NEW APPROACHES FOR THE LABORATORY DIAGNOSIS OF LEGIONNAIRES’ DISEASE

Summary of the study results and General Discussion

The key role of diagnostic tests in clinical microbiology is the accurate and timely detection of an etiologic agent(s) causing infection to enable clinicians to provide the most effective therapy in an early stage. Unfortunately, the ideal test for *Legionella* spp. does not exist. Although diagnostic methods have improved during the 30 years since *L. pneumophila* was first described, no currently available test is able to detect all *Legionella* spp. timely with optimal sensitivity and specificity.

Serological tests for Legionnaires’ disease (LD) were frequently used as diagnostic test, especially in the time after the discovery of LD. The need for testing paired serum samples collected at least 3 weeks apart has diminished the use of serology, although it remains a valuable tool for the definitive diagnosis [1,2]. Indirect immunofluorescent assays (IFA) and enzyme-linked immunosorbent assays (ELISA) are the most commonly used methodologies for *Legionella* serology [3]. The ELISA technique generally shows higher sensitivity [4,5,6] and better characteristics in terms of both automation and objective measurement than IFA. Diagnosis requires both acute and convalescent sera, because an antibody response may take up to 12 weeks (and sometimes longer) to develop [1]. The availability of commercial ELISA and IFA kits has resulted in an increasing use of these tests, despite that only few studies determining their sensitivity and specificity are available. In chapter 2 we evaluated commercial IFA and ELISA assays (Vircell, S.L., Santa Fé, Granada, Spain) in a well described population of patients with and without LD. We included 129 serum samples of 65 patients with proven LD (cases), and 50 serum samples of 29 patients with respiratory tract infections other than Legionella (controls). Also, we determined the
agreement, sensitivity and specificity of the different VIRCCELL assays in comparison to a validated ELISA assay (SERION classic ELISA L. pneumophila serogroup 1 to 7 assay). Samples were tested for L. pneumophila serogroup 1, IgM and IgG antibodies by an IFA procedure, L. pneumophila serogroup 1, IgM and IgG antibodies by ELISA, and L. pneumophila serogroup serogroups 1-6 in an IgM plus IgG combined ELISA assay, according to the manufacturer’s instructions.

Clinical sensitivity and specificity were respectively 75% and 97% for IFA IgM and 65% and 88% for IFA IgG, 92% and 100% for ELISA IgM, 43% and 97% for ELISA IgG and 91% and 100% for IgM plus IgG combined ELISA. If the new test to be evaluated, the Vircell assays, are compared to the alloyed standard, Serion ELISA, the calculated agreement, sensitivity, and specificity are 79%, 66% and 89%, respectively, for IFA IgM, and 75%, 59% and 84%, respectively, for IFA IgG, 90%, 97%, and 83%, respectively, for the Vircell IgM ELISA, 82%, 55%, and 96%, respectively, for the Vircell IgG ELISA, and 94%, 96%, and 92%, respectively, for the Vircell IgM and IgG combined ELISA.

That the ELISA techniques showed an equal or greater sensitivity than the IFA was not surprising, this was already reported in previous comparisons [3,5,6]. High sensitivity is important in an assay, since the assay should detect the greatest possible number of patients with LD. However, a more important test variable for an illness of low prevalence, such as LD, is the specificity [3,7]. In a study by Rojas et al.[4], Vircell ELISA for IgM, Vircell ELISA for IgG plus IgM, and Vircell IFA for IgM detected 72%, 61% and 51%, respectively, of outbreak-related patients. Our evaluation included patients from various sources in a non-epidemic setting and therefore differs from studies on patients in an epidemic setting, where only a single L. pneumophila strain is evaluated. In addition, outbreak-related studies, with a relatively high prevalence of the disease, provide insufficient information regarding the positive predictive value of serological assays. Our results indicate that the predictive value of
a positive diagnostic result obtained by the Vircell IgM IFA and the Vircell IgG IFA will be low when used in a population with a low prevalence of LD. The Vircell IgM ELISA and the IgM-plus-IgG ELISA show high sensitivity and specificity and therefore a reliable serodiagnosis can be made using these assays. The Vircell IgG ELISA shows moderate sensitivity, but high specificity, and could be included together with IgM or IgM-plus-IgG for optimal clinical decision making.

