CHAPTER TWELVE

Summary

The outbreak of Legionnaires’ disease (LD) at a flower show in Bovenkarspel in 1999 was the first time the Netherlands were confronted with the potential consequences of inadequate control of Legionella. Comprehensive epidemiological investigation identified a whirlpool spa as the major source of the outbreak. Conclusive proof was furnished by demonstrating that a Legionella pneumophila serogroup 1 strain could be cultured from the filter and inner tubing of the whirlpool that was genotypically indistinguishable from the strain that was isolated from several patients. The different approaches to identify the source are described in Chapter 2. The microbial investigation of this outbreak took our laboratory many weeks, partly due to the complexity of the situation (equipment was moved, in some instances even abroad, immediately after the exhibition), but also because of our relative inexperience with this kind of investigation. Completion of the epidemiological and microbial investigations into the cause of the outbreak was at the same time the starting point for several lines of research that have been and continue to be carried out in different collaborating institutes. In this thesis, parts of this ongoing research are presented.

In Chapters 3 – 6, emphasis is put on the diagnostic aspects of Legionnaires’ disease. One of the possible explanations for the enormous size of the outbreak in 1999 was the lack of availability of the urine antigen test in most Dutch laboratories. In our laboratory, we had planned a trial with pulmonologists from our local hospitals to assess the added value of the urine antigen test as an early diagnostic parameter. The outbreak interfered with our intended trial by offering us an immediate opportunity to evaluate three commercially available urine antigen tests (Binax EIA, Biotest EIA and BinaxNow) on urine from patients who had visited the flower show. The results are described in Chapter 3. What we were able to demonstrate is that the overall sensitivities of the different tests are comparable (around 70%) and that concentrating the urine samples led to an increase of sensitivity of 5-10%. More important is the conclusion that there is a significant correlation between clinical severity and the sensitivity of the tests. This means that – for patients with a serious CAP that requires admission to the ICU – the urine antigen test is a very reliable parameter for diagnosing LD caused by L. pneumophila serogroup 1 (responsible for more than 90% of LD cases). In contrast, LD patients with a mild pneumonia may go underdiagnosed if urine antigen tests alone are used. Since most patients with a Legionella pneumonia are not producing sputum, the urine antigen test is often the only available diagnostic tool in the acute phase of the disease. A negative test does not rule out the
possibility of LD, and in a patient with a pneumonia that is not responding to conventional beta-lactam antibiotics it is important to keep this in mind. Although not very helpful in the acute phase, serology may be important to confirm a suspected LD. In Chapter 4 we have investigated the sensitivities of three serum antibody tests on available sera from patients of the Bovenkarspel outbreak. In general, serology is only accepted as confirmative for LD in case of a fourfold rise in antibodies or a seroconversion in a paired serum sample. What we saw was an unacceptably low sensitivity of less than 50% for the rapid micro-agglutination test (RMAT) and a sensitivity of just above 60% for the immunofluorescence antibody test (IFAT) and the ELISA. When we included standing titres, the sensitivity of the IFAT rose to 86% and of the ELISA to 75%. We came to the conclusion that – especially for the IFAT – our acute phase serum samples were taken too late to establish seroconversion. Therefore, we tried to estimate the time of seroconversion by modeling using a computer program for nonparametric estimation of interval censored data (DPpackage). With the IFAT, seroconversion can be expected within three weeks after onset of disease (Chapter 5). The results of the ELISA, although less sensitive, are in line with these findings and emphasize that it is essential to get an acute phase serum sample, and that clinicians have to be advised to take a second sample not later than 14 days after the first one. This advice is remarkably different from the current policy in most laboratories.

In Chapter 6, an overview is presented of the various diagnostic possibilities for LD. According to the literature, culturing Legionella spp. from a respiratory specimen is not considered a very sensitive diagnostic test (25-75%), primarily due to inexperience in medical laboratories. Moreover, most patients with LD do not have a productive cough and in these patients obtaining specimens for culture requires invasive techniques. Due to this limitation and to the introduction of urinary antigen tests, culture-based diagnosis of LD has decreased remarkably. A major drawback of this current trend is that it limits the recognition of L. pneumophila non-serogroup 1 disease and hence impairs the investigation of outbreaks.

