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SEROPREVALENCE OF ANTIBODIES TO *CHLAMYDIA PNEUMONIAE* AND *LEGIONELLA PNEUMOPHILA* IN MINE WORKERS, FACTORY WORKERS AND PNEUMONIA PATIENTS

CATHELEEN BARTIE

A Dissertation Submitted to the Faculty of Medicine of the University of the Witwatersrand, Johannesburg, for the De ee of Master of Science in Medicine

Johannesburg 1994

FOR MY MOTHER AND SONS

NEILL AND ETIENNE

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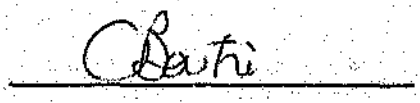
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DECLARATION

I declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine in the University of the Witwatersrand, Johannesburg, and has not been submitted before for any degree or examination at any other university.



CATHELEEN BARTIE

ABSTRACT

South African gold miners work under stressful conditions and live in communal hostels. Respiratory infections are common in these workers and several cases of Legionnaires' Disease have previously been diagnosed in workers at a gold mine on the East Rand. The prevalence of antibodies and the rate of seroconversion within a period of six months, to *C. pneumoniae* and *L. pneumophila*, both common causes of atypical pneumonia, was studied in relation to several risk factors including age, smoking habits, previous underground experience and past exposure to dust and humidity in the environment. Factory workers from a rural area in Natal and hospitalised patients with community acquired pneumonia were tested for comparison. Water samples were collected from several areas at the mine, including both surface and underground samples.

Antibodies to *C. pneumoniae* were present in 66% of the mine workers, compared to 50% of pneumonia patients and 22% of factory workers, a statistically significant difference ($P<0.001$). Seroconversion was demonstrated in 17% of the mine workers within a period of six months working underground, and in 22% of pneumonia patients, with convalescent stage sera taken 1-6 weeks after onset of symptoms. None of the risk factors studied influenced the prevalence of *C. pneumoniae* antibodies in the mine workers, but a significant association between the presence of respiratory symptoms in the six month period and seroconversion was demonstrated ($P<0.025$).

Using heat-killed antigens, antibodies to *L. pneumophila* serogroups 1-4 were demonstrated in 36% of the mine workers, in 16% of the pneumonia patients and in 10% of factory workers ($P<0.001$). Seroconversion occurred in 18% of mine workers and in 14% of pneumonia patients. An association could not be demonstrated between any of the risk factors studied and the prevalence of antibodies or the rate of seroconversion to *L. pneumophila* in the mine workers.

No legionellae were cultured from the water samples, and the presence of these organsims in chlorinated water from both surface and underground samples could not be confirmed.

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PRESENTATIONS

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CHAPTER ONE

INTRODUCTION

1.1 THE GENUS CHLAMYDIA

The order Chlamydiales consists of one family, Chlamydiaceae, one genus, Chlamydia, and three species: *C. psittaci*, *C. trachomatis* and the more recently discovered *C. pneumoniae*. Chlamydiae are differentiated from other bacteria by their morphology, by a common group antigen and by the unique developmental cycle which involves two morphologic forms - one adapted to extracellular survival and the other to intracellular multiplication within cytoplasmic vesicles commonly termed inclusions (Nichols et al 1980). Chlamydiae were long thought to be viruses due to their small size and the fact that they can only multiply within susceptible cells. They have, however, many characteristics in common with other bacteria. They contain both RNA and DNA, although their DNA is not surrounded by a nuclear envelope. Their cell envelope resembles that of other gram negative bacteria, consisting of both outer and inner membranes. The membranes do not contain muramic acid, and peptidoglycan has not been detected (Volk et al 1986). Chlamydiae contain ribosomes similar to those of other bacteria and are susceptible to antibiotics. The reason why the organisms can only grow intracellularly is not certain, but they depend on their host cells for a supply of energy-rich compounds, of which ATP is the most important (Volk et al 1986).

Chlamydiae are visible by light microscopy. The genome is about one third the size of that of *E. coli*. The species have been known by a variety of names in the past, Miyagawanella and Bedsonia being two of the more commonly used ones (Schachter 1978). The three species are differentiated by their inclusion morphology, the presence of glycogen in their

inclusions and their susceptibility to sulfonamides (Campbell et al 1987). Chlamydiae share certain rRNA sequences and the unique developmental cycle, but they share only 10% DNA homology and can therefore easily be distinguished by DNA restriction endonuclease analysis (Campbell et al 1987). The characteristics of the three Chlamydia species are presented in Table 1.1. C. *trachomatis* has been divided into 15 strains known to cause human infection. The species is sensitive to sulfonamides, contains glycogen in its inclusion bodies, and can be stained with iodine. The main infections caused by *C. trachomatis* are trachoma, inclusion conjunctivitis of the newborn, infant pneumonitis and several genital tract infections of which nongonococcal urethritis (NGU), chlamydial epididymitis and lymphogranuloma venereum (LGV) are the most common (Abrams 1968, Birkelund 1992, Griffin et al 1990, Richmond and Sparling 1976, Schachter 1978). *C. psittaci* is common in most bird and animal species and causes psittacosis (ornithosis), a respiratory disease, in humans. Psittaccsis is usually a mild disease, but severe or even fatal cases have been reported. *C. psittaci* is resistant to sulfonamides and it's inclusions contain no glycogen, thus they will not stain with iodine (Stephens et al 1982). The exact number of strains is still unknown (Grayston et al 1992).

C. pneumoniae was described for the first time in 1986 by Grayston and coworkers (Grayston et al 1986). It was originally thought to be a strain of *C. psittaci*, until 1989 when it was formally established as a third species on the basis of differences in immunological characteristics, DNA structure and morphology (Grayston et al 1989). It's inclusions contain no glycogen and do not stain with iodine. No animal or avian host (other than man) has been found for the organism and it causes several upper and lower respiratory tract infections ranging from mild to severe (Li et al 1989). The species consists of at least two strains of which the most important is called TWAR, from the laboratory designation of the first two isolates namely TW-183 (from 'TAIWAN') and AR-39 (from 'acute respiratory') (Marrie 1993). The other is called FML-10 (Berdal et al 1991). Black et al (1991) analysed four *C. pneumoniae* strains, CWL-011, CWL-029, CWL-050 and CM-1, that were found to be antigenically different from TW-183 and FML-10. Another strain, called IOL-207, was isolated for the first time in 1985 from the conjunctiva of a trachoma patient and was shown to be distinct from *C. trachomatis* and *C. psittaci*, but similar to TW-183 (Saikku et al 1985). Antibody to the IOL-207 strain was also frequently found in blood donors in London and in school children over the age of 5 years in England (Kuo et al 1986).

TABLE 1.1

DIFFERENCES BETWEEN CHLAMYDIA SPECIES

	CT	CPS	CPN
Elementary body	Round	Round	Pear-shaped
Inclusions	Oval Vacuolar	Variable Dense	Oval Dense
Glycogen	Present	Absent	Absent
Folate biosynthesis	Yes	No	No
G+C content (mol%)	40-43	41	40
% DNA homology to CPN	<5-10	<10	94-100
Plasmid DNA	Present	Present	Absent
Number of strains	15	Unknown	2 known
Natural hosts	Humans	Birds Mammals	Humans
Sulfonamide sensitivity	Sensitive	Resistant	Resistant
Species-specific antigen present on MOMP-molecule	Present	Present	Absent
Type specificity	Present	N/A	Present
Major human diseases	Trachoma LGV	Pneumonia Psittacosis	Pneumonia Bronchitis

CT : C. trachomatis
CPS : C. psittaci
CPN : C. pneumoniae
N/A : Not applicable
Table adapted from Marrie 1993

TW-183 was first isolated from a conjunctival swab from a Taiwannese child taking part in a trachoma vaccine trial in October 1965. AR-39 was the first strain to be isolated from a pharyngeal swab, from a student with acute respiratory disease in Seattle, Washington, in 1983 (Grayston et al 1989). *C. pneumoniae* has however been found to have been associated with respiratory disease long before 1983: retrospective studies on sera collected between 1963 and 1975 in Seattle showed antibodies to the organism, as well as changes in antibody titres, suggesting infections and re-infections during this period (Foy et al 1979). Several outbreaks of respiratory disease, retrospectively shown to have been caused by *C. pneumoniae*, occurred between 1977 and 1985 in Finland, Norway and Denmark (Fryden et al 1989, Grayston et al 1989, Kleemola et al 1988, Mordhorst et al 1986, Saikku et al 1985). During the 1970's extensive studies showed that there was no association between *C. pneumoniae* and eye infections.

1.1.1 MORPHOLOGY

The genus Chlamydia has been divided into two subgroups on the basis of the morphological appearance of their inclusion bodies, the presence or absence of glycogen in the inclusions, their sensitivity to sulfonamides and the extent of DNA homology between related strains (Jones et al 1974). Organisms in subgroup A form compact inclusion bodies containing glycogen and are generally susceptible to sulfonamides. *C. trachomatis* strains causing trachoma, paratrachoma and lymphogranuloma venereum therefore belong to this subgroup. Organisms in subgroup B are resistant to sulfonamides and have diffuse inclusion bodies that do not contain glycogen. Nearly all chlamydiae of avian or animal origin are in this subgroup, including *C. psittaci*. Although not documented, *C. pneumoniae* would therefore also be in subgroup B.