A strength of the evaluation is that we used a well described population of patients with proven LD. An important shortcoming is the inclusion of only a small group of patients with respiratory tract infections other than LD as control patients, which could influence the specificity figures. Also, the majority of LD-positive patients were infected with *L. pneumophila* serogroup 1, making it impossible to conclude anything for *L. pneumophila* infections caused by other serogroups. Although most serological assays yield good sensitivity and specificity data [1,3,8,9], the delay in the development of a measurable antibody response constitutes a major drawback for clinical decision making in the acute patient.

**Chapter 3** describes a case of probable LD in which the diagnosis was complicated by the presence of cross-reacting antibodies to *C. burnetii*, emphasizing the need for cautious interpretation of antibody titers for members of the family *Coxiella* and *Legionella*. In the acute stage of disease, results of ELISA for *L. pneumophila* were negative, but elevated antibody levels of IgM (74 U/ml) were found. These were higher compared to what can be expected from the prevalence of measured Serion ELISA IgM antibodies in the normal population [10]. Because *Legionella* and *Coxiella* are responsible for similar clinical syndromes, this may lead to an incorrect diagnosis and probably less effective treatment. This underscores the importance of a laboratory professional that reports laboratory results with
both awareness of the laboratory procedures that have been performed and knowledge of the consequences of these results for the patients involved.

A urinary antigen test for the diagnosis of LD caused by *L. pneumophila* serogroup 1 was made available in the early 1980s [11,12,13], and its use has increased considerably in recent years. This test is sensitive and highly specific, and it allows a rapid diagnosis of LD. Rapidity of diagnosis is an important advantage of the urinary antigen test, because it means that cases can be detected early in the course of infection, when treatment decisions can be influenced. There is still a need to develop an antigen capture assay that for detection of infections with any species and serogroup of *Legionella*. The urinary antigen test has replaced other diagnostic methods and is now the major test both in the Netherlands and in Europe [2,14]. Its use may have led to a selection towards diagnosis of *L. pneumophila* serogroup 1 infections [15] with concomitant underestimation of the number of infections caused by other *L. pneumophila* serogroups and other *Legionella* spp. The most important feature of this test is its >99% specificity, a requirement for a test for a relatively rare disease.

In **Part 2** the evaluation of several newly developed immunochromatographic urinary antigen tests for the detection of *L. pneumophila* serogroup 1 in urine is described. The test was evaluated on stored (frozen) urine samples from a well-described sample of patients with and without LD. The results were compared with the Binax NOW urinary antigen test.

In **chapter 4** the sensitivities and specificities were estimated to be 71% (95% confidence interval [95% CI], 60 to 80%) and 97% (95% CI, 90 to 99%), respectively, for the Rapid U test (Diamondial, Sees, France); 32% (95% CI, 22 to 43%) and 99% (95% CI, 92 to 100%), respectively, for the SD Bioline test (SD Bioline test; Standard Diagnostics, Inc., Kyonggi-do, Korea); and 92% (95% CI, 83 to 97%) and 100% (95% CI, 95 to 100%), respectively, for the Binax NOW test. The sensitivities of the Rapid U test and Binax NOW
test increased to 81% (59/73; 95% CI, 70 to 88%) \( (p = 0.25) \) and 95% (69/73; 95% CI, 86 to 98%) \( (p = 0.75) \), respectively, if the tests were reexamined after an hour of incubation. Prolongation of the incubation time did not affect the specificity for the Rapid U and Binax NOW test.

The manufacturer of the Rapid U test adapted their test, and we evaluated this new test (Rapid U Plus test), described in chapter 5. Sensitivity and specificity were estimated to be 92% (65/71; 95% CI 82%-96%) and 96% (4/91; 95% CI 89%-99%) for the improved test. The sensitivity of both the Rapid U Legionella Plus Test and Binax NOW urinary antigen test increased to 93% (66/71; 95% CI 84%-97%) if tests were reexamined after 45 min. Prolongation of the incubation time significantly affected the specificity of the Rapid U Legionella Plus Test: it decreased to 80% (18/91; 95% CI 70%-87%). Again, prolongation of the incubation time did not affect the specificity in the Binax NOW urinary antigen test in this evaluation. We can conclude that the Binax NOW urinary antigen test is superior to the Rapid U test and the SD Bioline Legionella urinary antigen tests for the diagnosis of infections caused by \textit{L. pneumophila} serogroup 1. The performance of the SD Bioline test is below the acceptable level for any diagnostic assay. However, false positive test results occurred in all three new tests, with poor positive predictive values as a result. Given the potential impact of misdiagnosis of LD, it would seem unwise to rely on these tests for the diagnosis of LD.

