactions because of their greater user friendliness.

2.2. Real-time NAATs

2.2.1. Single target real-time NAATs (Table 3)

The combination of the use of capillary glass tubes heated by air, shortening significantly the cycling times, together with the use of fluorogenic probes allowing on-line fluorescence detection of the amplification, results in a considerable increase in the speed of RT-PCR. Since amplification and detection are performed simultaneously in sealed tubes there is no need for further manipulation, eliminating the risk of carry-over contamination. For the probes the simplest chemistry uses SYBR green dye that binds to ds DNA generated during PCR, however, with lower specificity due to unspecific binding to all ds DNA.

Alternatives are TaqMan probes or molecular beacons in which a quencher molecule is removed from the vicinity of the fluorescent marker probe upon binding to RNA or DNA generated during amplification. In what is called FRET technology two probes, one with a fluorescence donor and one with a fluorescence acceptor molecule, are designed to bind to adjacent sequences of the amplicons to generate a signal (Mackay et al., 2002). RT detection by molecular beacons is also applicable to NASBA (Ieven and Loens, 2006; Loens et al., 2006a). For RNA viruses the preliminary synthesis of cDNA was originally done in a separate tube but is now done in a single tube together with the amplification–detection.

The traditional NAATs are gradually replaced by real-time formats in which the same targets may be used.

The sensitivity and specificity of RT-PCR is identical to that of conventional PCR for *L. pneumophila* (Hayden et al., 2001), for *M. pneumoniae* (Templeton et al., 2003a; Ursi et al., 2003) as well as for RSV (Mentel et al., 2003). In some studies a superior sensitivity of RT-PCR versus conventional PCR is mentioned for some agents: rhino-viruses (Dagher et al., 2004) and sars-coronavirus (Poon et al., 2003). Comparisons between different RT-PCRs for sars-coronavirus showed no significant differences in sensitivity nor specificity (Chui et al., 2005; Hourfar et al., 2004; Mahony et al., 2004).

2.2.2. Multiplex real-time NAATs (Table 4)

The number of agents that can be detected simultaneously in one RT reaction tube is restricted by the number of available wavelengths in existing equipments mostly three at present. But several reaction tubes can be run in parallel. The major drawback of this approach is the reduction of the amount of NA that can be introduced in each amplification and the higher hands-on-time required to manipulate all

the tubes. Once more the thermocycling may be suboptimal for some agents involved in the assay. In all simultaneous assays a compromise will have to be made between the optimal temperature cycling requirements and the sensitivity of each component.

RT multiplex PCRs have been applied to two to three agents simultaneously, mostly influenza A and B together or not with RSV. One of the first multiplex PCRs was developed by Welti et al. (2003). The PCR was done in two separate reactions: in the first reaction *M. pneumoniae* and *C. pneumoniae* were detected and in the second reaction *L. pneumophila* together with a commercialized internal control (IPL Applied Biosystems). A RT multiplex NASBA for the diagnosis of *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila* was developed by Loens et al. The multiplex NASBA was performed in one tube. Both groups compared the multiplex assays with the corresponding mono-assays. The sensitivity of the multiplex PCR was identical to the conventional PCR but the multiplex NASBA assay was less sensitive compared with the corresponding RT mono NASBA procedure. A loss of sensitivity was also mentioned by Tong et al. (1999) in a conventional multiplex PCR for these agents as mentioned above. Templeton et al. (2004) developed a two-tube RT multiplex PCR for the diagnosis of influenza A and B and RSV in a first tube and the four parainfluenzaviruses in a second tube. The sensitivity was higher than culture or DIF test but no comparisons were made between multiplex reactions and monoreactions on the same samples. Gruteke et al. (2004) applied four multiplex reactions to detect 11 agents, Templeton et al. (2005) covered 15 agents by six multiplex real-time reactions and Gunson et al. (2005) targeted 12 agents through four real-time multiplex reactions. More research is needed to identify those reactions that can be combined with a minimal loss in sensitivity.

