A Review on Bacterial Respiratory Infections and Optimization of Their Identification

**ABSTRACT**

**Background:** The current narrative review aims to describe microbial agents causing pneumonia briefly. In addition, the ongoing review tries to introduce the diagnostic methods from biochemical to molecular tests used routinely and the promising molecular methods which will be used in near future.

**Materials & Methods:** PubMed was searched for all review and original articles related to the lung infection. Studies providing insights into clinical symptoms, microbiology, risk factors, and diagnosis were included.

**Conclusion:** Untreated respiratory infections are one of the most common health care problems worldwide. We tried to provide a collective view of new aspects of bacteriology and diagnosis methodology of lung infection detection.

**Keywords:** Respiratory infection, Pneumonia, Molecular diagnosis, Multiplex PCR

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Introduction

Respiratory infections are one of the most critical medical infectious diseases. Delay in diagnosis and lack of suitable cure lead to adverse outcomes. Different bacterial and viral agents contribute to the development and expansion of respiratory infections. So, the recognition between bacterial and viral agents is vital to determine therapy protocols. The respiratory system is divided into two parts: upper and lower. Infection occurs in both of these two parts. The upper respiratory tract consists of the nose, throat, larynx, and trachea, and the lower respiratory tract consists of the lungs and their sections, including bronchial tubes, bronchioles, and alveoli [1].

The most common type of lung infection is pneumonia. It has been known since ancient times. By definition, pneumonitis is a parenchymal inflammation of the lung, and if a microbe causes the inflammation, it is called pneumonia. These microbial agents could be bacterial, viral, and even parasitic agents. Lower respiratory tract infections (LRTI) are one of the most frequent causes of severe and fatal diseases in children and adults worldwide [1, 2].

In 1901, William Osler, in the fourth edition of his book "The Principles and Practice of Medicine," pointed out that pneumonia is the most murderous, and at the same time, the most pervasive disease. Despite passing more than a century since then, pneumonia has remained as a critical clinical syndrome. It is one of the ten main reasons of death amidst entire age groups in the United States, the sixth leading cause of death in people over 70 years old, and the most common infection leading to death [3]. As stated by the World Health Organization (WHO) [4], approximately 56 million deaths due to pneumonia were reported in 2001. Among which 10.6 million were children, and the majority of them lived in developing countries. Therefore, the burden of acute infectious respiratory diseases, especially pneumonia, seems to be high in children [5].

Three types of pneumonia have been identified based on the pathogens, their acquisition methods, and the clinical outcomes, including: i) Community-acquired pneumonia [6], ii) Hospital-acquired pneumonia (HAP) and iii) Pneumonia in patients with suppressed immunity [7]. About 40 to 60% of mentioned diseases remain unidentified due to the lack of patient referral to physicians, lack of referring patients to the laboratory, and the exclusion of samples from further analysis. Another reason is the variability in the sensitivity and specificity of conventional diagnostic methods. Antimicrobial agents could successfully treat most of these infections. Identifying the causative agents is of particular importance, especially in connection with public health and planning for the disease treatment. Diagnostic tests play a crucial role in choosing appropriate antimicrobials and achieving the desired clinical outcomes [2, 7].

In several studies, Streptococcus pneumonia has been responsible for most of community-acquired pneumonia. The highest mortality rate due to this kind of pneumonia has been reported to be in patients requiring hospitalization. The reported mortality rate in these patients was 23% for 30 days. On the other hand, the mortality rate in all patients with community-acquired pneumonia [8] reached 28% during a year after the start of infection [9, 10].

The etiology of the community-acquired pneumonia varies depending on the geographical region. Although in undeveloped and developing countries, pneumonia is a reason for childhood death and the major cause of admission to hospitals and one of the most important causes of death in adults, but inadequate basic epidemiological studies have ended to inability to connect etiological...
and prognostic factors in mentioned societies (9, 11-13). Unfortunately, few studies have been done in our country on the clinical features of this common health problem and its etiological factors so far. Hospital-acquired pneumonia (HAP) is a parenchymal lung infection which is not caused by infectious agents existing at the time of admission, instead acquired by a patient during a period for at least 48 hours after hospitalization. HAP is considered as a common and serious problem in hospitals (14). In hospital-acquired pneumonia (HAP), Gram-negative organisms (e.g. Klebsiella pneumoniae and Pseudomonas spp.) and Gram-positive bacteria (e.g. Staphylococcus aureus) have been observed in most cases (1, 15).

