Progress in diagnostics and prevention of Legionnaires' disease
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CHAPTER ONE

General introduction

THE CASE OF THE BOVENKARSPEL OUTBREAK

One of the world’s largest outbreaks of Legionnaires’ disease occurred in the Netherlands in 1999, when more than 20 people died (1). This event triggered significant efforts on the part of researchers and medical doctors to explore the background of this outbreak. Several months later, microbiological and epidemiological studies revealed that a spa pool display at the annual flora show in Bovenkarspel was responsible for the tragic event. At the same time, this event provided a tremendous opportunity for increasing our knowledge about a relatively new disease. Now, ten years later and thanks to the cooperation of patients, relatives and other people involved, three PhD theses and more than 30 articles have been completed, where most of these were based on findings that resulted from the scientific exploration of the Bovenkarspel outbreak. We hope and expect that the published data will contribute to avoiding similar tragedies in the future.


LEGIONELLACEAE

The Legionellaceae are obligately aerobic Gram-negative bacilli. They are nonsporeforming and unencapsulated. Most species are motile by means of one to three polar or lateral flagellae. Motility may be lost during growth under artificial circumstances. Bacterial length varies depending upon growth conditions, stage of growth, and whether the bacterium is grown in eukaryotic cells or extracellular environments. When grown on solid media, the bacterium is usually a rod 5–40 µm in length, with a cell width of 0.3–0.9 µm. All are weakly catalase-positive. The oxidase reaction is usually weakly positive, but may be negative. Amino acids are used as an energy source rather than carbohydrates. L-cysteine is required for growth, and iron is required for initial isolation from the environment or clinical specimens. The pH and temperature optima for in vitro growth are 6.8–7.0 and 25–42°C, respectively, with optimal growth occurring in vitro between 35°C and 37°C.

Forty-eight Legionella species comprising 70 distinct serogroups have been confirmed (2). An additional 20 species have been named, and at least 10 more
species without names have not yet been fully characterised (3-5). Twenty *Legionella* spp. have been reported to cause human pneumonia, although more then 90% of all infections are caused by *L. pneumophila*. The rest have been isolated only from water sources.

**HABITAT**

Legionellae are omnipresent in natural and manmade aqueous ecosystems. In nature, they appear to be at least facultative, but may even be obligatory, parasites of free-living amoebae and protozoa (6-8). Legionellae have shown to be present in water with temperatures between 5°C and 63°C (7). The production of potable water from surface or ground water leads to the presence and subsequent colonization of waterworks, waterway systems in buildings, air-conditioning systems and comfort and industrial cooling towers. The adhesive substance glycocalyx and the microbial community it envelopes at the interface between liquid and surface is called biofilm. In these systems, a complex but normally balanced ecosystem develops, with a rich variety of interactive microorganisms including *Legionella* (9-12). Legionellae are present throughout the biofilm, but their concentration is highest at the surface, where the main activity of the ecosystem takes place, in a well-balanced equilibrium with the planktonic population (10;13). Disturbing the ecosystem by manipulation can lead to a sudden increase in free legionellae (14). This means that, in the complex water systems of buildings, the quantitative recovery of legionellae in water samples can vary enormously and thus does not provide a realistic reflection of the local biofilm-related ecosystem (15;16).