2.3. Quantitative tests

In RT NAATs the cycle threshold (Ct) is related to the quantity of virus present in the sample. Provided samples are standardized, comparison between Ct values allows a relative quantification of viral load and can be useful to follow the evolution of an infection in a particular patient. For absolute quantification several standards were used in the past, such as high titer virus preparations from tissue cultures, a quantified bacterial suspension, and virus suspensions quantified by electron microscopy. The most popular are in-house cloned plasmids. DNA is measured and the corresponding number of target molecules is calculated. The construction of standard curves (Fronhoffs et al., 2002) allows absolute quantifications expressed as the number of viral particles (Vijgen et al., 2005).

There is still a lack of information on the significance of viral loads in respiratory infections. Higher amounts of RSV were found in children less than 1 month of age, and as far as quantitative studies go, higher viral loads correspond to more severe clinical disease. Quantitative data differentiating colonization and infection are lacking entirely (DeVincenzo et al., 2005).

3. Needs for improvement

3.1. Sample type and automation of nucleic acid extraction

For the molecular diagnosis of respiratory infections the preferred clinical specimens are nasopharyngeal aspirates (NPA) and sputum (Covalciuc et al., 1999) as well as bronchoalveolar lavage specimens if available. The superiority of NPA for the detection of all viruses was clearly illustrated in the study by Gruteke et al. (2004); the percentage of diagnosed episodes was 84% on NPA compared with 58% when only swabs were available. Nasal swabs were not suited for the detection of RSV in the study by Heikkinen et al. (2002). The Copan flocked swabs and universal transport medium collection and transport system is a universal system compatible with antigen kit, DFA, culture and PCR (Castriciano et al., 2005, 2007; Daley et al., 2006). Nasal swabbing with the new-flocked swabs is equivalent to traditional rayon nasopharyngeal swabs (NPS) with less patient discomfort. Significantly more epithelial cells are collected by these flocked swabs providing better specimens for diagnosis. Furthermore, NPS collected with flocked swabs detect a higher number of positives than NPS collected with dacron swabs.

Specimens intended for NASBA and reverse transcription PCR should be introduced immediately after collection in an appropriate buffer to maintain RNA integrity.

Isolation and purification of NA have been the most labor-intensive parts of the molecular diagnostic tests. NA extraction originally performed with phenol–chloroform has been widely replaced by the Boom method (Boom et al., 1990) and by commercial sample preparation kits. These methods are time consuming, labor intensive and susceptible to contamination. Especially for the detection of *L. pneumophila*, contamination problems have been described using filters/columns which are flushed with water in their preparation; these have been shown to be potentially contaminated with non-pneumophila *Legionella* spp. (Evans et al., 2003; Van der Zee et al., 2002a,b).

The probability of false positive results because of contamination increases with the number of manipulations involved in sample processing. Lately complete automatization was introduced performing RNA as well as DNA extraction within 20–40 min on small or on high numbers of specimens. Although robotic automated sample preparation has been shown to perform equally and more consistently than manual techniques only a limited number of studies are available on a variety of respiratory specimens. In studies on *L. pneumophila*, *M. pneumoniae* and *C. pneumoniae* in respiratory specimens several authors showed that the performance of the automated MagNaPure and the NucliSens extraction

procedures (EasyMAG and miniMAG) was superior compared with manual extraction methods (Loens et al., 2007; Wilson et al., 2004).

For reactions exclusively directed on DNA viruses (adenoviruses and herpes viruses) simple heating of throat swab extracts is feasible (Faix et al., 2004) and has been applied on *M. pneumoniae* (Ieven et al., 1996). A multiplex reaction directed at adenovirus, *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila* after simple heating of the samples might be explored.

3.2. Detection of amplification inhibitors and contamination control

Suitable controls should monitor the NA extraction and amplification procedures as well as the quality of the specimens and detect laboratory contaminations. Such controls are at present not always included in test panels.