HAP is the second most common nosocomial infection standing after urinary tract infection (which is accounted for 30% of all nosocomial infections). Some studies have estimated that the incidence rate of HAP is between 15 and 20% of all nosocomial infections. In patients admitted to ICU, hospital-acquired pneumonia is the most quotidian nosocomial infection leading to death in 20 to 70% of cases. Various studies have reported 10 to 20% nosocomial pneumonia in ICU (15-18). In a study carried out in Jordan, the overall rate of HAP infection in internal and surgical ICUs was reported as 17% (17, 19). The results of a prospective multicenter study on 46 hospitals in Central and North America, India, Morocco, and Turkey indicated that the HAP infection rate might be higher in developing countries (19, 20).

The pathogens involved in the LRTIs were classified into three groups:

1. Typical or conventional bacteria, including Haemophilus influenzae, S. pneumoniae, S. aureus, and Gram-negative bacteria.

2. Respiratory viruses, including respiratory syncytial virus (RSV), Influenza and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

3. Non-typical bacteria, including Chlamydia pneumoniae, Mycoplasma pneumonia and different Legionella species.

The atypical term represents the difference in clinical signs associated with each of the three mentioned pathogens groups. Of course, other factors could also be involved in LRTIs, including fungi, anaerobic bacteria, and Mycobacterium tuberculosis.

The mortality rate due to bacterial pneumonia is 2.7 times higher than viral pneumonia (1, 2, 21). Viruses could also lead to other respiratory infections, including Severe Acute Respiratory Syndrome (SARS) (a type of pneumonia or infection of the lung) and bronchitis whose chronic form could result in COPD (chronic obstructive pulmonary disease). Pharyngitis is an inflammation and infection of the throat and tonsils, called sore throat; in addition to viruses, S. pyogenes alone accounts for about 30% of all sore throats (22).

Nowadays, world experience a pandemic crisis due to the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). In late December 2019, SARS-CoV-2 triggered the outbreak of pneumonia from Wuhan, China; which is known as a global health threat (23). SARS-CoV-2 is a Coronavirus belonging to the betacoronavirus. COVID-19 is Corona’s third known zoonotic virus disease after SARS and Middle East Respiratory Syndrome (MERS), both belong to the betacoronaviruses (24). The SARS-CoV-2 shows an extremely high transmission power. The angiotensin-converting enzyme 2 (ACE2) is the receptor for the SARS-CoV-2. Comprehensive expression of ACE2 in various cells, such as airway epithelia, lung parenchyma, the upper esophagus, and absorption enterocytes of the ileum and colon, may play a role in SARS-CoV-2 multifactorial infection (25, 26). The virus particles transmit via droplets, close
contact, aerosols, and possibly faecal-oral transmission \(^{(27,28)}\). The diagnosis of COVID-19 relies on radiological and laboratory findings. Radiological examination is critical for early diagnosis. Conventional diagnostic testing methods, such as antiviral antibody testing or viral antigen have been clinically developed and used \(^{(27-29)}\). Samples used for laboratory testing include nasopharyngeal swab samples, sputum, or lower respiratory tract. The confirmatory diagnostic test is usually Real Time-PCR, which detects the existence genome of the ribonucleic acid virus \(^{(30)}\).

In this review, the bacteria causing respiratory tract infections are introduced, and in the next step, the experimental methods used to recognize the cause of infections are explained.

**Bacterial agents of respiratory infections**

**Nocardia spp**

*Nocardia* spp. are Gram-positive, aerobic, non-motile, and catalase-positive bacteria. Many *Nocardia* species have been reported to be responsible for different human and animal illnesses. They belong to aerobic actinomycetes. *Nocardia* infections usually require an early diagnosis. *N. asteroides* complex and *N. brasiliensis* are considered as common causes of nocardiosis in immunocompromised individuals. Pulmonary nocardiosis (PN) is an uncommon but potentially life-threatening and opportunistic infection in humans \(^{(31-33)}\). On standard laboratory media, after incubation at 35–37°C for several days, they develop as heaped, irregular, and waxy colonies.

**Mycobacterium tuberculosis**

*Mycobacteria* are rod-shaped and aerobic bacteria. *M. tuberculosis* is the cause of tuberculosis (TB) in humans and the first cause of death associated with a single pathogen worldwide. This bacterium is aerobic and resistant to antibiotics. It could stay hidden in humans and is the main mycobacterial pathogen in terms of the global number of patients and the severity of the disease. It uniquely interacts with innate immune cells through different pattern recognition receptors \(^{(34-36)}\).