Chapter 6. Another Urinary antigen test, the SAS Legionella Test (SA Scientific, San Antonio, Texas) showed a high degree of sensitivity (83%; 95% CI, 72 to 90%) and specificity (99%; 95% CI, 94 to 100%). The sensitivity increased significantly (97%; 95% CI, 90 to 100%) after a prolonged incubation time. Because prolongation does not affect specificity, we recommend to use an incubation time longer than what is currently recommended by the manufacturer. A drawback of the studies described in chapter 4, 5 and 6 is that we evaluated a relatively small group of patients; this means that the calculated
sensitivity and specificity figures for the assays are more uncertain. However, the studies provide relevant data related to the clinical sensitivity and specificity levels of these new commercial kits for the detection of *L. pneumophila* serogroup 1 antigen in urine.

The diagnosis of pneumococcal infections in patients with community-acquired pneumonia (CAP) is relying heavily on culture of *Streptococcus* (*S.*) *pneumoniae* from blood or other normally sterile fluids, and is therefore limited by prior administration of antibiotics. Given the difficulty in identifying precise microbial etiologies in patients with pneumonia, there is a clear need for new diagnostic tests. An immunochromatographic test, the NOW *S. pneumoniae* urinary antigen test (Binax, Inc., Portland, Maine), has been developed; the test is simple to perform, detects the C polysaccharide cell wall antigen common to all *S. pneumoniae* strains, and provides results within 15 min. Chapter 7 describes an evaluation of this test using unconcentrated urine samples from adults admitted to hospital with community-acquired respiratory tract infections. Antigen was detected in 40 of 58 cases overall, resulting in a test sensitivity of 69% (95% CI, 58 to 78%). Antigen detection was greater in patients with bacteremia (38 of 52 [73%]) than in those without bacteremia (2 of 6 [34%]) (*p* = 0.07). Urinary antigen was also detected in 3 of 136 adult patients with community-acquired respiratory tract infections caused by other organisms, giving a test specificity of 98% (95% CI, 93 to 99%). There were no false-positive results among tested patients (n=98) with LD. Detection of urinary antigen could be a valuable, sensitive, and rapid test for the early diagnosis of pneumococcal infections in adult patients [16,17,18]. Further prospective studies are warranted. This research should focus on the utility of a positive test result for safe, targeted use of a small spectrum penicillin for immunocompetent individuals with severe CAP.
Isolation of *Legionella* from respiratory secretions is considered the gold standard to define a case (100% specificity), but its reported sensitivity varies from 10 to 80%, and a positive result is not available until at least 3 days of incubation. Another limitation of sputum culture is that less than 50% of patients with LD actually produce sputum [3,19]. PCR techniques have the potential to provide a rapid diagnosis of LD on readily obtainable specimens such as serum or urine. The use is appealing, since it may provide an answer within a relatively short period of time. In accordance with our own findings, other investigators have tried to amplify *Legionella* spp. DNA in urine, but the test proved of low sensitivity (0-30%) [20,21,22]. In chapter 8 we describe two patients with LD diagnosed by both the urinary antigen test and PCR on serum samples. Quantification of *L. pneumophila* DNA using real-time PCR during the course of illness was carried out. The results of real-time PCR mirrored both the clinical condition and the C-reactive protein values during the course of illness. The detection of *Legionella* DNA in serum probably reflects changes in the quantity of bacteria in the bloodstream over time, and may, in theory, allow assessment of the response of the patient to treatment. However, the clinical course is also closely reflected by the CRP course, which has already been shown to bear prognostic potential in patients with pneumonia [23]. Future investigations might help to determine associations between amount of bacteria in blood or serum samples and clinical signs and symptoms. Due to the high cost and unproven (cost)-effectiveness, PCR is not recommended at the moment as a marker to monitor treatment response in patients with LD.