Positive controls assure that correct amplification has taken place. Internal controls should be added to the samples before NA extraction to monitor the efficiency of the extraction, and to detect inhibitors and possible laboratory contamination. A negative result may point to the absence of the target organism in the sample or to its presence in low copy numbers or to the presence of amplification inhibitors or to poor quality specimens. Different types of internal controls (IC) are available (Loens et al., 2003b). A homologous extrinsic control is an amplicon modified by a non-target-derived sequence insert. It is added to each sample prior to NA extraction and is co-amplified with the same primers used for the target (Ursi et al., 1992, 2003). This allows to monitor with precision the combined effect of extraction and amplification. The quantity of IC added should be low to prevent competition with the real target. Homologous extrinsic controls have to be constructed for each amplification target separately. Heterologous extrinsic controls are DNA or RNA derived from viruses that cannot infect humans such as seal herpes virus and phocine distemper virus as proposed by Niesters (2002, 2004). They are non-competitive, can be added to any amplification reaction but require the addition of specific primers. Dingle et al. (2004) proposed a modified hepatitis deltavirus for this purpose. Finally a heterologous intrinsic control certifies the presence of human NA and thus possible virus containing cellular material in the sample. Examples are the beta-globin, the gamma interferon, the glyceraldehyde-3-phosphate dehydrogenase and the U1A genes (Loens et al., 2003b). They are particularly important for throat swab specimens that may have inadequately scraped the mucosal surface and for sputum specimens that may contain too few leucocytes. Apfalter et al. (2005) discussed all steps in NAAT procedures that need proper attention, from the pre-analytical procedures over the assay design, interpretation of the results and quality control.

The use of an internal control is mandatory and the assurance that human NA is present in the sample is highly recommendable.

3.3. Validation of amplification tests

For the synthesis of primers and probes genomic evolution and geographical genomic diversity have to be taken into account, particularly for RNA viruses. The matrix of the positive control, whether whole virus or nucleic acid should be of the same nature as that of the clinical sample.

When traditional culture techniques are compared with NAATs the latter in general detect considerably more positive specimens, only a small number of culture positives being missed by the NAATs. With a few exceptions NAATs are always more sensitive than culture and DIF or other antigen detection procedures (Henrickson, 2004, 2005; Jennings et al., 2004; Murdoch, 2004). Numerous in-house PCR assays for the detection of atypicals have been developed but as is the case for many viral assays as well, proper validation and standardization are often lacking. Validation must be performed at several levels, including sample preparation, amplification and detection as was concluded in a standardization workshop on *C. pneumoniae* assays (Dowell et al., 2001). The conclusion of this working group was that more studies need to be conducted using proper controls and a large number of clinical specimens to compare and evaluate more adequately the usefulness of different PCR tests for the diagnosis of *C. pneumoniae* infection.

In a few instances positive and negative predictive values of NAATs were calculated. PPV and NPV for NAATs for rhinoviruses compared with culture were 83.3% and 98.5%, respectively (Loens et al., 2006c). For two adenovirus NAATs, Vabret et al. (2004) found a PPV of 84% and 87.8% and a NPV of 91% and 98.8%, respectively, and Gueudin et al. (2003) registered a PPV and NPV for RSV NAATs compared with culture of 92% and 100%, respectively.

The absence of a gold standard requires adapted statistical techniques to evaluate properly the different diagnostic techniques and especially the more sensitive NAATs (Hadgu et al., 2005).

Nevertheless, molecular techniques are gradually replacing tissue culture as the gold standard for the diagnosis of respiratory infections (Arden et al., 2006; Kuypers et al., 2004; Murdoch, 2004; Templeton et al., 2003a; Van de Pol et al., 2007; van Elden et al., 2002).