Both selective and non-selective media must be used to primarily culture mycobacteria. Agar-based (solid) media are used for observing morphology and detecting mixed cultures in antimicrobial susceptibility testing \(^{(37)}\).

**Bordetella pertussis**

*B. pertussis* is a Gram-negative, obligate, and aerobic microorganism. It is the causative agent of pertussis, and exclusively a human pathogen. At present, two types of vaccines exist to prevent the disease. These bacteria attach and proliferate extracellularly in the airways and secrete several toxins with a variety of local and systemic effects on the host cells \(^{(38-41)}\).

**Bordetella parapertussis**

*B. parapertussis* is a Gram-negative bacterium and the whooping cough etiological agent, which has the ability to elude the immune response stimulated by pertussis vaccines. This bacteria can cause an illness similar to whooping cough but with commonly less severity \(^{(42)}\). The primary isolation of this bacteria needs an enriched medium called Bordet-Gengou (potato-blood-glycerol agar); to which the addition of penicillin G could be useful. Besides, a charcoal-containing medium supplemented with cephalexin, horse blood, and amphotericin B (Regan-Lowe) is more preferred. The cultures are aerobically incubated at 35–37 °C for 3–7 days in humidity \(^{(37)}\).

**Mycoplasma pneumoniae**

*M. pneumoniae* is a unique cell wall-free
bacterium and a significant reason for respiratory tract infections in different ages and in different species. It can cause a variety of diseases and symptoms. *Mycoplasma* is predominantly transmitted through human-to-human contact. 

*Mycoplasmas* for culture needs to media supplemented by serum or cholesterol, a metabolic substrate such as glucose, growth factors such as yeast extract, and incubation at 37°C for 48–96 hour. There is no unique optimal medium for all species \(^{(37, 43)}\).

**Haemophilus influenzae**

*H. influenzae* is a Gram-negative coccobacillus containing a capsule which allows to resist phagocytosis. This organism causes epiglottis infection, and hospitalized patients are more at risk. It is the most common bacterial cause of lower airways infection, which occurs in adults with chronic obstructive pulmonary disease and facilitates the long-time retention of pathogen in the airways. Continuous airway infection contributes to the chronic inflammation. One of the prominent features of this disease is the progressive loss of lung function. Nasopharyngeal colonization provides a resource for strains which pollute the lower airways and lead to severe exacerbations. Continuous infection in the lower airways accelerates the progression of the disease. No vaccine is yet available to prevent the infection \(^{(44-46)}\).

The medium for culturing this bacterium is chocolate agar, and colonies appear as flat, grayish, and translucent with 1–2 mm diameters after 24 hrs of incubation at 37°C \(^{(37)}\).

**Klebsiella pneumonia**

*K. pneumoniae* is a Gram-negative, anaerobic, non-motile and opportunistic bacterium. This organism has a polysaccharide capsule mediating resistance against phagocytosis, which is important in immunocompromised people. *K. pneumoniae* causes a wide range of infections like urinary tract infection, pneumonia, meningitis, and liver abscesses. Studies have shown that clinical specimens of *K. pneumoniae* have been isolated from bronchi, urea, blood, catheter, rectal, bile, trachea, and ulcer \(^{(47-48)}\). This bacterium is plated on both blood agar and differential media \(^{(37)}\).

**Moraxella catarrhalis**

*M. catarrhalis* is a Gram-negative, oxidase-positive, and aerobic bacterium. This bacterium is a human pathogen which could infect the respiratory tract, middle ear, eye, central nervous system, and human joints. It is the leading cause of middle ear infection in children and chronic obstructive pulmonary disease in adults. There is no vaccine for *M. catarrhalis*. Most children (80%) under-3 years, experience at least one episode of Otitis media (OM), and half of them suffer from OM infection in different parts. OM is the most common infectious disease in Iran \(^{(49-52)}\).

**Acinetobacter baumannii**

*A. baumannii* is a Gram-negative, aerobic, opportunistic, and prevalent pathogen. *A. baumannii* causes a wide range of infections, including pneumonia, urinary tract infections, skin and soft tissue infections, meningitis, and bacteremia \(^{(53, 54)}\).