The application of PCR on serum samples is attractive, because this will circumvent the problem of patients who do not produce sputum and the inability of the urinary antigen test to reliably detect organisms other than *L. pneumophila* serogroup 1. In chapter 9.1 we assessed the performance of PCR as a rapid diagnostic method and compared the results of different PCR assays in serum samples from patients with LD. We performed a laboratory-
based study, with stored serum samples from patients with proven LD and patients with respiratory tract infections other than *Legionella*. Laboratory validation of several PCR assays was carried out using a panel of human-pathogenic and environmental *Legionella* spp. Three assays were used, targeted at specific regions within the 5S rRNA gene, the 16S rRNA gene, and the *mip* gene. The analytical sensitivity of the 5S rRNA based PCR assay was highest with a lower detection limit of 10 fg of chromosomal DNA. In the *mip* gene based assay, the lower detection limit was 100 fg, and 1000 fg was found as lower detection limit for the 16S rRNA based PCR. In the clinical evaluation, we included 68 patients with proven LD, with 151 serum samples taken in total. The key question of this evaluation was to know what the clinical utility of serum PCR is in the early phase of disease. Among the patients with proven LD, 54% tested positive in 5S rRNA PCR, 53% in *mip* gene PCR, and 31% in 16S rRNA PCR in the first available serum sample. Discrepant results between 16S rRNA gene, 5S rRNA gene and *mip* gene based PCR occurred, most probably due to differences in analytical sensitivity and because some samples might contain amounts of bacterial DNA at the limit of detection. In the majority (81%) of PCR positive patients of which both acute and convalescent serum was obtained, PCR was positive only in the acute serum sample. In contrast to the high sensitivity found by Lindsay *et al*. (80%), the sensitivity of PCR in our study was relatively low (31%-54%) [22]. In the PCR described by Lindsay [22], to confirm the specificity of PCR a Southern blot with a 50 basepair long digoxigenin-labelled probe is required. Due to its large size, as well as overlap in homology with species other than *Legionella*, this PCR assay may not be very specific. In 60 serum samples of patients with respiratory tract infections due to other pathogens than *Legionella*, we found 15 false positive results, using the same conventional 5S rRNA based PCR. This lack of specificity might explain the higher sensitivity described by Lindsay *et al.*
Because of the retrospective nature of our observations we were not able to investigate the relation between test sensitivity and severity of disease. However, the association between Ct value in 5S rRNA PCR positive serum samples (n=49) and CRP-value was determined and showed a strong negative correlation ($r = -0.63$, Pearson correlation coefficient, $p < .0001$).

The urinary antigen test is less reliable in milder cases of LD, and we think it is plausible that the same holds true for the detection of *Legionella* DNA in serum. In February 1999 an outbreak involving 188 cases of LD occurred in Bovenkarspel, The Netherlands. In chapter 9.2 we investigated the relationship between PCR test sensitivity and the severity of disease with a selection serum specimens from patients with outbreak-related LD. Severity of pneumonia was scored according to the criteria for severity of CAP advised by the American Thoracic Society. Patients were classified as category 1 (mild pneumonia and moderately severe) and category 2 (severe pneumonia). In patients with severe pneumonia, 49 % (19/39; 95 % CI 34–64 %) tested positive in the first available serum sample compared to 37 % (7/19; 95 % CI 19–59 %) in patients with mild and moderately severe pneumonia. An important limitation of this study is that we only tested a small number of patients in both groups and the fact that the serum samples were stored at -20°C and thawed repeatedly before the PCR assays were applied. The storage and thawing most probably influenced the stability of *Legionella* spp. DNA present in the samples.

Detection of *L. pneumophila* DNA in serum could be a valuable tool in addition to existing diagnostic tests for the rapid diagnosis of LD caused by any *Legionella* species and serogroup in the acute phase of disease. As our results show, the sensitivity of the detection of *Legionella* DNA in serum is relatively low (~50-60%) in LD patients, but will most probably be higher (~70-90%) in those patients with more severe disease. However, the ultimate proof for its presumed utility would lie in a prospective study to evaluate the value of *Legionella*-specific PCR on serum samples in patients with pneumonia.
In addition to *L. pneumophila*, 19 other *Legionella* spp. have been documented as human pathogens on the basis of their isolation from clinical material. Unfortunately, *Legionella* spp. infection does not present with a distinctive clinical syndrome; it cannot be reliably differentiated from pneumonia due to other bacterial pathogens on the basis of signs, symptoms or laboratory findings. Patients with non-*pneumophila* Legionella infections are more likely to be immunocompromised than are patients with *L. pneumophila* infection [24]. Infection with non-*pneumophila* spp. in immunocompetent patients is considered to be extremely rare. Chapter 10 describes the case of a patient who developed a severe CAP with unknown etiology. A single sputum culture and urinary antigen tested negative for *Legionella* spp. The sputum sample was sent to our laboratory and tested positive for non-*pneumophila* Legionella spp. Nucleotide sequence analysis of the 16S rRNA gene amplification product showed 100% match with *L. longbeachae*. In addition, an acute serum sample tested positive for non-*pneumophila* Legionella species, and showed an elevated IgM titer of 1:512 and an IgG titer of 1:64 for non-*pneumophila* Legionella spp. This case is highly suggestive for infection with a non-*pneumophila* Legionella species. A point of criticism could be the absence of a definite proof of *L. longbeachae* pneumonia; seroconversion or even better a positive culture. However, non-*pneumophila* strains are more easily missed in clinical specimens because the culture media that are used often are less sensitive for isolation of non-*pneumophila* species, especially if antibiotics are added. Instructive for the difficulty of culturing legionellae was a case of CAP due to *L. pneumophila* serogroup 3, reported by Herpers et al. [25]. In this case, urinary antigen testing and the first culture of a bronchoalveolar lavage fluid sample were negative for *Legionella* spp. *L. pneumophila* DNA was detected by PCR in the BAL washing sample. Eventually, after repeated culture, *L. pneumophila* serogroup 3 was isolated from this specimen. Because of the shortcomings of
available diagnostic tests, and because culture of sputum samples for *Legionella* spp. is not a standard practice in the evaluation of CAP, it is possible that cases of both community-acquired and nosocomial non-*pneumophila* *Legionella* spp. infection remain undiagnosed. In patients with severe pneumonia of unknown etiology, repeated culture on *Legionella*-selective media or PCR should therefore be considered.