The outer membrane of chlamydiae plays a major role in their interaction with their surroundings. It does not contain peptidoglycan, but other classes of molecules that make up the cell envelope are generally the same as in other gram negative bacteria. These molecules include phospholipid, lipid, protein and lipopolysaccharide/glycolipid. It is not certain whether the outer membrane of Chlamydiae also contains a protein analogous to the 'Braun' lipoprotein found in other gram negative bacteria or whether it contains

flavoprotein conjugates (Allen 1986). Chlamydiae can synthesize their own proteins, lipids, and macromolecules but have no mechanism for production or trapping of energy.

The morphology of Chlamydiae vary during their developmental cycle, which alternates between two forms: the elementary body (EB) and the reticulate body (RB). Both these forms can be stained with Giemsa, Machiavello, Castaneda and Gimenez stains (Schachter 1990).

1.1.1.1 ELEMENTARY BODIES (EBs)

Elementary bodies of Chlamydiae are specialised for survival when released from the host cell, and can induce phagocytosis when attached to a host cell membrane and contain outer and inner membranes detectable by electron microscopy. The outer membrane is rigid, does not contain a peptidoglycan layer with muramic acid, but penicillin-binding proteins are present. EBs are resistant to sonic treatment and trypsin and are relatively impermeable to macromolecules. The rigidity of the EB depends on disulfide cross-linking of MOMP molecules with each other and with other cysteine-rich proteins that appear late in the growth cycle when the EBs are formed (Schachter 1990).

The structure and morphology of the EBs of the three Chlamydia species are different. While the EBs of *C. psittaci* and *C. trachomatis* are round and dense with narrow, nearly invisible periplasmic spaces, those of *C. pneumoniae* are pleomorphic, but typically pear-shaped with large periplasmic spaces (Grayston et al 1989, Grayston et al 1990) in which small, round, electron dense bodies can be seen by electron microscopy (Chi et al 1987). Explanations for the pear shaped structure and the large periplasmic space of *C. pneumoniae* are speculative. It may be caused by a disturbance in the regulation of outer membrane synthesis or by degradation, so that excess outer membrane continues to be made or is not cleaved off when the cytoplasmic mass shrinks during RB-to-EB transformation (Chi et al 1987). Another possibility is that the outer membrane of *C. pneumoniae* is more rigid than that of the other chlamydiae and that this rigidity may prevent the membrane from collapsing on the shrinking cytoplasmic mass and may suggest a difference in the

chemical structure of the outer membrane between *C. pneumoniae* and the other chlamydiae.

The EBs of *C. pneumoniae* possess polarity and the loose outer membrane appears to assist in the attachment and anchoring process. The pointed end may serve as the primary site of attachment, or the EB may actively seek targets by directing the tip end toward the host cells (Kuo et al 1988). The EBs may attach either to the smooth surface of the host cell, or as is more often the case, to the microvilli. The outer membrane of the EB is then rearranged to form several protruding structures, unique to *C. pneumoniae*. Grayston, Wang and coworkers (1989) described these stem-like structures (peduncles) by which the EBs attach to the cytoplasmic mass of host cells in more detail. It is not clear if these structures have any effect on infectivity. During attachment, the outer membrane reshapes, suggesting that it is more fluid than that of the other chlamydiae. After attaching to the microvilli, the EB is transported toward the base where it enters the host cell by invagination. The process is completed when the host cell completely encircles the EB. In his latest article Marrie (1993) however stated that additional studies in two different cell lines showed no unique morphological features in *C. pneumoniae* and that it undergoes the same developmental cycle as *C. trachomatis*.

1.1.1.2 RETICULATE BODIES (RBs)

Reticulate bodies (also termed initial bodies) are adapted for intracellular multiplication. The cell wall is fragile, highly permeable and contains no hemagglutinin or subunit envelope, is sensitive to sonication and can be lysed by trypsin (Shachter 1990). The RBs of all three Chlamydia species are round and undergo the same intracellular development cycle (Chi et al 1987, Grayston et al 1989). Unlike the EBs, the outline of the cytoplasm is in conformity with the outer membrane.

1.1.2 DEVELOPMENTAL CYCLE

Chlamydiae show an affinity for the epithelial cells of mucous membranes. Attachment of chlamydiae to epithelial cells appears to involve a heat-labile surface component on the organism, and a trypsin-sensitive receptor on the host cell, but the uptake of chlamydiae by macrophages or granulocytes does not seem to depend on specific attachment mechanisms. Electron-micrographic studies suggest that entry into host cells may take place via clathrin-coated pits by a mechanism similar to receptor mediated endocytosis.

The developmental cycle of chlamydiae is as follows: EBs are taken up into the host cell by phagocytosis. A microcolony develops within the phagosome, inhibiting phagolysosomal functions and the rest of the life cycle takes place within the phagosome where host cell ATP is used by the EB, and ADP is released. The cell envelope of the EB now becomes less rigid, the subunit layer is disrupted and disappears, the cell envelope becomes highly permeable to macromolecules and the EBs change into RBs with internal strands of nucleic acid and ribosomes. The RBs are are non-infective and start dividing by binary fission at about 10-15 hours after infection. After 20-48 hours some of the RBs reorganize into small dense EBs again. During this stage the cytoplasmic contents of the RBs condense and decrease until they are typical EBs again. It is not certain what triggers the reorganisation of the RBs back into EBs, but this reduction in size is accompanied by loss of large amounts of RNA from the RBs. Most of the RBs will however continue to multiply until the host cell bursts and the EBs are released by a mechanism which is not yet fully understood (Schachter 1990), thus completing the developmental cycle. Studies have shown that the mechanism whereby *C. pneumoniae* binds to cells is different from that seen in the other chlamydiae. Kuo and coworkers (1988) suggested that initial contact with cells is usually made by the pointed end of the organism. The points of attachment are the tips of the cell wall protrusions although, infrequently, a stretch of EB outer membrane is seen next to the host cell plasma membrane. The Chlamydial EBs have a diameter small enough to be inhaled into the alveolar sac where they encounter the alveolar macrophage, the major immune-response regulating cell of the lung, which synthesizes and secretes various chemical mediators directing the inflammatory response to the chlamydial antigens.

1.1.3 CHEMICAL COMPOSITION

Chlamydiae are chemically complex. The ribosomes of RBs appear to be similar to those of other bacteria and the cell walls of EBs resemble those of gram negative, but not gram positive bacteria. The amino acid content of chlamydial EBs is similar to that of *E. coli*. Of the common amino acids, only arginine and histidine are not present in their cell walls. The dry weight of both intact cells and cell walls is approximately 35% protein, with a lipid content of 40-50% The carbohydrate content ranges from 1-2%, the RNA content from 2-7% and the DNA content from 3-4% The DNA has a guanine plus cytosine ratio of approximately 45% for *C. trachomatis*, 41% for *C. psittaci* and 40% for *C. pneumoniae*. Unlike the other chlamydiae, *C. pneumoniae* contains no plasmid DNA (Chi et al 1987, Grayston et al 1989). It shares ≤10% homology with the other chlamydiae, but approximately 94% with other strains of *C. pneumoniae* (Campbell et al 1987, Grayston et al 1989, Marrie et al 1987). Chlamydiae have no mechanism for the production or trapping of energy, therefore they have to parasitize their host cells for metabolic energy (Schachter 1990). They can only metabolize a few intermediates of the tricarboxylic acid cycle and glucose, through a portion of the pentose cycle, to pyruvic acid. Cytochromes and flavoproteins are absent and the organism therefore appears to grow anaerobically. Although chlamydiae are unable to synthesize their own ATP, they possess an ADP/ATP translocator that functions as a kind of 'reverse mitochondrion', taking in host cell ATP and releasing ADP. Chlamydiae therefore need well-nourished cells to be able to multiply. It has been suggested that chlamydiae possess bacteriophages.

1.1.4 ANTIGENIC COMPOSITION

Chlamydiae do not share antigens with any other organisms, but the antigenic composition of all three Chlamydia species is similar. They contain group-, species- and type-specific antigens that are exposed throughout the developmental cycle of the organism (Allen 1986, Reeves et al 1962, Stephens et al 1982). The physiochemical character of the antigens are not clear. Stephens et al (1982) developed monoclonal antibodies reacting with epitopes that occur in different strains of chlamydiae. The amount of antigen however increases about

six hours before the organism becomes infective, which explains why antibodies to EBs will also react with RBs (Stephens et al 1982). Chlamydial antigens may be either heat-stable or heat-labile (Marrie 1993) and are important in infectivity, inhibition of lysosomal fusion and the immunopathogenesis of disease (Stephens et al 1982). Antigenic activity can be extracted from the EBs with the use of ethyl ether, sodium deoxycholate or sodium laurel sulphate (Allen I 1986).