**Pseudomonas aeruginosa**

*P. aeruginosa* is a naturally widespread Gram-negative bacterium known as an opportunistic human pathogen. It is a type of bacteria known to produce strong biofilms. *P. aeruginosa* is the most common pathogen among *Pseudomonas* species. This bacterium causes mild infections such as OM or foreign otitis media. Although, in healthy people, it could rarely cause serious problems; *P. aeruginosa* is known to be the main leading cause of death in people suffering from cystic
fibrosis \(^{(55)}\). In addition, \(P. \text{ aeruginosa}\) causes pneumonia, endocarditis, meningitis, wound infection, and burning. These infections are associated with a high mortality rate \(^{(56, 57)}\). \(P. \text{ aeruginosa}\) and \(A. \text{ baumannii}\) simply grow on various media and occasionally produce specific odor. Some strains have hemolysis ability \(^{(37)}\).

**Staphylococcus aureus**

\(S. \text{ aureus}\) is an anaerobic, Gram-positive bacterium that often exists in the upper respiratory tract and on the skin. 50% of its cell wall is peptidoglycan by weight. It is an opportunistic pathogen causing many diseases. A number of \(S. \text{ aureus}\) strains are able to remain in macrophages and neutrophils during phagocytosis process, which can also play a role in pathogen release \(^{(58-60)}\). Culturing of this bacterium is done by inoculating sputum to blood agar and incubating the plate in \(\text{CO}_2\) at 37°C \(^{(37)}\).

**Streptococcus pneumoniae**

\(S. \text{ pneumoniae}\) is a Gram-positive, non-motile, non-spore forming, alpha-hemolytic (in aerobic), or beta-hemolytic (in anaerobic) bacterium. \(S. \text{ pneumoniae}\) strains are usually found in the form of diplococci bacteria \(^{(29)}\). It contains polysaccharide capsules which is crucial to prevent phagocytosis. As stated by WHO, \(S. \text{ pneumoniae}\) is the fourth most common microbial cause of death and the cause of mucosal and invasive diseases with a high mortality rate. This pathogen belongs to the Streptococcal mitis group and is part of the normal flora of the upper respiratory tract \(^{(61-64)}\). \(S. \text{ pneumonia}\) growth increases at 5-10% \(\text{CO}_2\) \(^{(29)}\).

**Streptococcus pyogenes**

\(S. \text{ pyogenes}\) is a Gram-positive, aerobic, non-motile, non-spore-forming cocci. It is extracellular bacterium. \(S. \text{ pyogenes}\) is a beta-hemolytic organism responsible for a variety of human illnesses, commonly referred to as purulent pharyngeal and nasal diseases. This bacterium shares common traits and similar strategies for causing infection in other organs of the host. Aggressive infection with oral \(Streptococcus\) is a “complete storm” that need the concerted function of several biotic and abiotic agents. \(Streptococcus\)-associated morbidity and mortality rate is significant in public health worldwide. It could cause mild illnesses, such as strep throat and sea burn, or less frequent but dangerous diseases, such as necrotizing fasciitis and streptococcal toxic shock syndrome \(^{(65-68)}\). \(Streptococcus\) growth on the media is weak unless enriched with blood. By adding several growth factors and incubation at 37°C with 10% \(\text{CO}_2\), its growth and hemolysis are boosted \(^{(37)}\).

**Fungal respiratory infections**

Respiratory tract fungal infections and invasive fungal diseases (IFD) are the most common causes of death in patients with immunosuppression. This is particularly true in patients undergoing cytotoxic or radiotherapy treatment for neoplastic diseases, patients who undergo bone marrow or organ transplantation, or patients with acquired immunodeficiency \(^{(69)}\). Fungi could cause pulmonary infections in humans by various mechanisms. Some fungi such as \(Histoplasma capsulatum\), \(Coccidioides immitis\), and \(Blastomyces dermatitidis\) are primary pathogenic therapies which could cause infection in healthy people. These fungi are found in specific geographical areas and usually cause mild or subclinical infections. Acute or chronic primary pulmonary diseases with or without systemic diffusion may sometimes lead to death \(^{(70, 71)}\). The second group of fungi includes opportunistic fungi such as some species of \(Aspergillus\) and species of \(Candida\), which cause problems in patients with weakened immune systems or with systemic...
or pulmonary disease. These fungi may appear to be saprophytes, called mycetoma, invading the organism and weaving them to destruction (72-74). Breathing a certain amount of fungal mass could be the cause of a non-allergic toxic pulmonary reaction, such as pulmonary mycotoxicosis (75).

