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Multiplex PCR theranostics of severe respiratory infections

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Bruno Pozzetto

Author for correspondence
Laboratory of
Bacteriology–Virology–
Hygiene, University of
Lyon – GIMAP EA 3064,
University-Hospital of
Saint-Etienne, 15 rue
Ambroise Paré,
42023 Saint-Etienne
cedex 02, France
Tel.: +33 477 828 434
Fax: +33 477 828 460
bruno.pozzetto@univ-st-etienne.fr



Florence Grattard

Laboratory of
Bacteriology–Virology–
Hygiene, University of
Lyon – GIMAP EA 3064,
University-Hospital of
Saint-Etienne, 15 rue
Ambroise Paré,
42023 Saint-Etienne
cedex 02, France



Sylvie Pillet

Laboratory of
Bacteriology–Virology–
Hygiene, University of
Lyon – GIMAP EA 3064,
University-Hospital of
Saint-Etienne, 15 rue
Ambroise Paré,
42023 Saint-Etienne
cedex 02, France

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Scope of the problem

Acute respiratory tract infection is the leading cause of hospitalization for infants and young children in developed countries, and a major determinant of death in developing countries. In 2002, lower respiratory tract infections accounted for approximately 4 million deaths worldwide and 7% of all deaths that year [1]. Therefore, there is a huge need for new diagnostic tools that enable the detection of a broad range of respiratory pathogens, especially those that are difficult or impossible to obtain by conventional culture methods.

“A collateral consequence of the current influenza pandemic has also been to emphasize the difficulty of the diagnosis of influenza on the basis of clinical symptoms alone...”

The recent emergence or re-emergence of different viral threats is an additional reason for the development of accurate diagnostic methods allowing the detection of the causative agents; for example, SARS coronavirus [2], which was responsible for an epidemic in over 29 countries in five continents in 2002–2003, influenza A/H5N1, which first emerged in Hong Kong in 1999 and has caused 450 official human infections worldwide in 7 years, approximately 60% of them being fatal [101], and the current A/H1N1 influenza pandemic that arose in April 2009 in Mexico and has already killed at least 10,000 people in the USA alone [102].

Besides emerging agents, many new viruses have been identified as significant respiratory pathogens during recent years, all of them by molecular methods, and it is

important to take them into account in the diagnostic algorithms; they include human metapneumoviruses [3], new human coronavirus NL63 [4,5] and HKU1 [6], human bocavirus [7] and the novel polyomavirus WU [8]. With the development of nucleic acid screening in human samples from patients with infection of unknown origin, new respiratory agents will probably be added to this list in future years.

A collateral consequence of the current influenza pandemic has also been to emphasize the difficulty of the diagnosis of influenza on the basis of clinical symptoms alone, and the necessity to propose an alternative identification when the search for influenza virus is negative, at least in the most severe clinical cases.

Theranostic paradigm

Molecular theranostics for infectious disease is an emerging concept in which molecular microbiology tools are needed to provide accurate and informative data, thus enabling better therapeutic intervention [9]. In the field of respiratory infection, the availability of rapid information regarding the putative agent(s) would have two important consequences. First, the differentiation between viral and bacterial pneumonia is often difficult, especially when atypical bacteria are involved, including *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae* or even *Legionella* species; a rapid identification of the agent would allow an adequate use of antibiotics, since macrolides, quinolones or tetracyclines are indicated in case of atypicals whereas antibiotics are not indicated in viral pneumonia, thus saving resistance and reducing costs. Second, the availability of antiviral drugs active against influenza

viruses, especially at the initial phase of infection, makes the rapid confirmation of influenza useful in order to decide whether or not to continue with antiviral treatment.

Concept of multiplex PCR assays in a panel of respiratory pathogens

Conventional microbiology tests, including bacterial culture, cell culture for viruses, rapid detection of bacterial antigens in urine (for *Streptococcus pneumoniae* and *Legionella pneumophila* serogroup 1) or viral antigens in respiratory secretions (using immunofluorescent, enzymatic or immunochromatographic assays), and serological testing, have been used to target a specific pathogen (or a subgroup of pathogens) but are not convenient to use, for either the clinician or the microbiologist, when the diagnosis must be broadened to a large number of pathogens. In addition, many viruses, including all the recently described ones listed earlier, are poorly or not at all detected by conventional testing.