3.4. External quality control

All in-laboratory developed tests have to be verified for their analytical and clinical performance. As illustrated by a number of studies, there is a need for standardized material, particularly for quantitative tests, and participation in external quality control programs. Commercialized kits are hardly available for respiratory agents, with the exception of a sars-coronavirus RT-PCR (Bio-Mérieux). This situation may evolve rapidly: a research-use-only kit was already available for *C. pneumoniae* (Chernesky et al., 2002) as well as standardized reagent kits and internal controls (IPL Aplied Biosystems) with some commercialized multiplex tests, both

conventional such as Pneumoplex and Hexaplex (Prodesse), the ID-Tag respiratory viral panel from Tm Bioscience Corp. (Toronto, Canada) as real time formats such as Chlamylege (Argene) (Ginevra et al., 2005). The greatest problems to overcome with molecular methods is false positivity caused by contamination, although automatic sample preparation and real time NAATs represent a significant progress in this matter. The false negativity is associated with the great differences in sensitivity of home made assays (Apfalter et al., 2005; Loens et al., 2006c; Templeton et al., 2006; Van Vliet et al., 2001). The task of QC is being taken up by internationally collaborating laboratories such as QCMD (Quality Control for Molecular Diagnostics) endorsed by the European Society for Clinical Virology (ESCV) and the European Society for Clinical Microbiology and Infectious Diseases.

4. The clinical usefulness of NAATs

To evaluate the usefulness of NAATs in the diagnosis of acute respiratory infection the objectives of an etiologic diagnosis in this condition should be remembered. These are:

(1) to avoid empirical start of antibiotic treatment and to allow narrow spectrum targeted antibiotic treatment;
(2) to allow appropriate use of antiviral drugs;
(3) to allow cohorting of patients in case of hospitalization, preventing nosocomial spread;
(4) to provide more accurate epidemiological information to formulate preventive and therapeutic recommendations;
(5) to decrease duration of hospital stay and to reduce management costs.

To answer the first three objectives, diagnosis should be available rapidly, preferably within about 2 h. The traditional NAATs are unable to fulfill this requirement, but recent technical progress has brought NAATS to age, through the development of kinetic or "real-time" (RT) tests, coupled with automatic NA extraction.

A multitude of reports have appeared on the epidemiology of ARI but most are restricted to a few viruses (influenza, sometimes together with RSV, to rhino-, metapneumo- or corona-viruses) and/or to some population groups, e.g. children, adults or old age people. Great variations occur in function of time, place and the age groups studied (Esper et al., 2004; Guittet et al., 2003; McAdam et al., 2004; Monto, 2004; Tsolia et al., 2004). It appears that RSV is the most common cause of viral LTRI in very young children (Drummond et al., 2000; Freymuth et al., 1987; Ieven et al., 1996) with an increasing importance of rhinoviruses in young children (Miller et al., 2007). RSV, rhino- and influenza-viruses are common in older people (Falsey and Walsh, 2006; Nicholson et al., 1997). Coronaviruses and *M. pneumoniae* are also more prevalent than previously thought (Billaud et al., 2003; Loens et al., 2003b; Waites and Talkington, 2004). As time progresses the importance of the more recently discovered

human bocavirus (Allander et al., 2007; Kesebir et al., 2006; Kleines et al., 2007; Kupfer et al., 2006; McIntosh, 2006), human metapneumovirus infections (Boivin et al., 2007; Dare et al., 2007; Ordas et al., 2006; van den Hoogen, 2007), and coronaviruses (Koetz et al., 2006; Vabret et al., 2005) are becoming more evident. Although the role of some of these new viruses becomes more clear in specific patient populations, more studies are needed to identify the clinical relevance of some others such as the bocavirus.

The traditional NAATs require at least 1–2 days. Therefore all former studies were done "a posteriori", i.e. the results were not available to the clinician in time to have any possible impact on patient management.

To cover the wide spectrum of etiologic respiratory agents a number of uni- and/or multiplex reactions are to be performed simultaneously (Lee et al., 2006). Combined with traditional bacteriologic techniques to diagnose *Streptococcus pneumoniae* infections, only 24% of the infections remained etiologically undefined in the multiplex study by Templeton et al. (2005) and only 14% in the study by Gruteke et al. (2004). All studies were limited in time and were pilot trials.