The most common fungus isolated from among Candida spp. (74.7%) is the fungus causing pulmonary candidiasis. Pulmonary candidiasis may be caused by primary bronchopneumonia or may be secondary that spreads through the blood flow. Primary Candida bronchopneumonia may occur in patients severely debilitated due to tumorigenesis, neutropenic patients with extensive chemotherapy, and very low birthweight infants (75). Aspiration of infectious oral secretions in the bronchi and their dissection into pulmonary parenchyma are the main contributing factors to Candida bronchopneumonia. Secondary pulmonary candidiasis that spreads through the blood flow is a common pulmonary infection in neutropenic patients with diffused infection (70-72). The second most common fungus is Aspergillus (17.3%). The outbreak of pulmonary epithelial aspergillosis has been reported in immunosuppressed patients. Aspergillosis is a name encompassing a wide range of pulmonary diseases in different forms (72, 76).

Diagnosis
The patient's history assists in recognizing specified risk factors and conditions associated with specific pathogens (77). For example, depending on the stage, human immunodeficiency virus (HIV) infection would raise concern about infections caused by S. pneumonia, M. tuberculosis, Pneumocystis carinii, and other opportunistic pathogens, or in a patient with alcoholism history, S. pneumoniae, K. pneumoniae, or perhaps anaerobic bacteria could be suspected as the causes of pneumonia (77, 78).

Bacteria are recognized to entail primary infections or superinfections, which in most cases need a direct therapy. Respiratory tract infections respond to antibiotic treatment, although antibiotics misuse might often lead to resistance since respiratory tract infection is particularly widespread in middle-income countries. Monitoring the patterns of antimicrobial resistance in causative microorganisms is necessary not only to help physicians to treat cases requiring antibiotic therapy but also to regard the tendency of these infections (79).

Unfortunately, for most pathogens, the best test does not yet exist. Identifying the cause of infection allows clinicians to choose proper therapy. These methods include sputum Gram staining and culturing, serological tests, antigen detection tests, blood cultures, and molecular techniques (80).

Microbiologic studies
The specimen should be collected by a professional medical staff and transported directly to the lab for examination before any antibiotic therapy. Monitoring the cytological state of the sputum should be done under a low-power magnification before performing bacteriological evaluations. For more assessments, the infectious disease society of America/American thoracic society (IDSA/ATS) guidelines recommended performing complete liver function tests, chemistry panel, blood count, oxygen saturation, and HIV antibody screening (81).

Standard methods of detecting respiratory pathogens
Culture
Culturing is the most common method used for detecting bacteria causing respiratory infections. It is pursued by identification and antiprogram tests via different methods (82).
When a sample is not specified by Gram stain or collected after antibiotic administration, bacterial culture can be insensitive. Bacterial culture is laborious and requires significant technical experience and takes 48–96 hrs if antibiogram testing is also performed; hence, it could be considered as insufficient for optimum patient care and ineffective for antimicrobial therapy (82-85).

Serological Tests
Serological testing is exclusively incentive for bacteria, especially for recognizing atypical bacteria (e.g. M.pneumoniae). Serological testing is not as clinically beneficial as PCR, with limited sensitivity and specificity as opposed to PCR (87,88). The clinical usefulness of serological tests is also limited because they need both acute and convalescent sera of a patient to recognize a four-fold rise in antibody titer (88).

Determining isolated species
The determination of the desirable isolates is performed by standard microbiological tests, including Gram staining responses, morphological scrutiny of colonies, and a series of biochemical tests (89).

Antibiotic susceptibility testing
Antimicrobial sensitivity testing (antibiogram testing) was performed by the disc diffusion or Kirby-Bauer method. Antimicrobial agents used for the following tests on Mueller Hinton agar plates were as follows: cefuroxime, chloramphenicol, gentamicin, co-trimoxazole, erythromycin, ceftriaxone, penicillin, ceftazidime, amikacin, ampicillin, ciprofloxacin, aztreonam, cefoperazone-sulbactam, cefepime, piperacillin-tazobactam, ticarcillin-clavulanic acid, and imipenem. The cultured plates were incubated at 37 ºC for 24 hrs to determine the susceptibility or resistance of specimens (90,91). Gram-stained smears and culture are of little importance; since in 25% of patients suffering from chronic obstructive pulmonary disease, bacterial colonization of the upper airway presents no symptoms (92).