1.1.4.1 GROUP-SPECIFIC ANTIGENS

The group-specific antigen, shared by all the members of the genus (Schachter 1978, Stephens et al 1982) is a lipoprotein-carbohydrate complex containing a periodate-sensitive acid polysaccharide as the antigenic determinant, similar to the lipopolysaccharide (LPS) of Gram-negative bacteria such as *A. calcoaceticus* (Brade et al 1987) and Re mutants of *S. typhimurium* (Nurminen et al 1983, Schachter 1990). This acidic, high molecular weight polysaccharide is insoluble in water, diffuses readily in agarose and is responsible for fixing complement. It can be extracted with sodium laurel sulphate or by alkaline saponification of the lipid fraction. The group-specific antigen is heat-labile although heat-stable forms have been demonstrated (Schachter 1990), is pronase-resistant (Stephens et al 1982) and can be detected throughout the growth cycle by direct and indirect complement fixation, agglutination, hemagglutination inhibition, radioisotope precipitation (RIP) and intradermal tests (Jones et al 1974, Reeve et al 1962, Ross MR et al 1962).

The lipopolysaccharide (LPS) molecule consists of three different domains, the lipid A domain, the core polysaccharide and the O-polysaccharide (Nurminen et al 1983). The lipid A domain is responsible for endotoxic activity and is embedded in the outer membrane of the organism. It is composed of disaccharide glycoamine, containing 6 fatty acid residues like that found in *S. typhimurium* (Birkelund 1992, Saikku et al 1992). The core polysaccharide consists of a 2-keto-3-deoxyoctanoic acid (KDO) containing an innermost region, a heptose region and a hexose region (Brade et al 1987, Nurminen et al 1983). It is linked to the C-6 of glycoamine and is composed of two α 2-4 linked KDO moieties, with an additional 2-8 linked KDO that is not present in any other bacteria. The formula for the KDO molecule is α-KDO-2.8-α-KDO-2.4-α-KDO(Birkelund S 1992). The most distal part

of the LPS is called the O-polysaccharide, is variable in structure and is the site of the O (somatic) antigens. It is linked to the core oligosaccharide and protrudes from the surface of the outer membrane. The immune response is mainly directed to the O-polysaccharide.

1.1.4.2 SPECIES-SPECIFIC ANTIGENS

Although species-specific antigens are known to exist, they have not yet been fully characterized (Caldwell et al 1977), but appear to represent a spectrum of immunologic reactions (Schachter 1978). Species-specific antigens have been demonstrated in solubilized chlamydial suspensions and stimulate the formation of neutralizing antibodies. They are resistant to periodate oxidation and are mostly heat-labile.

The species-specific antigens of *C. psittaci* are difficult to immunotype (Schachter et al 1975). Caldwell et al (1977) was the first to purify and partially charac[illegible] *C. trachomatis* species-specific antigen. It is a heat-labile protein with a molecular weight of 155,000, called antigen-0.65. Unlike *C. psittaci*, strains of *C. trachomatis* are today relatively easy to immunotype. Strains causing trachoma and/or inclusion conjunctivitis will need mechanical assistance (eg. lysis of cells or centrifugation of inoculum onto new cells) for good growth. Their infectivity in tissue culture can be enhanced by treatment of the cells with diethylaminoethyl (DEAE) dextran, a positively charged macromolecule that increases the infectivity and transforming ability of a number of viruses, while treatment of the cells with neuramidase will decrease their infectivity. Treatment with DEAE dextran changes the surface charge of the cells and allows better attachment of the EB to the cell membrane after inoculation. LGV strains are capable of spontaneous cell-to-cell transmission in tissue culture and are not affected by treatment of the cells with DEAE-dextran or neuramidase during tissue culture (Schachter 1978). Antibodies directed to species-specific antigens will only react with homologous or closely related strains. Each species of the chlamydiae can be further divided by type-specific antigens. Not much is known about species-specific antigens of *C. pneumoniae*.

1.1.4.3 TYPE-SPECIFIC ANTIGENS

Type-specific antigens are situated with the sub-species and species-specific antigens in the major outer membrane protein (MOMP or Omp1) (Wang et al 1970). Antibodies directed to these antigens seem to prevent infection of host cells, but have little effect on chlamydiae that have already established an intracellular existence in the host cells. The MOMP forms part of the chlamydia outer membrane complex (COMC), has a molecular weight 39-45 kDa and accounts for 60% of the total protein in the COMC. It's main function is maintaining the cell wall rigidity (Melgosa et al 1991). The rest of the COMC consists of two cysteine-rich proteins, one that appears as a double band at 60 kDa, called Omp2, and one at 12-12.5 kDa, called Omp3. It also possesses a 96 kDa protein that can be demonstrated by polyacrylamide gel electrophoresis. The isoelectric point of the MOMP ranges from 5.3-5.5 and it is synthesized throughout the growth cycle of the organism (Schachter 1990). The location of the MOMP in the COMC and its size and pI values are similar to that of the porins found in other gram-negative bacteria. This suggests that it also functions as a type of 'pore' in the chlamydiae, allowing passage of molecules of up to 2.25 kDa. *C. trachomatis* antigens are heat-stable, periodate resistant and pronase sensitive, and are therefore protein in nature. Twelve trachoma serotypes (designated Ba and A-K) and 3 LGV serotypes (L-1, L-2 and L-3) have so far been identified in *C. trachomatis* (Schachter 1990). Several biotypes of *C. psittaci* have been identified to date (Marrie 1993).

The MOMP of *C. pneumoniae* is antigenically less complex than that of the other chlamydiae and is not the immunodominant antigen during infection (Marrie 1993, Melgosa et al 1991). Immunoblot analyses of the *C. pneumoniae* MOMP have shown that the recognition of this MOMP is only genus reactive, and that it has no other antigenic reactivities (Melgosa et al 1991). Campbell et al (1990) studied the humoral response to *C. pneumoniae* and concluded that the sera from their study population recognized immunoreactive proteins of 98, 68, 60 and 30 kDa as well as the 39,5-kDa MOMP, but that unlike sera from patients infected by the other Chlamydia species, which reacted strongly with the homologous MOMPs, the sera from *C. pneumoniae*-infected patients did not react strongly with the *C. pneumoniae* MOMP. They also demonstrated cross-reactions between *C. pneumoniae*-infected sera and the MOMPs of the other species. The *C. pneumoniae* MOMP gene is similar to the *C. trachomatis* and *C. psittaci* Omp1 and has been designated

'ompA' (Melgosa et al 1991). The 60 kDa protein found in *C. pneumoniae* is similar to the delayed type hypersensitivity antigen of *C. trachomatis* and the heat shock protein of *E. coli* (Marrie 1993). Black et al (1991) studied the antigenic profiles of six strains of *C. pneumoniae* and concluded that antigenic diversity among the strains of *C. pneumoniae* are found mainly with the 65-70 kDa proteins. Although strain variation can also involve the MOMP, this protein is not commonly recognized by serum from infected patients in immunoblots. The importance of antigenic variation in diagnosis by immunofluorescence is therefore uncertain. Convalescent serum samples were found to react with the 98, 68, 60, 39.5 and 30 kDa proteins of chlamydiae (Campbell et al 1990). According to Marrie (1993) the 98 kDa protein seems to be specific for *C. pneumoniae*. Overall conservation of the *C. pneumoniae* MOMP gene is 68% with *C. trachomatis* and 71% with *C. psittaci*.

1.1.4.4 TOXINS

The type-specific antigens have been described as either toxins or type-specific lipid haptens in the past (Stephens et al 1982). Although a specific toxin has not been identified in chlamydiae, a large number of infectious EBs inoculated intravenously into young mice have been demonstrated to cause death within 24 hours, after damaging the vascular endometrium, and infection of cell cultures with high numbers of EBs resulted in their death without a productive infectious process. This toxic effect can be prevented by antibodies that prevent entry of the chlamydiae into the host cells (Schachter 1990). It was also found that mice can be immunized against the toxin and that antiserum prevents death. The toxin is pro[illegible] type-specific antigen located in the RB of the organism. Antibodies to the toxin ca[illegible] absorbed by EBs but not by RBs. Recent evidence indicated that, when relatively large numbers of EBs are phagocytosed by macrophages or L-cells, early death and lysis of the cells occur and they release lysosomal enzymes (Nichols et al 1980). The toxicity associated with this release probably plays a role in pathogenesis.

1.1.5 IMMUNE RESPONSE TO CHLAMYDIA INFECTIONS

Diseases caused by chlamydiae tend to be chronic and relapses frequently occur. For example, the same serotype of *C. trachomatis* can be isolated from eyes or genital tracts of patients for up to 5 years. This implies either reinfection, or continuing infection, suggesting that chlamydial infections may evoke an ineffective immune response. Infection or immunization results in humoral, secretory and cell-mediated immune responses. Lymphokines, particularly gamma interferon, restrict chlamydial replication (Schachter 1990). Neutralizing antibodies that combine with the cell envelope prevent spread of infection to other susceptible host cells but do not appear to inactivate chlamydiae already localized within the cells. The role of circulating antibodies, secretory antibodies and the cellular immune response in chlamydial infection is not yet clear. The immune response to chlamydiae varies in the three species and can be harmful because re-exposure may lead to even more severe disease (Schachter 1990). Asymptomatic carriers have been documented and may be important in the spread of infection (Schachter 1990), but the cellular mechanisms underlying the carrier state are unknown.