**TRANSMISSION**

If the presence of the bacterium were restricted to its natural habitat, confrontation with man could only occur by aspiration of colonized water. In reality, the evidence is overwhelming that the vast majority of legionellosis patients contract the disease by inhalation of *Legionella* bacteria floating in the air, although in many cases the actual source of the infection remains undiscovered. The phenomenon of inhalation seems not to be limited to patients that develop Legionnaires’ disease. A seroprevalence study in the Netherlands has shown that with aging, the presence and amount of measurable IgG antibodies against *Legionella* increase, while IgM antibodies decrease (17). These results indicate that encountering *Legionella* is a much more common experience than is generally thought. Aerosols floating through the air are considered as the vehicles responsible for transport of bacteria. Particles 5 µm or less in diameter are capable of reaching the
alveoli of the lungs, which is thought essential for establishing full-blown pneumonia. However, the hypothesis of aerosols as the primary mode of transmission seems difficult to maintain if one considers the evaporation time of water droplets in unsaturated air at 22°C (18). Under these circumstances, droplets 12 µm in diameter evaporate in 0.02 seconds. On evaporation, aerosols are converted into the dried residue of larger droplets, the so-called droplet nuclei. These nuclei behave very much like minute particles of smoke. In absolutely still air, the sedimentation rate is approximately one meter per hour, but in normal atmospheric circumstances, the particles remain suspended in the air for an indefinite period and are dependent on prevailing air currents for transportation. Survival of bacteria within airborne particles is determined by a multiplicity of factors (19-21). One of the factors for *Legionella* bacteria is their sensitivity to drying (22); their survival in droplet nuclei for longer periods of time depends mainly on the relative humidity of the air. Survival of airborne *L. pneumophila* as a function of relative humidity has been investigated by the US Army (23). Data indicated that under artificial circumstances, *Legionella* was relatively stable at 80% relative humidity (t1/2, 15.6 min), less so at 50% (t1/2, 10.3 min), and least stable at 30% relative humidity (t1/2, 3.2 min). If one combines these data with the results from microbial air sampling around cooling towers, which have shown concentrations of *L. pneumophila* as low as 0.02 colony-forming units per litre of aerosol, then it seems essential that airborne legionellae are kept protected from drying in order to be able to cause infection (which can sometimes occur even miles away from the primary source). This has led to the unproven hypothesis that legionellae remain alive and infective via packets of bacteria contained within amoebal or protozoan vacuoles (24;25). It may also elucidate a gap in our understanding regarding the infective dose. Animal experiments suggest that a high dose is required; this is supported by the absence of person-to-person spread. In monkeys, for example, 6.10^6 micro-organisms can induce fever, but this amount is not capable of causing pneumonia (26). In humans, the clinical form of pneumonia may occur as a result of exposure to legionellae packaged in amoebae. The number of bacteria that can be transmitted this way is not known exactly, but probably does not exceed a maximum of a few thousand. To explain these environmental and epidemiological observations, it seems that humans, especially those who are immunocompromised, are unusually susceptible in comparison to the susceptibility to legionellae of investigated animals. There are no data on the minimal amount of bacilli required to cause disease in humans (27). The pathology of Legionnaires’ disease is a pneumonia with intracellular microorganisms and no freely prevalent microorganisms in the airways or sputum; this probably contributes to the fact that there is no transmission between humans.
CLINICAL PRESENTATION

Legionnaires’ disease is spread primarily via aerosols of contaminated water, although microaspiration also may be an important mode of spread, especially in nosocomial cases (28-30). After an incubation time of about 2 – 20 days (31), clinical presentation generally starts with fever, fatigue, headaches, muscle aches and/or a cough. Chest pain, diarrhoea, confusion, shaking chills, and shortness of breath may be seen before full-blown pneumonia is present. Chest X-ray usually demonstrates alveolar filling, focal infiltrates, and lung consolidation with or without pleural effusions. These features are indistinguishable from other common forms of bacterial pneumonia, such as pneumococcal pneumonia (32-36). There is some suggestion that a combination of factors such as diarrhoea, hyponatremia, and increased serum creatine kinase is more consistent with Legionnaires' disease than with other pneumonic diseases, but no study has shown this unequivocally. Extrapulmonary infection occurs rarely, either as a disseminated infection in patients with pneumonia or, very rarely, as a primary infection (37;38). Isolated primary disease of prosthetic heart valves, respiratory sinuses, and wounds has been reported (39-42). Pleural empyema, myocarditis, meningitis, encephalitis, pericarditis, peritonitis and colitis have been documented very rarely during the course of Legionella pneumonia (43-55).