In the next part, the molecular techniques assessing the DNA and RNA will be reviewed.

Polymerase Chain Reaction (PCR)
Polymerase Chain Reaction (PCR) is a fast technique for in vitro amplification of specific DNA fragment by two oligonucleotide primers, which anneal to opposite strands in the flanking region of target DNA. The cycles involve repetitive denaturation, annealing of primers, and elongation or extension of fragments by DNA polymerase, resulting in the accumulation of a particular product exponentially (93,94). The basic protocol of PCR is as follows: at the beginning, the DNA template, primers, DNA polymerase, deoxyribonucleoside triphosphates (dNTPs), and Taq DNA polymerase are mixed. The solution is cycled repeatedly (commonly 30 times) at temperatures permitting denaturation, annealing, and extension. The product is then loaded on a suitable concentration of agarose gel and analyzed in terms of size and specificity (95).

Multiplex polymerase chain reaction
The multiplex polymerase chain reaction is a kind of PCR, in which two or more target sequences are amplified using more than one pair of primers together in the same reaction mixture. The advantage of Multiplex PCR is the time and laboratory efforts’ saving. The method was first described in 1988, and has been applied in several fields of DNA analysis such as gene deletion, polymorphism, mutation, and reverse-transcription (RT)-PCR (96,97). Efforts were made to increase sensitivity and specificity and to facilitate automation, which led to the appearance of several publications.
on the use of multiplex PCR in the diagnosis of infectious agents, in particular those which target viral nucleic acids \(^\text{98}\). The change in other PCR components; including PCR buffer combinations, dNTPs, MgCl\(_2\), and enzyme; Multiplex PCR generally results in a substantial improvement in the sensitivity and/or specificity of the test by using concentrations higher than those used in most uniplex PCRs \(^\text{99}\).

In multiplex PCR, one of the most critical parameters is the Annealing temperature. Though many single loci could be explicitly amplified at 56–60 °C, it has been proven that in multiplex mixtures, annealing temperature needs to be lowered by 4–6°C to co-amplify the loci. A problem that is faced during running a multiplex PCR happens when the more effectively amplified loci show an adverse effect on the amplification of less effectively amplified loci. This is because of restricted availability of DNA polymerase enzyme and deoxynucleotides \(^\text{99,100}\).

Optimization of parameters can, however, provide a simple approach for some of the typical PCR multiplex problems (such as fake amplification products, undesirable or no amplification of specific target sequences and challenges in procreating those results) \(^\text{96,100}\).

With the increase in the number of bacterial agents detectable by PCR, it would be beneficial for practical purposes to simultaneously diagnose multiple agents which cause similar clinical syndromes and/or share similar epidemiological factors \(^\text{101}\).

**New approaches for diagnosing respiratory infections**

Regarding the early diagnosis of pneumonia, Britain, European, and US standard guidelines stated that there is a need for new tests and strategies to identify bacteria, causing respiratory infections, via molecular techniques rather than the gold standard culture method \(^\text{102}\). Table 1 demonstrates common methods available for diagnosis of bacterial respiratory infections as stated by CDC (Center for Disease Control and Prevention). Multiparametric molecular diagnostic tests are a new approach for identifying causative agents of complex infections such as RTI. In the following section, the most recent and widely used multiparametric molecular diagnostics tests currently used for RTI were introduced.

**Real-time PCR**

The real-time PCRs is considered as a sensitive, specific, and rapid detection method. In this method, post-PCR manipulations have been eliminated, and decrease the contamination risk. Another advantage of using real-time PCR to measure gene expression is its sensitivity, which means that it could even detect a single copy of the transcript in question \(^\text{103}\).

Two general methods of real-time PCR were presented as follows:

1. The first technique is the use of SYBR green which could be added to a color PCR mixture and bound to DNA to generate the fluorescent signal. SYBR green is cheaper and easier to use. This technique reports the total amount of double-stranded DNA available during PCR at any time. During each cycle of real-time PCR, the fluorescent signal is increased more and more by double-stranded DNAs binding to SYBR green.

2. Another technique is the use of a reporter probe that binds to the PCR products to generate a fluorescent signal, which is more common to use. This technique is known as the TaqMan 5’ nuclease assay. The probe used in this process is called reporter because it indicates the product in question. This method is less prone to errors resulting from incorrect connections, such as primer dimers \(^\text{104,105}\).