1.1.6 LABORATORY DIAGNOSIS OF CHLAMYDIAL INFECTIONS

The laboratory diagnosis of chlamydial infections involve techniques such as direct examination of clinical material, isolation of the organism and serological tests. Special skills are required and routine laboratory tests are not useful for identification of the organism.

1.1.6.1 HANDLING AND STORAGE OF SPECIMENS

Chlamydiae are relatively unstable in laboratory conditions, therefore correct handling and storage of specimens is vital. Infectivity may be lost within 48 hours at 37°C, within 5 minutes at 56 °C and within a few weeks at 0°C. *C. pneumoniae* is rapidly inactivated at room temperature and only 1% of the organisms are viable after 24 hours. If stored at 4°C,

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70% of the organisms will be viable after 24 hours. The organisms can also be inactivated with 0.1% formalin, 0.5% phenol or organic solvents such as ether and ethanol that will destroy their infectivity within 30 minutes at room temperature. The pH range of stability is narrow and the rate of inactivation by ultraviolet irradiation is similar to that of *E. coli*. Suspensions are best preserved at -70°C or lower, or in some cases by lyophilization. Frozen suspensions will maintain their titre for several years. Rapid freezing at -70°C may however inactivate 50-60% of *C. pneumoniae* organisms (Grayston et al 1990) but if frozen slowly, by cooling at 4°C for up to 4 hours before freezing, only 23% of the organisms are inactivated. Ideally, specimens for isolation of the organism should be stored at refrigerator temperature and isolated witnin 24 hours, or specimens should be frozen below -65°C after 1-4 hours storage in the refrigerator (Grayston et al 1989, Grayston et al 1990).

1.1.6.2 DIRECT EXAMINATION OF CLINICAL MATERIAL

Cytological techniques are helpful in the diagnosis of ocular Chlamydia infections, but not for the diagnosis of human psittacosis. For ocular infections, the Giemsa stain is recommended, although the fluorescent or iodine stains can also be used. Correct thickness of the impression smears is very important for visibility of inclusions using cytological techniques. *C. pneumoniae* infections have not been diagnosed by cytological techniques so far.

1.1.6.2.1 GIEMSA STAIN

The Giemsa stain will provide permanent preparations. It allows assessment of the inflammatory response and is therefore useful for the diagnosis of mainly sporadic adult inclusion conjunctivits cases that can easily be confused with other forms of acute follicular conjunctivitis. Viral infections have a predominantly lymphocytic response while in chlamydial infection, a mixture of polymorphonuclear leukocytes, lymphocytes and other cells can be seen. In cases of atopic conjunctivitis, eosinophils and free granules are seen in the smears, while trachoma cases usually present with polymorphonuclear leukocytes,

lymphocytes and macrophages. In active trachoma, immature lymphoid cells and plasma cells can be seen together with necrotic material. Sometimes Leber cells and giant macrophages containing phagocytosed material are also seen. Disadvantages of the method are that it is time consuming, proper examination of one slide may take up to an hour, and should be carried out by experienced workers. An oil immersion lens should be used to avoid inclusions being overlooked or confused with artifacts and cellular structures that could be present. The Giemsa stain is not recommended for genital tract specimens but can be used for demonstration of particles in histologic sections, impression smears and tissue cultures.

1.1.6.2.2 FLUORESCENCE STAINING

Either direct or indirect techniques can be used successfully. This is the most sensitive cytologic method and has been applied in the diagnosis of trachoma, mainly in mild cases, and in occulogenital infections. Although comparisons of Giemsa and immunofluorescence staining sensitivity for the detection of *C. trachomatis* inclusions in cell culture were inconclusive, immunofluorescence proved to be more sensitive and rapid than the Giemsa method for detection of inclusions of the organism produced from clinical specimens inoculated into cell cultures. Disadvantages of the technique are that it is time consuming, requires skilled interpretation and fades rapidly. Since the development of monoclonal antibodies to *C. pneumoniae*, immunofluorescence has also been used for the diagnosis of infections by this organism.

1.1.6.2.3 IODINE STAIN

Chlamydial inclusions contain a glycogen-like material that can be stained with iodine. It is a very simple and quick technique to use and entire slides can be screened in a matter of minutes. Inclusions can be recognised even in thick areas of the smears. The slides can be kept for permanent record or can be counterstained with Giemsa for confirmation. Iodine staining can however not be used for staining *C. psittaci* because the organism does

not produce sufficient glycogen to show up in the stain (Stephens et al 1982). It is the least sensitive of the cytologic techniques and cannot be used to stain scrapings from the genital tract since cells containing glycogen may also be present in normal specimens.

1.1.6.3 ISOLATION TECHNIQUES

Chlamydiae can be isolate. by either the yolk sac or the cell culture (tissue culture) techniques. Cell culture is more sensitive and less time consuming than the yolk sac method (Gordon et al 1969). Also, a higher level of bacterial contamination can be tolerated in cell culture than in the yolk sac method. The isolation of *C. pneumoniae* is difficult as it grows poorly and is inactivated by repeated freezing and thawing, but is important in patients whose antibody response may be unreliable, eg. those infected with HIV (Augenbraun et al 1991). Chlamydia transport medium SPG or 2SP should be used for storage of specimens (Grayston et al 1989).

1.1.6.3.1 YOLK SAC CULTURE

The yolk sac technique is the method of choice for isolation of *C. trachomatis* strains causing LGV, but is not sensitive for isolation of trachoma strains. For yolk sac procedures nutrient broth containing streptomycin, neomycin and nystatin is normally used. Seven-day old embryonated chicken eggs are inoculated and incubated. The yolk sac membranes are harvested when the eggs are killed either by growth of chlamydiae or on day 12 or 13 before hatching. Smears from the yolk sac membranes are made on microscope slides and stained with the modified Machiavello or immunofluorescence stains (Kuo et al 1986). The method is not recommended for *C. pneumoniae* which shows low virulence in egg culture.

1.1.6.3.2 CELL CULTURE

The cell culture technique has been used for decades for the isolation of *C. trachomatis* and

C. psittaci, and lately also for *C. pneumoniae*. The best medium to use for cell culture is complete cell culture medium supplemented with gentamycin, vancomycin and amphotericin B. Strains of *C. trachomatis* that cause LGV grow easily in cell culture, but not strains which cause trachoma. This can be overcome by increasing the contact between the organism and host cells by centrifuging the culture. Alternatively, irradiated McCoy cells, or cells treated with iodo-deoxyuridine or DEAE-dextran can be used. DEAE-dextran increases and neuramidase decreases the infectivity of trachoma-causing strains, but do not affect strains causing LGV. *C. psittaci* grows well in a variety of cell lines, including Chang human liver cells, fetal-mouse-lung cells, human diploid cells, L-cells and chicken fibroblasts. The cell line most frequently used is HeLa-229 cells because the organism will not only grow well in cell monolayers, but will also grow in suspended cells in spinner culture. Any of the three cytologic staining techniques mentioned earlier can be used for screening of the cell cultures for inclusions.

C. pneumoniae grows poorly in cell culture and produce smaller inclusions than the other two species (Grayston 1992). However, with multiple passages of cultures and the newer cell lines, isolation of the organism is becoming easier (Campbell et al 1991). Various cell lines can be used for isolation, of which HeLa-229, L-cells and McCoy cells are the most commonly used (Grayston et al 1989). Cles and Stamm (1990) discovered a human cell line called HL-cells that can be used for the culture of *C. pneumoniae*. These cells are more sensitive than HeLa-229 cells and can be used for both laboratory adapted and wild strains of the organism. A kinetic study of *C. pneumoniae* growth in HL-cells showed a three day cycle, similar to that in HeLa-229 cells. In the HL-cells, the inclusions ruptured on day 4 and a second growth cycle occurred in previously uninfected cells (Kuo et al 1990). This has not been observed in any of the other cell lines. The most sensitive cell line for culture of *C. pneumoniae* was however recently reported to be HEp-2 cells (Marrie 1993). Inclusions can be stained with either of the staining procedures mentioned earlier but the immunofluorescence stain is recommended for *C. pneumoniae* due to the small number of inclusions normally present (Grayston et al 1990).

Experiments with TW-183 and AR-39, using egg grown organisms as inoculum showed that the inclusion counts were increased by addition of DEAE-dextran, centrifugation or cycloheximide treatment in any of the cell lines mentioned (Kuo et al 1988), although less cycloheximide is needed for isolation of the organism in HL-cells than in the other cell

lines. The HL-cells also grow more slowly than the others, and addition of DEAE-dextran will not increase the infectivity of the organism. Centrifugation alone yields 3600 times more inclusions in HeLa-229 cells, 1400 times more in McCoy cells and 60 times more in L-cells. The best results can however be obtained by combination of all three techniques. Titres of 1.5-2.5 times greater were demonstrated with the combined methods than with centrifugation alone. Incubation of cell cultures is more effective at 35°C than at 37°C for inclusion formation as well as burst size of the organism. Kuo and coworkers (1988) showed that inclusion counts were between 1.5 and 2.5 times higher and the burst size 1.2 - 4.6 times greater at 35°C. Although the inclusions of *C. pneumoniae* in HeLa-229 cells resemble those of *C. psittaci*, they do not have the variable shape (Kuo et al 1986) or the growth around the cell nucleus found in *C. psittaci* (Marrie 1993).