PATHOGENESIS

*Legionella pneumophila* infects humans after inhalation of contaminated aerosols or droplet nuclei or after aspiration of contaminated potable water (29;30;56-61). Once the organisms have reached the alveoli, they enter resident macrophages by coiling or conventional phagocytosis. The basic pathway for phagocytosis in human cells involves opsonization of bacteria with complement component C3 and subsequent binding to complement receptors CR1 and CR3 (62). Entry via this pathway appears to limit the phagocyte's oxidative burst and thereby may help to facilitate subsequent bacterial intracellular survival (63). Others, however, have stressed the importance of opsonin-independent phagocytosis (64-66). Within macrophages, the bacteria reside within a unique phagosome that does not follow the endosomal pathway (67). The early *Legionella* phagosome lacks alkaline phosphatase, major histocompatibility complex (MHC) class I and II markers, transferrin receptors, Rab 7, LAMP-1 and cathepsin D (67). After 4–6 hours, the phagosome becomes associated with ribosome-studded membranes that derive from the host cell endoplasmic reticulum. *L. pneumophila* avoids lysosomal fusion and starts to proliferate (68-69). Late in the intracellular cycle (i.e., 12–24 h), the *Legionella* phagosome does fuse with acidic lysosomal compartments, but bacterial growth continues until host cell death and release of bacteria (70). As a result of this intracellular multiplication, polymorphonuclear leukocytes (PMNs),
additional macrophages (i.e., differentiated monocytes), and erythrocytes infiltrate the alveoli, and capillary leakage results in local oedema (71). Release of tissue-destructive substances from the bacteria contributes to pathology (68;71). More host defence responses are triggered at least in part by chemokines and proinflammatory cytokines (i.e., IL-1, IL-6, GM-CSF, MCP-3, MIP-1α, MIP-2 and TNF-α) released by infected macrophages (72-75). When cell-mediated adaptive immune response is functioning normally, further bacterial amplification is usually limited (69;76-79). Dendritic cells are likely to play an important role in initiation of adaptive immune response. Immature dendritic cells are phagocytic and able to internalize bacteria (80). Maturation of dendritic cells is triggered by bacterial components, such as lipopolysaccharide (LPS); as they mature, their phagocytic capacity is reduced (81). Mature dendritic cells secrete IL-12 and other cytokines that play an important role in skewing the development of naïve T-cells towards a Th1 phenotype (82). The ability of dendritic cells to restrict intracellular growth of Legionella could be an important property that facilitates priming of protective T-cell mediated immune responses to vacuolar pathogens (83). Most of the evidence points to a critical role in the clearance of L. pneumophila for the Th1 T-cell response and its associated cytokines (84-90). When adaptive immune response is impaired in certain immunocompromised individuals, bacterial proliferation and extrapulmonary dissemination are pronounced, and the disease may be fatal (71;79;91).

VIRULENCE FACTORS

Since its discovery, many virulence factors have been described for L. pneumophila. The first virulence-associated gene was detected in 1989 and designated mip for macrophage infectivity potentiator. This gene encodes a 24-kDa surface-exposed membrane protein (mip), that possesses peptidyl-prolyl cis/trans isomerase (PPIase) activity (92;93). Mip-like genes have also been detected in other species of Legionella. The mip-gene is required in the early stages for efficient infection of phagocytic cells and protozoa. The exact function of the PPIase activity of mip remains unknown (94).

Another L. pneumophila surface structure is the genus-wide, peptidoglycan-linked, outer membrane porin (95-97). This 28-kDa protein, which is also known as the major outer membrane protein of L. pneumophila, is a binding site for complement components and thus mediates opsonophagocytosis (69;98;99).

L. pneumophila LPS, in addition to bearing the serogroup-specific O antigen, contains endotoxin (100-104). However, LPS-lipid A of Legionella possesses a relatively weak endotoxic activity that appears to be due to its low affinity for the CD14 receptor on macrophages (105). Interestingly, a particular LPS epitope, recognized as MAb 2 by typing with an international panel of seven monoclonal antibodies, is more frequently expressed on clinical versus environmental isolates
of serogroup 1 strains (106-109). However, mutational analysis has determined that loss of that epitope itself does not diminish the ability of *L. pneumophila* to infect macrophages or protozoa (110;111). On the other hand, several studies have correlated other antigenic changes in LPS expression with reductions in serum resistance, intracellular growth, and virulence (112;113). Mutation of a gene (*rcp*) that appears to modify lipid A structure reduces the ability of *L. pneumophila* to resist cationic antimicrobial peptides (i.e., polymyxin and defensin C18G) and infect host cells (114).