The respiratory MWSR-GENE® kits are ready-to-use molecular kits to detect...
causative agents of the respiratory infection by multiplex real-time PCR, which can detect 35 causative agents of respiratory infections in just 1.5 hours, separately. This test is only available in the USA. This method uses modular multiplex TaqMan® technology (106,107).

**PCR microarray technology**

A DNA microarray (also known as a DNA chip) consists of a collection of DNA oligonucleotides attached on a slide. Microarrays have been extensively used in infectious disease research and have helped us better understand environmental responses and overall gene expression in microorganisms. So far, much research has been done on infectious agents, and microarrays of some species have been designed. Multiplex PCR along with amplified PCR hybridization linked to specific probes in a microarray chip, is a multiparametric technology to diagnose bacteria causing the infection (104,108). The Respiratory Multiplex Array is used to detect 22 respiratory pathogens simultaneously in people suffering from the respiratory tract infection. The capability of determining up to fifteen respiratory viruses and seven respiratory bacteria in one sample provides a detailed patient profile for clinicians and allows them to make more effective and rapid therapeutic decisions. The respiratory multiplex array is based on multiplex PCR, microarray hybridization, and chemiluminescence detection by the Evidence Investigator analyzer. This array could simultaneously run 54 samples in five hrs (102).

**PCR Mass Spectrometry**

Mass spectrometry (MS) examines the mass-to-charge ratio of molecules using electric and magnetic fields. One of the new techniques based on MS is MassTag PCR (109). MassTag PCR is a combination of multiplex PCR with different MS technologies to identify a broad range of infectious agents. The difference with conventional PCR is that the primers used for MassTag PCR are identified with MassCodes. This method uses several primers instead of a single pair of primers, converting it into a multiplex system. Unlike the conventional multiplex PCR, more than 15 pairs of primers could be used in this method. The DNAs of all primer panel agents are amplified. Each amplified product has its own specific MassCodes. Then the PCR product is purified to eliminate the remaining primers and other impurities. Specific PCR products emit light due to chemical bonding between the nucleic acid and the primers. Since MassCodes are released from PCR products, they are identified by mass spectrometry. The existence of specific MassCode represents the presence of a particular pathogen (104,110). The PLEX-ID system is based on Electrospray Ionization MS (ESI-MS), which make it possible to diagnose and characterize a wide range of respiratory bacteria and viruses without focusing on the determination of each pathogen, separately (102).

**Luminex platforms**

The Luminex xTAG platforms are based on the use of liquid-phase array technology to amplify multiplex real-time PCR. The first FDA-approved multiple tests was the xTAG RVP method used to detect respiratory tract viruses. Protocols include nucleic acid extraction, multiplex real-time PCR, single-array hybridization, and detection in the Luminex 100/200 or Magpix (111). Two commercial kits, including Resplex I and II (Qiagen), were designed to detect bacterial and viral RTIs. The Resplex I kit is used to identify one virus and six bacteria, and the Resplex II kit is specifically designed to determine 13 viruses (107).
Respifinder SMART 22
The Respifinder SMART 22 is a multiplex real-time PCR used for the simultaneous determination of 22 respiratory pathogens (18 viral and four bacterial agents of RTI) within 2.5 hrs. The sensitivity and specificity of this technique is the same as monoplex real-time PCR. Smartfinder diagnostic technology is a multiplex-based probe-amplified real-time PCR that hybridizes two probes specific to each target into cDNA; thus, in LightCycler 480 or the Rotor gene Q / 3000/6000 (Qiagen / Corbett), real-time systems are analyzed (112).

Film array technology
The basis of the Filmarray system (Biofire Diagnostics) is a nested RT-PCR, pursued by a multiplex PCR based on its melting curve. The technology is highly sensitive and used to identify three bacteria and 17 viruses of respiratory infections (113,114).

Future Perspectives
Nucleic acid amplification tests (NAATs) are developed in laboratories to detect respiratory infections agents. In addition to their excellent performance (NAATs) because of their multiplex ability, accuracy, and specificity, they provide all the information needed for clinical decision making in the shortest time. They are performed with increasing complexity and high cost and require skilled personnel.