The wider application of multiplex reactions during recent years also resulted in the detection of numerous simultaneous viral infections with widely varying incidences: from 3% (Scheltinga et al., 2005) to 9% (Guittet et al., 2003) to 23% (Bellau-Pujol et al., 2005) and 35% (Templeton et al., 2005). In the latter study bacterial agents were also included. The differences in incidence may result from the variety of diagnostic panels applied. There were no preferential combinations of the viruses. Only a few studies found combined infections to be associated with a more severe clinical status. Semple found severe bronchiolitis associated with the combination of hMPV and RSV (Semple et al., 2005) and Templeton et al. found significantly more mixed infections, involving also bacteria, in patients with more severe pneumonia (Templeton et al., 2005). The clinical significance of combined infections remains to be further clarified.

Respiratory viruses have also been increasingly recognized as causes of severe lower respiratory tract infections in immunocompromised hosts (Ljungman, 1997; van Elden et al., 2003; Whimbey et al., 1997; Watzinger et al., 2004). Respiratory infections are more common in solid organ recipients, particularly in lung transplant recipients (Dare et al., 2007; Kotloff et al., 2004). Infections are especially dangerous prior to engraftment and during 3 months after transplantation, in the setting of graft versus host disease. The origin of the infections is community-acquired as well as nosocomial (Barron and Weinberg, 2005).

As more epidemiological information on the role of a panel of respiratory viral pathogens becomes available, it is clear that screening for these viruses in specific patient populations such as transplant patients, very young children or elderly is desirable and preventive and therapeutic recommendations may take this information into account.

4.1. Reevaluation of serological tests

The availability of the very sensitive NAATS has in recent years also put the often-used serological tests in their right perspective.

The most reliable serologic evidence of an ongoing infection is a fourfold rise or seroconversion in IgG antibodies during an illness. Therefore paired samples, collected at an interval of 3–4 weeks, are required. In practice, however, often only one serum sample, from the acute-phase of the illness is available or the two samples are collected within a too short time interval to detect a titre rise.

Since IgM antibodies appear earlier than IgG antibodies the detection of IgM in serum is a widely used approach for the early serologic diagnosis of many acute infections. It should be realized that IgM antibodies are often not produced in children under 6 months of age, in a proportion of primary infections and during reinfections. The IgM response may also appear late.

Solitary high IgG titers have no diagnostic meaning for an acute infection since the moment of the seroconversion is unknown and necessarily took place some time before the illness under observation started. Single high titres, for which a cut-off value has to be determined by a local evaluation, are useful only in prevalence studies among population groups.

The clinical significance of a serologic test, for both IgM and IgG, should be defined by studies of patients with a documented infection and for whom detailed information concerning the time lapses between onset of disease and the collection of the serum specimens are known.

Rothbarth detected a fourfold IgG antibody rise by EIA in only 24/29 (82%) of patients at 28 days after an influenza infection (Rothbarth et al., 1999). For RSV Meddens et al. (1990) found a significant increase in IgG antibodies by EIA in 33/36 (92%) of patients studied only at 20–30 days after the onset of disease. Serological tests for respiratory infections usually lack specificity (Beersma et al., 2005; Nir-Paz et al., 2006; Petitjean et al., 2002). Many test formats for *M. pneumoniae* have been proposed. Several studies illustrate a lack of standardization of antigens of *M. pneumoniae* (Beersma et al., 2005; Talkington et al., 2004) In one study 6/12 and 9/12 of PCR-documented *M. pneumoniae* infections were diagnosed in acute and convalescent phase sera, respectively (Talkington et al., 2004). In another study anti *M. pneumoniae* IgM antibodies were detected in 7–25% (depending on the test applied) of acute sera and IgG antibodies in 41–63% of convalescent sera depending on the timing of the second sample (Beersma et al., 2005). These studies illustrate the low incidence of IgM antibodies in the acute phase serum specimens and importance of the delay between the two serum samples.

Legionella antibody tests have a sensitivity of 61–64% depending on the assay applied and also do not substantially improve the diagnosis of legionellosis (Yzerman et al., 2006).