1.1.6.4 SEROLOGICAL TECHNIQUES

No single serological test is generally applicable for the diagnosis of all chlamydial infections. Currently two techniques are recommended: the complement fixation test (CFT) and the micro-immunofluorescence test (IFT). With either of the two techniques a fourfold rise in antibody titre (seroconversion) is usually considered to be diagnostic of disease. This is however not always applicable and the clinician must interpret the results in terms of the disease pattern and the background prevalence of antibody titres.

During the primary response to *C. pneumoniae*, usually seen in children, teenagers and young adults, complement fixation antibodies usually appear first. A relatively long time is required for development of *C. pneumoniae* antibodies detectable by the micro-immunofluorescence test.

1.1.6.4.1 COMPLEMENT FIXATION (CFT)

The complement fixation test detects antibodies to group-specific antigens. It is therefore not specific and will detect antibodies to all three Chlamydia species as well as antibodies to *M. pneumoniae*, influenza A and B, adenovirus, and respiratory cyncytial virus (Grayston

et al 1990). Complement fixation cannot be used for the diagnosis of trachoma, but will detect antibodies to psittacosis and LGV.

The antigens used in the test are prepared by inoculation of embryonated hens eggs, incubation, grinding and suspension in nutrient broth. The suspensions are standardized and refrigerated before use. Ether-acetone-extracted antigens are also routinely used for Chlamydia species as well as influenza A and B, parainfluenza, respiratory cyncytial virus, adenovirus, herpes simplex, coxackie B5, cytomegalovirus and *M. pneumoniae*. There is an antigen commercially available that contains a purified and inactivated suspension of *C. psittaci* grown in yolk sacs (Kleemola et al 1988).

Reagents necessary for complement fixation include buffered saline, group antigen, antigen control, positive and negative sera, quinea-pig complement, rabbit-anti-sheep complement and sheep red blood cells. Serum is diluted in phosphate buffered saline and should be tested for reactivity to normal yolk sac antigens and for anticomplementary activity. The endpoint of the serum is the highest dilution producing less than 50% haemolysis.

Results are interpreted as follows: a fourfold or greater rise in antibody titre or a single titre ≥1:64 are considered to be evidence of a current or recent infection (Thom et al 1990). If at least one of the serum samples shows a titre of ≥1:16 and neither shows a titre of ≥1:64, it is considered to be evidence of a past infection. In primary infections a rapid rise in both complement fixation and IgM titres is usually seen. This may be lost at approximately 6-7 weeks post-infection (Berdal et al 1991). It should be noted that older people with *C. pneumoniae* infections usually have a negative result on the complement fixation test (Grayston et al 1990).

1.1.6.4.2 MICRO-IMMUNOFLUORESCENCE (IFT)

The micro-immunofluorescence test for chlamydiae was originally developed and reported in 1970 by Wang and Grayston for the diagnosis of *C. trachomatis* infections. It is both sensitive and type specific and can be used for detection of antibodies of the IgA, IgM and IgG classes (Wang et al 1975). The test is mainly used to distinguish between current, recent

and previous infections (Grayston et al 1989) and between primary and secondary immune responses to t' ; rganism. There is no evidence of false positive results due to viral or mycoplasma infections (Aldous et al 1992). Most authors regard micro-immunofluorescence as highly sensitive and specific, although cross-reactions have been demonstrated with the other chlamydiae (Verkooyen et al 1992). Rheumatoid factor (RF) is an autoantibody (usually of the IgM class) that will react to antigenic sites situated in the Fc region of IgG, thereby interfering in the detection of IgM antibodies, leading to false-positive results. Absorption of IgM antibodies with IgG is therefore recommended to prevent false positive IgM results (Verkooyen et al 1992).

Antigens used in the test are suspensions of EBs. Chlamydiae can be grown by either yolk sac or cell culture techniques for antigen production. Antigen slides are prepared by placing dots of the appropriate antigen on the slides and fixing them in acetone. The slides can be stored frozen until use. For the test, serial dilutions of the serum are made in phosphate buffer. Drops of dilutions are placed on the slides, which are then incubated, dried, stained with fluorescein-conjugated antihuman globulin (either IgA, IgM or IgG) and mounted. Antigen slides are examined by standard fluorescent microscopy. The test measures specific antibodies to antigenic determinants present in the cell walls of the EB particles and appears to be reproducible within a two-fold variation, but recent observations by Marrie (1993) suggest that the FML-10 strain should be included because some patients show a rise in antibody titre to FML-10 and not to TW-183.

1.1.6.4.3 OTHER IDENTIFICATION TECHNIQUES

A polymerase chain reaction (PCR) technique has been developed by Gaydos et al (1992) for diagnosis of *C. pneumoniae* infections and identification of the organism but is not used routinely yet (Gaidos et al 1992, Holland et al 1990, Marrie 1993). There is also an enzyme immunosorbent test available to detect chlamydial antigens (Marrie 1993). To differentiate between *C. pneumoniae* and *C. psittaci*, a *C. pneumoniae*-specific monoclonal antibody is used.

1.1.7 INTERPRETATION OF SEROLOGICAL RESULTS

Both the timing of collection and the response patterns are important in the interpretation of *C. pneumoniae* antibody results in paired sera. The period from the onset of symptoms and the convalescent stage of disease is more than 21 days in 68% of *C. pneumoniae* infections. Therefore the interval between acute and convalescent sera should be between 14 and 27 days.

There are two patterns of antibody response to *C. pneumoniae* infection. During primary infection, generally in children, teenagers and young adults, an immediate complement fixation antibody response is usual. These antibodies tend to appear first, followed by the development of antibodies detectable by the micro-immunofluorescence test. IgM antibodies appear about 3-4 weeks after onset of symptoms, start to decrease within 2 months and usually disappear 4-6 months after acute infection. IgG titres may not reach diagnostic levels before 6-8 weeks after onset of illness, but will persist for up to 2-3 years in 50% of patients (Grayston et al 1990, Saikku et al 1992). IgA will also increase but decreases more rapidly than IgG (Saikku et al 1992). Therefore, if the convalescent sample is obtained less than 3 weeks after onset of symptoms, the antibodies can be missed. During reinfection, the complement fixation and IgM reactions may be absent, IgG titres rise quickly, often within 1-2 weeks and may reach titres ≥1:512 (Grayston et al 1989, Hahn DL et al 1991). The abscence of complement fixation and IgM in cases of reinfection can make it difficult to demonstrate evidence of current infection. Diagnosis of a chronic *C. pneumoniae* infection is problematic as antibody levels in patients with chronic infections are not well known, but recent evidence suggested an association between raised IgA titres and chronic bacterial infections in general. Also, circulating antibodies con microbial components have been observed in several chronic infections (Saikku et al 1 he general population usually has a low titre IgG and IgA is rarely detected except during epidemics (Saikku et al 1988).

The criteria for establishment of acute versus previous infection as developed by the University of Washington are: a fourfold or greater increase in antibody titre to ≥1:16, an IgM titre ≥1:16 or an IgG titre ≥1:512 are termed acute antibody and are considered presumptive of a current or recent *C. pneumoniae* infection. An IgG titre ≥1:16 (some

authors use 1:32 as the lowest titre) and ≤1:256 are considered as pre-existing antibody due to past infection and are used to determine population prevalence (Li et al 1989, Thom et al 1990, Torres et al 1993). Hahn and coworkers (1991) suggested that IgG titres ≥1:64 may indicate an ongoing secondary infection in some individuals. Also, in some cases of reinfection there may be a slight increase in IgM, with or without an increase in IgG. In a person who had IgG in a previous sample, a fourfold or greater increase in IgG is also regarded as reinfection (Marrie 1993). An IgG titre between 1:8 and 1:256 may also be considered as chronic antibody (Puolakkainen et al 1993). These authors also consider seroconversion during acute *C. pneumoniae* infections as a fourfold increase *or decrease* in antibody titre. The percentage of patients with persistent antibody to *C. pneumoniae* over time is very stable in comparison with *M. pneumoniae* and other agents. This reflects the longevity of anti-*C. pneumoniae* IgG detected by micro-immunofluorescence test compared to the anti-Mycoplasma response as measured by complement fixation.

1.1.8 *C. PNEUMONIAE* INFECTIONS

1.1.8.1 PREVALENCE

Antibodies to *C. pneumoniae* are rarely present in children under the age of 5 years (Grayston et al 1986). The prevalence of antibodies increases sharply in older children, reaching a peak at 8-9 years and by early adulthood, approximately 30-50% of the general population has serological evidence of past *C. pneumoniae* infections (Marrie 1993). This figure may even be as high as 60% (Torres et al 1993). The prevalence of antibodies reaches a plateau of about 55% by the age 30-40 years and persists at this level through old age (Grayston et al 1990). Another peak was reported by Marrie et al (1993) in persons in their 70's. Acute lower respiratory tract infections by the organism have however been reported in children in developing countries where it causes considerable morbidity and mortality. In Denmark and Finland, *C. pneumoniae* infections have been reported in young children (Saikku et al 1988), while in Taiwan, 10% of children under 5 and up to 36% of children between 5 and 9 years are infected at some time with the organism. Aldous et al (1992) also reported a high infection rate in children aged between 5 and 14 years in Seattle. The

prevalence of antibodies in the general population varies. In American teenagers the prevalence was 20-30%, rising to 40-60% in adults (Shor et al 1992). Pre-existing antibodies to *C. pneumoniae* were also reported in 50% of adults from seven different countries, ranging from 40% in Nova Scotia to > 60% in Taiwan. Torres et al (1993) reported a prevalence of 25-77% in adults in the general population.