The unique growth requirements of legionellae, the ability to switch to a viable but nonculturable state and the association of *Legionella* Like Amoebal Pathogens (LLAPs) with amoebae complicates the detection of *Legionella* in potable water using standard microbiological techniques. **Chapter 11** describes an investigation on the occurrence and identity of *Legionella* spp. in Dutch tap water installations with culture, real-time PCR and sequence analysis. The PCR assays used were a 16S rRNA gene-based PCR, with both a *Legionella* species-specific probe and a *L. pneumophila* specific-probe, and a *L. pneumophila*-specific PCR based on the sequence of the *mip*-gene. A total of 357 water samples from 250 locations in The Netherlands were investigated. The detection rates of *Legionella* spp. were 2.2% (8 of 357) by culture, and 87.1% (311 of 357) by PCR. The majority of samples was found to contain *Legionella* species other than *L. pneumophila*. We conclude that *Legionella* spp. DNA is ubiquitous in Dutch tap water installations and uncultured *Legionella* spp. are part of the indigenous microbial community. We should be aware that *Legionella* is a very common colonizer of water distribution systems, similar to other potentially pathogenic bacteria and fungi. Our observations are in concordance with other studies [26,27,28]: more positive samples by PCR than by conventional culture are found. Wellinghausen *et al.* [26], using 16S rRNA gene PCR to quantify the genus *Legionella* and *mip*-PCR to quantify *L. pneumophila*, found a weak correlation with conventional culture. This may reflect the fact that PCR methods detect all legionellae, whereas culture only detects viable and culturable
cells. Conventional culture may underestimate the number of viable legionellae, owing to the use of selective media and pretreatment by acid or heating [3,29].

However, the PCR results must be applied with caution. Many assume that the nucleic acids being copied are from viable cells, but PCR can detect both viable and nonviable cells by amplifying the target nucleic acids in the sample. This can explain why no correlation was observed between culture and PCR results. Another possibility for this poor correlation is low specificity of the 16S rRNA PCR used. We have made a comparison of sequences and concluded that the region that we used has a high homology with known Legionella spp. However, amplification and detection of non-Legionella DNA cannot be completely excluded, since alignment of the target sequence of the primers can never include all environmentally occurring, known and unknown, bacteria. Although multiple species may colonize water-distribution systems, only a few species will cause disease in patients exposed to the water. L. pneumophila is by far the most pathogenic, accounting for more than 90% percent of culture confirmed LD worldwide. Although 15 serogroups of L. pneumophila have been described, serogroup 1 accounts for almost 85% of the reported cases of LD caused by L. pneumophila. Dutch guidelines mandate routine environmental surveillance for Legionella spp. in certain high risk buildings such as jails, hotels and nursing homes, regardless of the occurrence of cases. However, the necessity to sample water routinely is unclear or, at least, questionable. The linkage between specific levels of colonization and risk of LD remains controversial. When samples for culture are obtained routinely there is an obligation to try to eradicate Legionella if they are detected. The apparent infrequency of sporadic LD, the ubiquitous nature of the organism, the difficulty in eradicating the organism from water sources and the lack of data on preventing disease makes routine culturing questionable [7,30]. Using PCR to test water for Legionella may further overestimate the risk of infection. If disinfection is performed, it is likely that the water samples will contain nonviable Legionella cells which
were killed by the disinfection measures. The remaining nucleic acids in the dead cells may still be recovered and amplified by PCR. Therefore, implementation of PCR assays for the detection *Legionella* in potable water samples will probably lead to more unnecessary and expensive “emergency” decontamination procedures.