Two *Legionella* protein-secretion systems are essential for pathogenesis. The loci encoding the type IV secretion system comprise 25 genes in two separate regions of the Legionella chromosome and have been named icm/dot (intracellular multiplication/defective for organelle trafficking) (115-119). This type IV secretion system encodes factors that are involved in the regulation of conjugal transfer of plasmid DNA (67;117). It seems that the infectious process is initiated by transporting virulence proteins required for entering the host cell. The system is probably responsible for diverting the phagosome from the endocytic pathway during phagocytosis. Among the proteins that are part of this system, DotA has been recognized as an inner membrane protein that plays a critical role in the initial trafficking of the *Legionella* phagosome (120-123). Bioinformatic analysis of the regulatory regions of all genes revealed that several icm/dot genes as well as two genes encoding icm/dot translocated substrates contain the conserved CpxR regulatory element. The CpxR regulator is a fundamental regulator of the icm/dot type IV secretion system in *L. pneumophila* (124). The second exoprotein production system is encoded by genes within the loci for the type II secretion system. These genes are responsible for unrestricted intracellular growth of *L. pneumophila*. The two most extensively analyzed genes are *pilE* (pilin protein) and *pilD* (prepilin peptidase) (125-131). The pilin protein is most likely involved in the attachment of *Legionella* to the host cell. The prepilin peptidase is essential for pilus production and the secretion of proteins.

*L. pneumophila* possesses two catalase-peroxidases, or enzymes that convert H$_2$O$_2$ to innocuous water and oxygen (132;133). KatA catalase-peroxidase is located in the periplasm and is induced during the stationary phase (134). The KatB enzyme is cytoplasmic and has a role in intracellular infection (132).

A large number of other genes also promote intracellular infection (135-140). Collectively, these loci promote all the various stages of intracellular infection: entry, intracellular survival and trafficking, replication, and escape.

**DIAGNOSTICS**

There are four standardized methods for the specific laboratory diagnosis of *Legionella* infections: detection of antibodies, demonstration of the bacterium in tissues or body fluids by immunofluorescent microscopy, isolation of the organism
on culture media, and detection of antigens in the urine of patients. Detection of bacterial DNA using PCR is being used but has yet to be validated.

SCOPE OF THIS THESIS

In the first several chapters of this thesis, different possibilities for the diagnosis of legionellosis are the focus of evaluation. The outbreak that provided the opportunity to evaluate a number of diagnostic tools is described in Chapter 2. The Bovenkarspel outbreak stimulated the rapid introduction of urinary antigen detection tests in medical microbiological laboratories in the Netherlands. The sensitivities of three commercially available urinary antigen tests are compared in relation to the clinical severity of disease in Chapter 3. Despite introduction of the antigen tests, serology remains an important tool in the diagnosis of Legionnaires’ disease. The sensitivities of different serological methods, alone and in combination, are evaluated in Chapter 4. In general, seroconversion is expected to occur late in the course of legionellosis, and the timing of a second serum sample is often a subject of discussion. In Chapter 5, we estimate the time to seroconversion for different serological methods. Chapter 6 reviews the available diagnostic tools for Legionnaires’ disease.

The lectin pathway is one of the three pathways that activate complement and, as such, is part of innate immunity. It is initiated by the binding of mannose-binding lectin (MBL) to foreign surfaces such as bacteria. This outbreak offered the opportunity to investigate the role of MBL in patients and exhibition staff, using healthy blood donors as controls. This study is described in Chapter 7.

As the Bovenkarspel outbreak prompted us to initiate a project to find the source of legionellae, the results of this project are described in the next chapters. In these studies, special attention is paid to differences in strains with regard to human pathogenicity. In Chapter 8, the background of our source-finding project is explained, along with the results from the initial part of the study. The distribution of *L. pneumophila* genotypes of strains derived from patients is compared with environmental strains in Chapters 9 and 10. Based on the results of the distribution of *Legionella* strains, we tried to identify markers of pathogenicity, since if it is possible to differentiate pathogenic strains from non-pathogenic strains, this may help regulatory authorities improve *Legionella* control. Chapter 11 describes the results of our attempts to predict the pathogenic capacity of different strains by whole genome analysis. The final conclusions are found in Chapter 12.
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