C. pneumoniae infections occur both endemically and epidemically. Asymptomatic carriage has been documented, and there is no evidence of sexual transmission of the organism. The exact mode of transmission is unknown but the low prevalence in children before school-going age suggest that transmission occurs more often outside the home (Aldous et al 1992). Clusters of *C. pneumoniae* infections have however been demonstrated in families (Grayston 1992). Seasonality of infections does not occur, but periodic outbreaks, possibly with a 6-year cycle, have been reported (Grayston et al 1992). Outbreaks have been reported in Finland, Denmark, Norway, England and the United States (Marrie 1993).

1.1.8.2 SYMPTOMS

C. pneumoniae is a human pathogen and is transmitted from human to human without a bird or animal reservoir. Little is known about the mode and place of transmission, the incubation period and the infectiousness of the organism. According to Grayston and Thom (1992) the incubation period is long compared with common viral respiratory pathogens and the organism is not highly infectious. Hyman et al (1991) described infection of two laboratory workers through droplet aerosolization. Although asymptomatic, the organism was cultured from specimens taken from these workers. The clinical manifestation of an acute *C. pneumoniae* infection may range from asymptomatic to life-threatening pneumonia. Chronic infections have been reported and recurrence of disease is frequent, despite antibiotic treatment (Saikku et al 1988). Although *C. pneumoniae* causes more human infections than the other chlamydiae, the majority of these infections (probably as many as 90%) are mild or asymptomatic and are therefore not reported as patients with mild symptoms often do not seek medical care (Grayston et al 1986, Marrie 1993, Torres et al 1993). The organism is primarily a respiratory pathogen that commonly causes pneumonia, bronchitis, pharyngitis and sinusitis.

The clinical appearance of *C. pneumoniae* infection varies by age. Studies of chidren in the USA and Denmark suggested that infections in children and young adults are usually mild and can be treated on an outpatient basis while older adults have more serious symptoms and may have to be hospitalized with pneumonia (Grayston et al 1992).

There is no set of symptoms unique to *C. pneumoniae* infection, but several characters in the clinical presentation may help to distinguish it from other infections. The onset of disease may be prolonged with upper respiratory symptoms, mainly pharyngitis, fever and sore throat often with hoarseness and wheezing (Hahn et al 1991, Marrie 1993). Laryngitis may occur, but without culture of pathogenic bacteria. This is followed by increased cough and other symptoms of lower respiratory disease, mainly bronchitis or pneumonia, with pharyngitis and sinusitis frequently accompanying these symptoms (Grayston et al 1989, Kleemola et al 1988). The cough and malaise may be persistent for up to two months. There is usually no sputum production although Thom et al (1990) reported a productive cough in their study population. Some patients may suffer from chest pain. Symptomatic treatment usually resolves the initial symptoms, but after 2-3 weeks a relapse may occur, this time with symptoms of bronchitis or pneumonia. The erythrocyte sedimentation rate (ESR) in *C. pneumoniae* infections is usually high for an average of up to two weeks but the white cell count and differential count are usually normal although an increase in white cell count has been reported (Grayston et al 1986).

1.1.8.2.1 PNEUMONIA

Pneumonia is the most common syndrome caused by *C. pneumoniae* and is usually mild (Grayston et al 1990, Marrie et al 1987). It may be asymptomatic with a single, subsegmental infiltrate, although severe illness may occur in older people and in those with chronic disease. Hospitalization is usually not required except in severe cases (Kleemola et al 1988). Pneumonitis is seen in 100% of *C. pneumoniae* infections, compared to the 80% in mycoplasma and 20% in viral infections. Pneumonia associated with acute phase *C. pneumoniae* infection has no characteristic clinical or radiographical features. Patients with *C. pneumoniae* infections are more likely to be hoarse and afebrile than patients with *M. pneumoniae* or viral infections, and usually wait longer before they seek medical attention

(Marrie 1993). In the USA, several hundred thousand cases of pneumonia due to *C. pneumoniae* infection are reported each year, compared to only a few hundred due to infections by *C. psittaci* and *C. trachomatis*. The organism is associated with 6-12% of pneumonias in outpatients and 6-10% in inpatients (Grayston et al 1989, Gra...n et al 1992). These authors also reported that, between 1980 and 1981, 11% of hospitalized pneumonia cases were caused by Influenza A virus, 10% by *C. pneumoniae* and only 3% by *M. pneumoniae*. In Spain, *C. pneumoniae* was the most common cause of community-acquired pneumonia in the outpatients of three hospitals, followed by *S. pneumoniae* and *M. pneumoniae* (Almirall et al 1993).

1.1.8.2.2 BRONCHITIS

Bronchitis caused by *C. pneumoniae* usually has a subacute onset, may be prolonged and is often preceded or accompanied by pharyngitis. Approximately 4-5% of bronchitis ...ses in young adults are caused by *C. pneumoniae*. It causes mainly chronic bronchitis in older adults. Hahn et al (1991) reported asthmatic bronchitis after respiratory tract illness caused by *C. pneumoniae* infection. The symptoms usually respond to appropriate antibiotic treatment.

1.1.8.2.3 PHARYNGITIS

Pharyngitis caused by *C. pneumoniae* may be quite severe and is one of the symptoms in about 80% of lower respiratory infections caused by the organism (Grayston 1989), but is not often seen in cases without lower respiratory infections (<1%) Huovinen et al (1989) reported *C. pneumoniae* antibodies in 8% of patients with pharyngitis. Hoarseness associated with pharyngitis is seen in 30% of *C. pneumoniae* infections (Cooper et al 1991).

1.1.8.2.4 LARYNGITIS

Several authors reported cases of severe laryngitis due to *C. pneumoniae* infections (Grayston et al 1986, Hashiguchii et al 1992).

1.1.8.2.5 SINUSITIS

Approximately 5% of primary sinusitis in young adults is associated with *C. pneumoniae* infections. A study in Denmark reported that 5-7% of patients with lower respiratory infections due to *C. pneumoniae*, suffered from sinusitis at the same time (Grayston et al 1992). In Japan, where the population density is high, 19% of patients with sinusitis were reported to have antibodies to the organism (Hashiguchii et al 1992). *C. pneumoniae* infection may result in chronic sinusitis in older adults (Grayston 1989).

1.1.8.2.6 ASTHMA

An association has been found between *C. pneumoniae* infection and asthma during the period after illness (Hahn et al 1991). Past infections by the organism may predispose the lungs to bronchospasm during subsequent infections with other micro-organisms. Chronic infection or reinfection with *C. pneumoniae* may trigger an immunopathologic process in the lungs that may cause epithelial damage and mediator release or delayed hypersensitivity to chlamydial antigens. This may cause chronic airway inflammation characteristic of asthma. Hahn et al (1991) found antibodies to the organism in 81% of patients with asthmatic bronchitis, in 100% of patients older than 40 years and in 80% of patients with asthma.

1.1.8.2.7 ENDOCARDITIS

C. pneumoniae infection rarely results in endocarditis but cases have been reported (Grayston 1989). When it does occur, the blood culture is usually negative (Marrie et al 1990). So far eight cases of chlamydial endocarditis have been published of which six were caused by *C. psittaci*, one by *C. pneumoniae* and one by *C. trachomatis*. Three of the patients died as a result of the infection (Marrie et al 1990, Marrie 1993).

1.1.8.2.8 CORONARY HEART DISEASE

Bacterial and viral infections are known to contribute to the formation of atherosclerosis by causing damage to the endothelium of blood vessels. The chlamydial LPS binds to low density lipoproteins, modifying them and making them immunogenic or toxic for endothelial cells (Saikku et al 1992). These modified or antibody-associated, low density lipoproteins cause formation of foam cells in vitro, that is known to be the first phase in the development of atherosclerosis. The LPS of chlamydiae also induces tumour necrosis factor which inhibits lipoprotein lipase and causes changes in lipid metabolism. This results in the accumulation of triglycerides in the blood stream.

Investigators in Finland (1989) and again more recently in Helsinki (1992) studied the association between coronary heart disease (CHD) and infection with several bacteria and viruses. They found that only antibodies to *C. pneumoniae* can be associated with CHD (Thom DH et al 1991 and 1992). Shor et al (1992) demonstrated the presence of *C. pneumoniae* inclusions in coronary arterial fatty streaks by electron microscopy. Inclusions were also seen in 87% of South African post mortem sera from individuals with atherosclerotic changes in the coronary arteries (Puolakkainen M et al 1993).

Several other studies have also suggested chronic *C. pneumoniae* infection in both acute and chronic coronary heart disease (CHD). In Finland antibodies to the organism were found in 50% of patients with chronic CHD and in 68% with acute myocardial infarction, compared to only 17% of controls. The presence of antibody to *C. pneumoniae* is also

associated with coronary artery disease (CAD). The estimated risk of CAD is 2.6 times greater in persons with antibodies to the organism than in those without antibodies. Thom et al (1991) showed an association between CAD and *C. pneumoniae* antibodies in smokers. This association was identical for persons with and without prior myocardial infarction.