Acute respiratory tract infections (ARI) are very common and responsible for considerable morbidity in the general population. Most infections are caused by viruses, especially rhinovirus, influenza virus, and respiratory syncytial virus [31,32,33], although in 30-40% no etiological agent is found. In studies conducted in Israel, *Legionella* spp. were found to be important agents of ARI [34,35]. Lieberman *et al.* found evidence for *Legionella* spp. as an etiologic agent in 12% of cases both in general practice and in patients admitted to a general hospital. The etiological diagnoses in this study were based exclusively on an in-house IFA, detecting 41 “serogroups” of *Legionella* spp.

Given the difficulty involved in obtaining and handling appropriate material for the isolation of the pathogen, the technical complexity of the isolation, and the fact that most other tests only reliably detect infections due to *L. pneumophila* serogroup 1, a sensitive PCR test might enhance the ability to diagnose these infections in patients with ARI. Since 1970, the GPs from the Continuous Morbidity Registration of The Netherlands Institute of Primary Health Care have registered all patients who have consulted them about influenza-like illnesses (ILIs). An extension of the system with the registration of all patients consulting with other ARI (Acute Respiratoire Infecties in de Eerste Lijn, ARIEL studie) gave the unique possibility to estimate whether *Legionella* spp. play a role as etiologic agent in ARI. This study is described in chapter 12. The inclusion of control subjects made it feasible to investigate whether a causal relationship between detection of *Legionella* spp. and airway complaints exists. Nose and throat swab specimens were obtained from case patients and
control subjects, and PCR tests were performed for the detection of *Legionella* spp. The primers and probes of the Legionella PCR assay were based on the 16S rRNA gene. In real-time PCR, *Legionella* spp. DNA was not detected in any of the samples of the patients with ARI nor in the control patients. Thus, in contrast to reports based on serological observations we demonstrated that *Legionella* spp. are not present in the nose or oropharynx of patients with ARI in general practice. Observations made by direct fluorescent-antibody (FA) testing suggested that colonization with *Legionella* spp., although infrequently, does occur [36]. We found no evidence for asymptomatic carriage of *Legionella* spp. in the upper airways.

How to explain the results [34,35] found by Lieberman *et al.?* Although in theory local *Legionella* epidemiology may have influenced the results, we have serious doubts about the validity of serological testing using in-house IFA assays for a reliable diagnosis of *Legionella* spp. infection in patients with ARI. This may have lead to false-positive results. Specificity has only been acceptably established for *L. pneumophila* serogroup 1 and cross-reactivity between *L. pneumophila* serogroup 1 and other serogroups and species has been reported in the literature [3]. In their outbreak-related case-control study, Boshuizen *et al.* [37] observed that control seroconvertors did not show any statistically significant clinical difference when compared to non-seroconverters. Although previous studies associated respiratory symptoms with the presence of *Legionella* antibodies [38,39], Boshuizen *et al.* conclude that as most studies have looked at multiple symptoms, some statistically significant results are to be expected based on pure chance. The same is true in case of a patient serum that is subjected to 41 different in-house IFA tests for the detection of *Legionella* spp.; the use of a less specific diagnostic method for a low prevalent disease, increases the likelihood of false-positive reactions dramatically.