Molecular tools have been developed extensively during the past few years for the diagnosis of respiratory infections, including in-house and commercial tests. Excellent recent reviews are available on these topics regarding either viruses only [10,11], or viruses and atypical bacteria [12]. In addition to the tests targeting a single pathogen or a small group of pathogens (influenza viruses, rhino/enteroviruses or atypical bacteria), the concept of multiplex PCR assay has recently emerged; this enables the simultaneous detection of a wide range of viral and bacterial agents involved in respiratory infections. The multiplex approach was proposed a few years ago [13] but initially lacked sensitivity in comparison with monoplex techniques. The use of multiple PCR tubes may circumvent this lack of sensitivity [14]. Alternative strategies minimizing the competition between the probes have been developed to allow for the mixture of all the primer pairs in the same reaction tube without loss of sensitivity. The currently available tests, some of them from commercial source with US FDA or CE approval, allow the simultaneous detection of 12–23 pathogens, including viruses and bacteria [15–24].

Following the amplification step, different strategies can be used to detect the PCR product(s):

- Fragment analysis or capillary electrophoresis, each probe having a different size [20,22]
- Fluid microbead-based assay [17,18]
- Hybridization on line blots [15,21] or microarrays [16,20,23,24]

The number and range of selected pathogens are different from one technique to another and may evolve through time with the need for investigating additional pathogens. The main viruses targeted by these techniques are influenza viruses (including A/H5N1 and A/H1N1 pandemic strain 2009), human respiratory syncytial viruses, human pneumoviruses, parainfluenza viruses, human coronaviruses (including 229E, HKU1, NL63, OC43 and SARS-CoV), human rhinoviruses A, B and C, human enteroviruses (species HEV-A–D), human respiratory adenoviruses, human bocaviruses, and the novel WU polyomavirus. Regarding bacteria, some assays include culturable microorganisms (e.g., *Staphylococcus aureus*, *S. pneumoniae*, *Streptococcus pyogenes*,

Moraxella catarrhalis, *Haemophilus influenzae* and *Klebsiella pneumoniae*), while others target non- or fastidious-growing agents (*M. pneumoniae*, *C. pneumoniae*, *Legionella* species, *Bordetella pertussis* and *Mycobacterium tuberculosis*).

Clinical usefulness of multiple PCR assays

From a clinical point of view, multiple PCR assays offer many interesting features:

- They allow the correct use of antimicrobial drugs, including antibiotics and anti-influenza agents (as detailed earlier);
- They permit the set up of rapid isolation measures that can prevent the nosocomial spread of infection;
- They are cost beneficial since they contribute to reducing the duration of hospital stay [25];
- They allow the diagnosis of dual and even triple infections on the same clinical sample by using the same test [26], which can have consequences on the severity of the disease and on the therapeutic strategy;
- They bring epidemiological results that can be used to evaluate the seasonal circulation of some pathogens, even for patients who do not benefit from the test.

In addition, the diagnostic procedures are greatly simplified since most of the tests can be performed on the same respiratory sample in a single laboratory; all the results are available at the same time, allowing an early re-evaluation of clinical decisions (anti-infectious treatments and isolation procedures). In addition to the benefit for the patient, this global assessment has been shown to be very efficient in terms of money saving (CAD\$291 per child in [25]) despite the relatively high individual cost (CAD\$80 in [25]) of these tests in comparison with conventional ones.

Pending questions & concluding remarks

By now, the main constraint of the multiplex PCR assays is the high technology of these tests and the need for several hours of intensive laboratory work to generate the results. In most cases, samples cannot be treated individually and the results are usually given to the clinician 1 day after reception of the sample, which requires the onset of an empiric treatment in the meantime until the availability of the results. This also means that these tests could not be performed in emergency conditions if trained technicians are not present 24 h a day, 7 days a week. Another difficulty is raised by the interpretation of the results; whereas conventional methods or monoplex molecular techniques are limited by too many negative results, PCR multiplex assays have been shown to exhibit an important range of positive results, the difficulty being to recognize which agent is the cause of the clinical disorders and whether the association may play a role in the severity of the disease. As mentioned earlier, the role of coinfections has to be evaluated in light of these new diagnostic tools. Finally, in contrast to real-time PCR, these techniques are not quantitative and the persistence of residual genomes despite the absence of replicative activity may be a source of misleading interpretations.

Despite these limitations, the routine implementation of these new tools for the diagnosis of severe acute respiratory infections – in replacement of the conventional ones – will contribute to the re-evaluation of the clinical data in light of a list of first-line pathogens (whether bacteria or viruses). By now, this work is technically feasible and economically justified; it will contribute to building new algorithms for the diagnosis and treatment of severe respiratory infections.

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