In 1988, Saikku et al demonstrated an association between raised *C. pneumoniae* antibody titres and the presence of circulating immune complexes containing chlamydial LPS in patients with acute myocardial infarction. Grayston (1993) reported *C. pneumoniae* immune complexes in 41% of chronic coronary artery disease cases compared to 15% of the controls they studied.

1.1.8.2.9 OTHER SYNDROMES

An association between *C. pneumoniae* infection and several other syndromes have been reported. These include encephalitis, chronic obstructive pulmonary disease, tonsillitis, otitis media, erythema nodosum, thyroiditis and sarcoidosis.

1.1.8.3 REINFECTION

Reinfections by *C. pneumoniae* occur frequently and may be either milder or more severe than the primary infection (Aldous et al 1992). The precise role of reinfection with the organism has not been determined fully, but is occurs more frequently in older adults who appear to have more severe disease. Reinfection may occur with or without measurable antibodies and within months or years after the primary infection. The infections may be asymptomatic or may cause upper respiratory infection only. Compared to *M. pneumoniae* and other agents, IgG to *C. pneumoniae* persist for longer periods. This may indicate frequent reinfections, although Thom et al (1990) suggested that reinfections do not occur frequently.

1.1.8.4 CHEST RADIOGRAPHS

Chest radiographs show single pneumonic lesions but findings may differ from person to person (Grayston et al 1989, Marrie et al 1987). Infiltrates occur more frequently in patients with *C. pneumoniae* and *M. pneumoniae* infections than in patients with pneumonia not associated with these organisms. The infiltrates seen on radiographs are usually single lesions in either the middle or the lower lung (Kleemola et al 1988) but in some cases more than one lesion may occur. These are usually single lesions in both lungs and may persist for up to 30 days. In all the patients studied by Saikku et al (1985), a single definite pneumonic lesion, and pneumonic infiltrates were observed. Most of these lesions were peripheral and appeared to involve only one lobe. The smaller lesions were 2-3 cm in diameter and rounded, while the more extensive ones showed segmental consolidation. The lesions were found in all parts of the lung. Half of them were of homogenous density while the other half were of heterogenous density. There was no atelectasis or apparent excavation on the more extensive lesions. None of the patients they studied had pleural effusions or definite hilar adenopathy. The lesions usually disappeared without complications.

1.1.9 TREATMENT OF *C. PNEUMONIAE* INFECTIONS

Chlamydiae are sensitive to sulfonamides and penicillin in cell culture. Penicillin does not affect the penetration of host cells by EBs or reorganisation to form RBs, but it inhibits subsequent binary fission, synthesis of envelope subunits and maturation to form EBs again. Sulfonamides inhibit only chlamydiae which synthesize folic acid. In general, *C. trachomatis* is susceptible, while *C. psittaci* and *C. pneumoniae* are resistant. Both *C. trachomatis* and *C. psittaci* are highly susceptible to tetracyclines. This is the drug of choice for most chlamydial infections. Erythromycin is the alternative for patients who cannot tolerate tetracyclines, eg. pregnant women and young children. *C. pneumoniae* is susceptible to both these drugs, but prolonged, intensive treatment is needed. The recommended dose is 2 g daily for 10-14 days or alternatively 1-1.5 g daily for 21 days. Even after this vigorous treatment relapses in the infection may occur. For a second course of treatment, tetracycline is recommended. Rifampin and related compounds are very effective in cell culture but are

not used clinically because resistance to these compounds develop quickly. Drugs that penetrate host cells with difficulty, eg. aminoglycosides and cephalosporins are relatively ineffective against chlamydiae. Clinical experience of antibiotic treatment of chlamydial infections is generally consistent with in vitro testing, although no controlled trials have so far been performed. Sulfonamides, penicillin, ampicillin and sulfisoxazole are not effective against *C. pneumoniae*. The drugs of choice for *C. pneumoniae* infections are therefore clarithromycin (Ridgway et al 1991), azithromycin (Marrie 1993), tetracycline and erythromycin. A comparison of minimum inhibitory concentrations (MIC) of different antibiotics against *C. pneumoniae* infections is listed in Table 1.2.

TABLE 1.2

MIC'S OF ANTIBIOTICS TESTED AGAINST C. PNEUMONIAE

	MIC (MG/L)	
Clarithromycin	0.007	(0.03)
14-hydroxy-clarithromycin	0.015	(0.03)
Erythromycin	*0.01 - 0.06	(0.5)
Tetracycline	0.05 - 0.1	(0.5)
Oxytetracycline	0.25	
Josamycin	0.25	
Roxithromycin	0.25	
Sparfloxacin	0.25	(0.5)
Azithromycin	*0.5	
Ofloxacin	1.0 - 2.0	
Ciprofloxacin	2.0	(16.0)
Spiramycin	4.0	
Trospectomycin	10.0 - 20.0	
Penicillin	> 100	
Ampicillin	> 100	
Sulfisoxazole	> 400	

() : MIC when drug added to cells after infection

* : Shows a discrepancy between results from Cooper et al (1991) and other authors. Cooper et al found a MIC of 0.12 for erythromycin and 0.03 (0.06) for azithromycin

Table adapted from Ridgeway et al (1991) and Cooper et al (1991).

1.2 THE GENUS LEGIONELLA

Legionnaires' Disease occurs worldwide in sporadic and epidemic form and was first recognized after a pneumonia outbreak in Philadelphia in 1976. The causative organism was placed in the family Legionellaceae, genus Legionella, on the basis of microbiological, immunological and genetic characteristics, and was called *L. pneumophila* (Brenner et al 1979). Retrospective studies proved that the first *L. pneumophila* strain, OLDA, was cultured in 1947, and the first strain of *L. micdadei*, TATLOCK, in 1943 (Winn et al 1981).

The clinical syndromes caused by members of the Legionellaceae are collectively known as legionellosis and consist of a severe, often fatal pneumonia called Legionnaires' Disease, and a milder, flu-like illness called Pontiac Fever. Outbreaks of Legionnaires' Disease have been reported from as early as 1957 in Minnesota (Osterholm et al 1983), Washington DC (England et al 1981), Phildelphia (Terranova et al 1978), Vermont (Broome et al 1979) and Wadsworth (Haley et al 1979). The first Pontiac Fever outbreak was reported in Pontiac, Michigan, in 1968 (Glick et al 1978).

The South African Legionella Research Laboratory was founded in 1979 in Johannesburg, where, by the early 1980's, sixteen sporadic cases of Legionnaires' Disease had been identified. Among these were 2 cases from Johannesburg (Kaplan et al 1980), 8 from Port Elizabeth (Randall et al 1980) and one from Durban (Hariparsad et al 1981). The first Legionnaires' Disease outbreak in South Africa occurred in a Johannesburg teaching hospital in 1985 (Strebel et al 1988).

1.2.1 CLASSIFICATION

The causative agent of Legionnaires' Disease was originally placed in the family Legionellaceae and called *Legionella pneumophila*. The genus Legionella currently includes more than 30 species with a total of more than 50 serogroups. Known Legionella species are listed in Table 1.3. *L. pneumophila* is responsible for 90% of infections caused by the family and contains 14 serogroups (SGs) of which SGs 1, 4 and 6 are most often implicated

in human infections (Yu 1990). *L. longebeacheae, L. bozemanii* and *L. hackeliae* each contain two serogroups (Brenner 1986).

Two additional genera, Tatlockia and Flouribacter, have been suggested for the family Legionellaceae (Garrity et al 1980). In this classification, *L. pneumophila* stayed in the genus Legionella, while *L. micdadei* was transferred to the genus Tatlockia and the three blue-white autofluorescing species, *L. bozemanii, L. dumoffii* and *L. gormanii,* were transferred to the genus Flouribacter. The legionellae however all show more than 25% DNA homology, are isolated by similar techniques and antibiotic therapy is the same for all species. One genus, Legionella, is therefore generally accepted.

1.2.2 MORPHOLOGY

Legionellae are thin, slow-growing, slightly irregular organisms, 0.3-0.9 μm wide by 2-20 μm long. They often appear tapered at one or both ends, but without the needle-shaped appearance of Fusobacterium species (Winn et al 1984). Organisms cultured from tissue and clinical specimens are usually coccobacillary, but filamentous forms have been demonstrated after growth on certain media (Yu 1990). They contain an inner trilaminar membrane, a peptidoglycan layer and an outer trilaminar membrane like other gram-negative bacteria. The cytoplasm contains vacuoles and ribosomes and the cells divide by a pinching process in which no septa are formed.

Legionellae are obligatory aerobes, non spore-forming, unencapsulated and generally non acid-fast (Brenner DJ 1986) although partial acid-fastness has been demonstrated in *L. micdadei* and one strain of *L. pneumophila* SG 6 (Winn 1984). False acid-fastness may occur resulting in some strains being confused with *M. tuberculosis* (Yu 1990).

Legionellae are physically hardy and heat-tolerant. They will survive in distilled water for up to 139 days and in tap water for more than a year at room temperature. The organisms will grow and multiply in temperatures between 25°C and 42°C, with an optimum of 35-37°C. They cannot survive for long periods at temperatures > 50°C (Winn 1984).