Acute exacerbation of chronic obstructive pulmonary disease (AECOPD), is characterized by an acute sustained worsening of the patient's condition from a stable state,
beyond normal day-to-day variations, which occurs one to three times a year and may warrant additional treatment. The aetiology of AECOPD is heterogeneous and still under discussion. In several studies serological evidence of *C. pneumoniae*, *Legionella* spp. and *M. pneumoniae* playing a role as a pathogen or copathogen in acute exacerbations has been reported [40-50]. We investigated the presence of atypical pathogens in sputum samples in patients with stable COPD and those with AECOPD using real-time PCR, described in chapter 13. A total of 248 sputum samples, 122 samples obtained during stable and 126 samples obtained during exacerbations, from 104 patients were tested. All samples were negative for *M. pneumoniae* and *C. pneumoniae* DNA, whereas two samples (one stable state sputum and one exacerbation sputum) were positive for *Legionella* *non-pneumophila* DNA. In search for an association between the presence of atypical pathogens in patients with stable chronic obstructive pulmonary disease and in those patients with acute exacerbations of the disease, no indication was found of a role for *Legionella* spp., *Chlamydia pneumoniae* or *Mycoplasma pneumoniae* in stable, moderately severe chronic obstructive pulmonary disease and in its exacerbations. Our study results further indicate that nonstandardised serology might introduce a false association between atypical pathogens and acute exacerbations of chronic obstructive pulmonary disease. In our view, the serological evidence of *C. pneumoniae*, *Legionella* spp. and *M. pneumoniae* playing a role as a pathogen or copathogen in AECOPD simply reflects the principal methodological problems of diagnosing such infections. The use of a less specific diagnostic method for the detection of a pathogen, will, from a statistical point of view, increase the likelihood of false-positive reactions.

**Chapter 14.** Attempts to determine the relative roles of bacterial and viral pathogens as etiological agents of community-acquired pneumonia (CAP) have yielded widely divergent results. It is known that in studies on the etiology of CAP, in up to 50% of patients the
etiologic agent cannot be identified. For many respiratory pathogens, PCR has been shown to be more sensitive than conventional microbiological methods. We conducted a study on frozen throat swabs obtained from a group of hospitalised patients with community-acquired pneumonia (CAP) to evaluate if the use of real-time PCR for detection of respiratory viruses and *Legionella* spp. would increase the diagnostic yield. Throat swab specimens from 242 adults admitted to hospital with CAP were tested. Overall, viral pathogens were identified by conventional techniques (serology) in 7 (2%) patients, and real-time PCR in 50 (21%) patients (*p*<0.0001). The diagnostic yield increased from 137 cases (57% of patients using conventional microbiological assays) to 158 cases (65% of patients using real-time PCR assays and conventional microbiological assays; *p*=0.06). This increase was mainly a result of the detection of respiratory viruses, especially influenza virus, parainfluenza virus and human coronaviruses, which were not detected by serology. *L. pneumophila* PCR was positive in 3 out of 11 cases (27%) of Legionnaires’ Disease (LD). This study demonstrates that real-time PCR can increase the number of microbiological detection of respiratory pathogens in patients with CAP from a little more than a half of the cases to nearly two thirds of patients. Although detection of *Legionella* spp. is possible, our results show that throat swabs are not suitable as a reliable sample for *Legionella* PCR.

The promise of molecular diagnostics to revolutionize the diagnosis of LD is still unfulfilled [19]. Nucleic acid amplification tests (NAAT) have greatly expanded the capabilities of clinical microbiology laboratories and have created new paradigms for the diagnosis and management of illness in patients with many kinds of infectious diseases. Despite development over many years, none of the many published *Legionella* PCR assays is commercially available and in widespread routine use, and few studies have addressed the practical value of PCR in routine clinical microbiology laboratories. At the Regional Public
Health Laboratory, located at the St. Elisabeth Hospital, Tilburg, The Netherlands, we have used a repertoire of conventional diagnostic tests (serology, culture, urinary antigen testing) as well as *Legionella* specific PCR for several years. **Chapter 15** describes a comparison between results obtained with PCR with those of conventional tests on samples sent to our laboratory from patients suspected of having LD. The two main objectives of our study were the following. First, to determine the performance of PCR compared to that of conventional diagnostic tests in terms of sensitivity and specificity. An estimated sensitivity and specificity of 83%, and 93%, respectively, was found for 16S rRNA based PCR, and 88%, and 98%, respectively, was found for the *mip*-gene based PCR assay (*p* > .5). Eight potential false-positive results were obtained with the 16S rRNA-based PCR, and 2 with the *mip* gene-based PCR, respectively. Because sensitivity of conventional testing is not 100%, we suspected that estimates of PCR specificity might be biased downward since a number of PCR-positive specimens from infected persons would be misclassified as uninfected if the conventional test failed to detect *Legionella*. A proportion of PCR-positive results were confirmed, and these results are considered true *Legionella* infections; two patients were positive for *L. pneumophila*, and one patient was diagnosed with a *Legionella* non-*pneumophila* spp. With the results of the discrepancy analysis, and assuming a prevalence of 5% of *Legionella* spp. in patients with pneumonia, the sensitivity, specificity, positive predictive value, and negative predictive value were recalculated and found to be 82%, 95%, 44% and 99% for the 16S rRNA assay, and 86%, 100%, 100% and 99% for the *mip*-gene-specific PCR, respectively.