If material for culture is available then it is also possible to detect Legionella nucleic acid. Various PCR tests have been developed that aim at different parts of the genome. The macrophage infectivity potentiator (mip) gene is species specific, whereas the 16S and 5S rRNA genes are specific for L. pneumophila. The most recent publications on real-time PCR are promising and report a high sensitivity combined with a high specificity. However, in order for PCR to become a reliable diagnostic tool, the reproducible specificity needs to rise to values higher than 99%. One may expect that such a test will become accepted in the near future as the new gold standard for diagnosis of LD.

The complement system mobilizes numerous immune effector mechanisms when it detects infected or damaged tissues. Three pathways of complement activation
are known: the classical pathway, the alternative pathway and the lectin pathway. The latter two pathways are part of the evolutionarily old innate immune system. The lectin pathway is initiated by binding of the protein mannose-binding lectin (MBL) to mannose or N-acetylglucosamine on surfaces of microbes, which promotes innate opsonization. MBL deficiency is associated with an increased risk of infection, as opsonization by complement is compromised. In infections with intracellular micro-organisms the role of MBL is more ambiguous, as some of these pathogens use opsonization by MBL as a way to enter host cells. We determined MBL2 genotypes and MBL-mediated complement activation in a retrospective case-control study using the patients of the Bovenkarspel outbreak as cases (Chapter 7). Because of the absence of pathogen variability as a confounding factor, this patient cohort offered us a good opportunity to study the role of MBL-mediated immunity in legionellosis. We found that genotypic MBL deficiency was not a risk factor for LD. However, patients with LD displayed deficient MBL-mediated complement activation even with MBL-sufficient genotypes. After the infection had passed and the patients recovered from the disease, complement activation returned to normal levels. Together, these genotypical and functional data suggest that the observed deficiency of lectin pathway activation is an effect of legionellosis rather than a risk factor for acquiring it.

After the outbreak of LD at the Bovenkarspel flower show in 1999, early outbreak detection became a topic of national interest. In 2002 a national outbreak detection program (BEL) was installed, an initiative of the Municipal Health Service Kennemerland and the Regional Laboratory of Public Health Kennemerland. BEL is aiming at a short response time between notified cases of LD and at finding and eliminating the possible sources of infection. The program is based on the observation that outbreaks are often preceded by small clusters or solitary cases. In Chapter 8 the first two years of BEL are evaluated. We concluded that systematic sampling and cluster recognition can contribute to outbreak detection and lead to cost effective secondary prevention of LD.

One of the products of BEL is a well documented collection of Legionella strains. All L. pneumophila serogroup 1 strains in this collection are genotyped by amplified fragment length polymorphism (AFLP) according to the typing protocol of the European Working Group for Legionella Infections (EWGLI). We were the first to report the intriguing finding that around 25% of human infections are caused by L. pneumophila EWGLI type 004 Lyon and that this genotype is only very rarely found in environmental locations that we consider to be the natural habitat of legionellae (Chapter 9). We have extended this study with epidemiological data and have looked at possible inclusion bias, but it did not effect our findings. In Chapter 10 two possible explanations are proposed. First, our source investigations may not reveal the true sources of LD. A second explanation is that genotype 004 Lyon is present in the sources sampled, but at
undetectable concentrations. Until now we have been unable to resolve this puzzle, and we still remain in the dark about the hiding place of genotype 004 Lyon. As a result, we have little clue to the transmission route into humans. One of our current research goals is aimed at solving this mystery. Based on the differences we found in the distribution of *L. pneumophila* strains in patients and environmental sources we concluded that there is a great variability in pathogenicity between *Legionella* strains, and we tried to discriminate between pathogenic and non-pathogenic strains by performing whole genome analysis. Using our collection we performed a micro-array test on patient strains and on strains derived from environmental sources that could not be linked to notified cases (Chapter 11). With genetic programming five markers were identified that correctly predicted 100% of the pathogenic strains and 62% of the environmental strains. This novel approach to identifying predictive markers for pathogenicity can be applied to all bacterial species. At the moment this technique is being translated into a commercially available tool to establish the presence of potentially pathogenic *Legionella* strains in water samples.