Serologic tests can never offer an early diagnosis and are therefore rather an epidemiological than a diagnostic tool.

5. Optimization of laboratory strategy

With the armamentarium available it is, however, hard to conceive that every hospital laboratory would perform the broad spectrum of RT NAATs, even if standardized reagents at low cost become widely available. Strategies will have to be developed adapting the evolution of the technology of the NAATs, the population of patients served (children, elderly, and immunocompromised patients) the resources available (infrastructure, staff, full-time service or service limited during some hours of the day, or some days of the week), the number and nature of the agents that can be covered. Permanent consultation between laboratorians and clinicians is becoming more necessary than ever.

Nolte (2005) proposed to consider three levels of services to be provided by clinical laboratories: level 1 to perform only FDA approved tests; level 2: performing FDA approved and research-use-only tests and protocols adequately approved by other laboratories; level 3 that design, develop and verify in-house tests.

However, laboratories belonging to levels 1 and 2 will, for the time being, continue to rely for some applications, on viral culture, immunofluoresence and immunochromatographic tests, recognizing the inherent lower sensitivity of these tests when applied on certain specimens or patients. Viral culture is a prerequisite for the study of viral isolates, and is particularly important for influenzavirus.

Alternatively, to cover public health needs, a reference laboratory functioning in close contact with an in- and out-patient clinic and a group of general practitioners could apply the broad spectrum diagnostic panel on their group of patients and produce the required global epidemiologic information. The reference laboratory should make its results available on a daily basis. Regional and local laboratories might limit their investigations to the antibiotic treatable, bacterial infections and the most important viral infections such as influenza and RSV, avoiding unnecessary antibiotic treatment. For community-acquired bacterial pneumonia the Gram staining of a sputum specimen remains a fundamental and rapid low cost diagnostic procedure. It could be combined in a first approach with a multiplex NAAT for the diagnosis of the slowly growing, antibiotic sensitive bacteria, *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila* and *Bordetella pertussis*. A positive result may lead to adaptation of antibiotic therapy, when these results are negative, tests for viral causes may be initiated although at present most clinicians do not stop antibiotics in patients negative for a bacterial cause.

Falsey proposes different protocols during the summer and the winter months. During the summer months PCR and viral testing is performed in cases of severe illness only. During the winter months the strategy is different whether influenza epidemic is ongoing or not (Falsey et al., 2007). Since in our

region important ARI viral agents are active mostly during the winter months, the diagnostic procedures to detect, e.g. influenza and RSV could be limited between November and March. In the presence of an influenza epidemic, efforts could be entirely concentrated on transfering the local isolates to the reference laboratory for subtyping. During the summer months PCR and viral testing could be performed in specific patient populations and in cases of severe illness only.

Thoroughly investigated specimens from infections remaining without a known infectious cause should be stored for studies aimed at the discovery of yet unidentified pathogens. Indeed all studies on the etiologic spectrum of ARI leave a considerable proportion, 40–50%, of cases without a known cause, although in some studies as those by Gruteke et al. (2004) and by Templeton et al. (2005) this fraction was reduced to 14% and 24%, respectively. Since the organisms discovered more recently multiply poorly in tissue cultures it may be surmised that agents remaining to be discovered will also grow poorly or not at all in tissue cultures, as illustrated by the recent discovery of previously unknown coronaviruses (Yam et al., 2003), human metapneumovirus (Maertzdorf et al., 2004; van den Hoogen et al., 2001), bocavirus (Allander et al., 2005), and the new polyomaviruses (Allander et al., 2007; Gaynor et al., 2007). Further studies will lead to the discovery of more 'hidden' causal agents.

NAATs are not always required for every purpose. For cohorting RSV-infected pediatric patients the DIF test can provide a result within 60 min. Chromatographic tests producing results within 15 min with a sensitivity of 80.9–93.3% for RSV (Reina et al., 2004; Ohm-Smith et al., 2004) are available that can be done in the laboratory outside the virology laboratory working hours (Cazacu et al., 2004). These tests, however, lack sensitivity when applied on respiratory samples of adult patients (Landry et al., 2000).