Although it is clear that NAAT offer new opportunities to improve the diagnosis and management of illness in patients with infectious diseases, the introduction of new diagnostic tests does not always lead to an improved diagnostic yield. Our second and main objective was to evaluate the additional value of a *Legionella*-specific PCR for the diagnosis of LD, compared to the urinary antigen test. With the urinary antigen test 35 patients were diagnosed
as having LD, the same number as diagnosed with the 16S rRNA-based PCR. The main problem with 16S rRNA PCR, however, was a high proportion of false-positive results, making confirmation necessary. A reliable “acute” diagnosis seems virtually impossible with 16S rRNA based PCR. Although the \textit{mip}-gene PCR was able to detect more cases of LD, enhancing the etiologic diagnosis of LD patients with 4\% in the acute stage of disease, this difference was not found to be statistically significant. However, with the combination of a urinary antigen test and \textit{mip}-gene PCR LD was diagnosed in 40 patients, eg. in 14\% (5/35) more compared to the use of the urinary antigen test alone (35/139 vs 40/139; \(p=0.58\)). We therefore conclude that the addition of a \textit{Legionella pneumophila} specific \textit{mip}-gene PCR is useful in patients with suspected LD who produce sputum, and might allow the early detection of a significant number of extra patients.

\section*{Conclusions}

When systematically sought, \textit{Legionella} species are consistently recognized as one of the more common causes of pneumonia. The failure to diagnose LD in routine clinical practice largely depends on 3 factors: the inability to clinically and radiographically distinguish LD from other causes of pneumonia, the omission to order specific diagnostic tests for \textit{Legionella} infection, and the shortcomings of available diagnostic tests. During an epidemic or in a setting with an unusual high prevalence, a specificity of 100\% is not an essential prerequisite for a diagnostic test. However, when the prevalence of infection is low, even a modest loss of specificity will result in many false-positive findings. As outlined in this thesis, this holds true especially for new (commercial) diagnostic methods for which clinical specificity is not yet well defined. The sensitivity of diagnostic tests for LD is usually in the 60-70\% range, and does not exceed 90\% for any test used. Therefore it appears that none of the individual
diagnostic tests fulfils the needs of both clinicians and microbiologists, and the examination of different specimen types with several tests in parallel is recommended.

The amount of microbiological work-up should be determined by the severity of the pneumonia. The incidence of LD is higher among patients with severe pneumonia, and all patients with pneumonia who are admitted to an intensive care unit should therefore be tested for this infection. Patients with pneumonia that does not respond to therapy with beta-lactam antibiotics or the combination with aminoglycosides, or patients with severe underlying disease, should also be tested for *Legionella* spp. Culture diagnosis remains the gold standard for diagnosis of LD and is the most specific diagnostic procedure, but its relatively low sensitivity and the reliance on the availability of a lower respiratory tract sample make it inadequate as a sole diagnostic test.

In The Netherlands, LD is a notifiable disease and a supplementary nationwide source identification program is operative (Bemonsterings Eenheid Legionella-pneumonie; BEL project). In this survey, 172 culture-confirmed LD cases were identified between August 2002 and Oktober 2006. *L. pneumophila* constituted 98.8% (170) of the isolates. Serogroup 1 was the predominant serogroup (86.6%), and serogroups 2 (2.3%), 3 (1.7%), 6(2.3%), and 2–14 (5.2%) accounted for the remaining serogroups. The *Legionella* non-*pneumophila* species isolated were *L. longbeachae* (1.7%) and *L. maeceachernii* (0.6%) (E. Yzerman, J. Bruin, personal communication). In regions, as The Netherlands, where *L. pneumophila* serogroup 1 are numerically the most important *Legionella* species causing disease, urinary antigen detection is recommended for patients with severe CAP and in patients where this infection is clinically or epidemiologically suspected. In case of a negative antigen test, *Legionella*-specific PCR should be considered in severe pneumonia of unknown etiology. In regions where legionellae other than *L. pneumophila* serogroup 1 are important pathogens, current urinary antigen tests are still useful but should never be used as the sole diagnostic tool.
The availability of a good diagnostic repertoire, suitable for accurately diagnosing LD, constitutes the basis for the early recognition and treatment of the individual patient as well as for effective measures for prevention and control.