Discussion
The frequent microbial agents causing respiratory infection were reviewed in current study, and the techniques using in laboratory to identify them were briefly explained.
Given the value of rapid and precise identification of pathogens in respiratory infections, the use of an efficient method that could accurately and precisely identify them is one of the necessities of laboratories. In many countries, the use of molecular methods for detection of pathogens and determining their antibiotic resistance in medical microbiology laboratories is increasing. Molecular techniques have high sensitivity and specificity and could help identify bacterial or viral agents, simultaneously (1).
PCR is a fast and sensitive reaction used to diagnose even minimal amounts of DNA as an indicator of the presence of a microorganism. This method is more responsive and precise than other approaches and needs no bacterial viability in the test sample. On the other hand, in infectious patients, the rapid detection of the pathogen is of importance because sometimes if left untreated, the patient would die or develop an irreversible complication in less than 12 hours (10,115).
Although the results of some research indicate that advanced diagnostic methods are more cost-effective, the imposition of expensive costs in Iran has limited the use of advanced PCR methods. For this reason, many efforts are underway to design a cheap and rapid method that, in the first step, could prove the existence of a wide range of organisms and, in the second step, could identify them, specifically (116).
One of the types of PCR methods is the multiplex PCR method, which has attracted much attention for a rapid and simultaneous detection of infectious agents, including bacterial agents of respiratory infections. This method could act as a suitable complementary technique; particularly when the results of staining, culture, and serological identification methods are vague (117, 118).
Although determination of the viral respiratory infection most of the time relied on real-time PCR; culture is the gold standard for a definite detection of bacterial respiratory infections. Unfortunately this procedure is affected by antibiotic treatment,
and the way of transferring the specimen could cause bacterial death \(^{(96)}\). In addition, this procedure is time-consuming, especially in the case of hard-growing bacteria, while the patient may have no chance to survive. In this regard, the molecular methods can provide good and reliable results; however, the labor-consuming and high expenses are the challenge that we faced when using the molecular methods in everyday laboratory activity. For instance, by sketching different primers, researchers expand a PCR method to identify bacteria causing respiratory infection, but the diagnosis of a bacterium is not necessary for a patient. Hence, the introduction of a multiplex PCR method that could cover the least number of common bacteria is of interest \(^{(119,120)}\).

In respiratory specimens due to high microbial load, the use of primers with low specificity could lead to false-positive results. Therefore, PCR reaction with primers designed from conserved regions could not provide exact results. Hence, in this research, specific primers were used to prevent undesirable responses in performing multiplex PCR \(^{(11,112,113)}\). In this particular technique, choosing the right target genes to amplify is crucial and can affect the specificity of the test \(^{(10,123)}\).

**Conclusion**

Convenience, simplicity, and low time needed for performing molecular methods are principal advantages for simultaneous diagnosis of aforementioned pathogens causing respiratory infections. These methods provide better specificity and sensitivity than other currently available biochemical methods in terms of cost and

| Name                     | Method of Diagnosis                                      |
|--------------------------|----------------------------------------------------------|
| *Mycoplasma pneumoniae*  | Real time PCR                                            |
| *Bordetella pertussis*   | Enzyme-linked immunosorbent assay                        |
| *Bordetella parapertussis* | Enzyme-linked immunosorbent assay                        |
| *Haemophilus influenzae* | Sequence based identification                             |
| *Staphylococcus aureus*  | 16S Sequencing, MALDI-TOF, phenotypic testing            |
| *Streptococcus pneumoniae* | Phenotypic testing, molecular testing                    |
| *Streptococcus pyogenes* | Phenotypic testing, molecular testing                    |
| *Klebsiella pneumoniae*  | PNA-based assay, PNA-based assay, fish                   |
| *Pseudomonas aeruginosa* | PNA-based assay, fish                                    |
| *Nocardia*               | MALDI-TOF, 16S sequencing                                 |
| *Moraxella catarrhalis*  | MALDI-TOF, 16S sequencing                                 |
| *Mycobacterium tuberculosis* | TMA, 16S sequencing, MALDI-TOF                           |

Table 1: Molecular Laboratory Methods for the Diagnosis of Respiratory Infection Bacteria Based on CDC Protocols (https://www.cdc.gov/laboratory/specimen-submission/cdc-lab-tests.pdf).
manpower in addition to reducing the risk of staff contamination and the spread of infection in laboratories. Therefore, these methods could also be used to detect these agents in clinical specimens. In fact, this study was an introduction to the application of this procedure for clinical specimens.

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