Practical issues in the laboratory may limit the theoretical possibilities of the rapid NAATs such as the necessity to handle specimens in batches, thereby losing some advantages of the rapidity of the tests. Moreover, virology laboratories at present do not operate 24/24 h, 7/7 days but the situation may change as more molecular tests may be required as an emergency, also outside the field of infectious diseases. The clinical laboratory should therefore also integrate among its activities the NAATs applied in fields other than respiratory infections such as arboviruses and emergency testing for meningo-encephalitits and intra-partum detection of *Streptococcus agalactiae*. Such testing might be performed in a permanently functioning and greatly automated laboratory section that might then include the molecular diagnosis of the most prevalent viruses of the moment and those respiratory infections susceptible to antibiotics.

6. Cost–benefit

Amplification techniques with their higher cost but improved sensitivity and more rapid results should lead to better streamlining of therapy and decreased antibiotic use. At present amplification techniques are more expensive than conventional approaches with the most expensive being the fluorogenic-based real-time detection systems. At the technical side improved standardization, more automation and more widespread use will result in cost reduction to rates competitive with conventional methods. In addition, to assess the overall benefit of amplification techniques, not only the direct costs of PCR reagents and equipment should be taken into consideration but also the indirect cost savings such as decreased antibiotic use or decreased hospital stay. This remains to be further studied.

Several studies, mainly based on antigen tests, tended already to show the cost efficiency of rapid diagnosis of ARI resulting from reduced antibiotic use and complementary laboratory investigations but most significantly from shorter hospitalization and reduced isolation periods of patients (Barenfanger et al., 2000; Falsey et al., 2007; Hueston and Benich, 2004; Rocholl et al., 2004; Welti et al., 2003; Woo et al., 1997). Oosterheert et al. (2003) pointed out that the lack of cost reduction in available studies results from the small impact of microbiological investigations on the therapeutic decisions.

During epidemics it may also be important to rule out certain infections. An important saving in further diagnostic procedures is possible by the abolishment of tissue cultures and serologic tests in ARI. In addition the improved diagnostics is not without an educative aspect as illustrated by the high frequency of rhinoviruses in bronchiolitis (Gruteke et al., 2004).

A closer collaboration between clinicians and the laboratory has a high priority.

7. Concluding remarks

A number of aspects remain to be investigated. The implementation of quantitative tests could shed further light on the relation between virus load and the seriousness of the disease (Adachi et al., 2004), produce useful prognostic information and help in the differentiation between colonization and infection. More information could be gathered on the length of the post-infection carrier state as well as on the importance of subclinical infections and how prone these are for spreading infection. The importance of ARI viruses in chronic respiratory diseases such as COPD and cystic fribrosis could also be better evaluated.

Several other agents responsible for respiratory infections should be considered separately because of the specific clinical picture for which they are responsible: *C. psittaci*, *B. pertusssis* and *B. parapertussis*, *Coxiella burnetii* and *Pneumocystis jeroveci*.

The rapid molecular characterization of the previously unknown sars-coronavirus within a few weeks after the appearance of the disease and the discovery of bocavirus

illustrate the potency of NAATs for broadening the knowledge on 'hidden' viruses remaining to be discovered.

Furthermore in the organizational framework of the diagnostic laboratory, NAAT panels directed at other clinical syndromes such as meningo-encephalitis, sepsis, sexually transmissible diseases, hepatitis and others will have to be included.

In the very near future NAATs will probably not be done at the point of care, but this must remain an objective for further development of the technology.

The need for the detection of an ever-expanding number of infectious agents will exceed the possiblitity of mixed RT-NAATs. The task will be taken over by the next generation of diagnostics, the array technology that opens a wide access to the infectious agents (Ambrose and Clenly, 2006; Lin et al., 2006; Wang et al., 2006; Mahony et al., 2007).

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