TABLE 1.3

LEGIONELLA SPECIES

PATHOGENIC	NON-PATHOGENIC
L. anisa	L. brunensis
L. birminghamensis	L. cherrii
L. bozemanii	L. erythra
L. cincinnatiensis	L. israelensis
L. dumoffii	L. jamestowniensis
L. feelei	L. moravica
L. gormanii	L. oakridgensis
L. hackeliae	L. qunilivanii
L. jordanis	L. sainthelensi
L. longbeacheae	L. santicrucis
L. maceachernii	L. spiritensis
L. micdadei	L. steigerwaltii
L. parisiensis	
L. pneumophila	
L. rubrilucens	
L. wadsworthii	

(Ratshikhopha 1990)

1.2.3 BIOCHEMICAL REACTIONS

Legionella species are not easily distinguished by routine biochemical tests, but methods such as gas-liquid chromatography, colony fluorescence on charcoal yeast extract (CYE) agar exposed to ultraviolet light, G+C base ratios and DNA homology can be used successfully. Carbohydrate fermentation tests are usually negative, but the API ZYM system (Bio Mérieux, Lyon, France) has been used successfully in the past. Most strains and serogroups of *L. pneumophila* and *L. feelei* are hippurate hydrolysis positive. This reaction is variable in *L. bozemanii, L. micdadei, L. dumoffii* and some of the other legionella-like organisms (Hebert 1981).

Catalase, peroxidase and superoxide dismutase (SOD) are not present in all legionellae (Pine et al 1986). *L. pneumophila* contains only peroxidase, *L. bozemanii* only catalase and *L. dumoffii* both catalase and peroxidase. The other legionellae are all catalase positive or weak positive (Winn 1984), asaccharolytic, urease and nitrate reduction negative and gelatinase and starch positive. Oxidase is negative in *L. pneumophila* but variable in the other species (Yu 1990). All species except *L. oakridgensis* are motile (Brenner 1986). The beta-lactamase reaction is positive in *L. micdadei, L. feeleii* and *L. maceachernii* (Yu 1990). Thorpe et al (1981) detected extracellular protease, phosphatase, lipase, ribonuclease and deoxyribonuclease and ß-lactamase in *L. pneumophila* while elastase, collagenase, lecithinase, hyaluronidase, chondroitinase, neuraminidase and coagulase were absent, and α-amylase activity was weak. Legionellae appear to possess decarboxylases for lysine and ornithine, or an arginine dehydrolase (Brenner et al 1979).

1.2.4 NUTRITIONAL REQUIREMENTS

Legionellae are chemo-organotrophic and use amino acids, catabolized through the Krebs cycle, as their main source of carbon and energy (Yu 1990). These amino acids include arginine, isoleucine, leucine, methionine, threonine, valine, phenylalanine and tyrosine. Most strains require serine that can be used as the sole source of carbon and energy and some also require proline (Rowbotham 1980, Tesh et al 1983). In addition to the amino

acids, several other carbon sources were shown to stimulate oxygen uptake, for example glutamate, pyruvate, lactate, acetate, succinate, fumarate and citrate (Tesh et al 1983). Pyruvate and acetate are incorporated into the lipid-containing cell fractions. Threonine and tyrosine stimulate oxygen uptake of legionellae, depending on the medium used for culture. Bacteria such as Neisseria, Campylobacter and Legionella are susceptible to the action of peroxides and superoxides that are generated by exposure of the culture medium to light.

L-cysteine and iron salts are critical for growth of all legionellae, although *L. pneumophila* can grow over a broad range of iron concentrations. Under conditions of iron deprivation, the lag phase of the organism is increased and cellular morphology is affected, giving rise to short, coccobacillary forms. Long slender rods are formed when the iron concentration in the medium is high (Ristroph et al 1981). The activated charcoal in buffered charcoal yeast extract (BCYE) and charcoal yeast extract (CYE) agar can absorb and detoxify fatty acids and oxygen radicals, thereby preventing the oxidation of cysteine (Yu 1990), while the addition of α-ketoglutaric acid and pyruvate in CYE agar and broth media stimulates the production of oxygen-scavenging enzymes to promote growth (Winn 1984, Pine et al 1986). The presence of selenium in broth cultures will increase and the elimination of magnesium and potassium will decrease growth (Winn 1984). Catalase, peroxidase and superoxide dismutase (SOD) in broth medium does not affect the growth of legionellae, and hydrogen peroxide and oxygen have no effect on their metabolism (Pine et al 1986). Although the requirement of glutamate for metabolism is controversial, radiolabelling studies have shown that it is rapidly metabolized and evenly distributed in all the cell fractions (Tesh et al 1983). Sugars are metabolized primarily by the pentose phosphate and Entner-Doudoroff pathways. Glucose and glycerol are present in both the lipid and polysaccharide fractions of the cell.

1.2.5 CELLULAR MEMBRANES

The cellular membranes of legionellae are similar to those of other Gram negative bacteria and consist of two trilaminar membranes, each 75 Å wide (Chandler et al 1979, Neblett et al 1979). Between these two membranes a fine peptidoglycan layer is visible by electron microscopy after plasmolysis or papain treatment. The two layers can be separated by

sucrose density gradient centrifugation but not by treatment with detergents (Gabay et al 1985). The outer- and cytoplasmic membranes of legionellae have different protein profiles, but the enzymatic activity and 2-keto-3-deoxyoctonate (KDO) content is similar.

L. micdadei contains an unusual, electron-dense layer between the two trilaminar membranes. The significance of this layer is unknown, but may be related to the acid-fastness of the organism. Complex surface structures, resembling capsules and containing mainly polysaccharides, have been demonstrated in *L. pneumophila*. The superficial antigens on these structures are different from those normally found in capsules and migrate in the opposite direction from most bacterial polysaccharides when subjected to counter immunoelectrophoresis (Winn 1984). Membrane-bound granules that resemble beta-3hydroxylbutarate granules have also been demonstrated in legionellae (Winn 1984).

1.2.6 FLAGELLAE

Most Legionella species and strains possess single polar flagellae and fimbriae on primary isolation (Hebert 1981, Yu 1990). Flagellated organisms are usually short forms (Chandler et al 1980) and dividing organisms are seldom flagellated. Curled flagellae have been demonstrated by light microscopy, but not by electron microscopy (Chandler et al 1980). Fimbriae are about half the size of flagellae (approximately 10-11 nm in diameter), are slightly coiled and bent at irregular intervals, similar to fimbriae found in *P. aeroginosa*, and have been demonstrated on *L. pneumophila* strains Togus 1, Philadelphia 1, Bloomington 2, Indianapolis 1 and Pontiac 1 after growth on CYE agar (Chandler et al 1980).

1.2.7 CHEMICAL COMPOSITION

The cell walls of Legionellae contain distinctive fatty acids, of which 80 90% are branched-chain, containing large amounts of ubiquinones with more than 10 isoprenes in the side chair, that are relatively uncommon in gram negative bacteria except in *Thermus aquaticus*

and a thermophilic flavobacterium (Brenner 1986, Ehret et al 1986, Moss et al 1977). These fatty acids are more common in gram positive bacteria like Listeria, Porpionibacterium, Bacillus and Staphylococcus species (Moss et al 1977). The high lipid content present in legionellae is also unusual for gram negative bacteria and contains phosphatidylcholine. Predominant fatty acids found in legionellae include C^{i} 16:0, C^{a}15:0 and C^{a}17:0 (Lambert et al 1989, Ehret et al 1986). Trace amounts of hydroxy fatty acids are present in 50% of Legionella species. Ubiquinones Q11 and Q12 are major components in *L. pneumophila* and Q13 is present in small quantities (Brenner 1986, Ehret et al 1986).

1.2.8 ANTIGENIC COMPOSITION

The antigenic composition of Legionella species is complex. At least 85 different family-, genus-, species- and type-specific antigens have been described (Collins et al 1983). The majority of these antigens are located in the capsular-like envelope material, on the cell surface, that contains 35% carbohydrate, 2.6% protein, 1.8% phospholipid and 1% KDO (Smith et al 1981). The major outer membrane protein (MOMP) is similar in all legionellae. It has a molecular weight of 24-29 kDa, accounting for approximately 90% of the sarkosyl-insoluble membrane fraction (Ciecielski et al 1986, Ehret et al 1986). The Legionella MOMP is associated with the peptidoglycan layer (similar to the *E. coli* K-12 OmpF, OmpC and LamB porins) and forms ion-permeable channels in contact with the lipid membranes (Gabay et al 1985). It can be partly dissociated from the peptidoglycan layer in the presence of high salt concentrations (Hindahl et al 1984).

The Legionella antigens fall in the molecular weight range between 20 and 70 kDa (Ehret et al 1986). The role of individual antigens in pathogenesis is still unknown, but they are implicated in attachment to mucosal surfaces, antibody binding and phagocytosis (Pearlman et al 1985). The complete chemical structure of species- and type-specific antigens is not known, but it is suggested that they are responsible for the coiling phagocytosis characteristic of Legionella species (Ciecielski et al 1986).

1.2.8.1 GROUP-SPECIFIC ANTIGENS

Antigens of 24, 43, 58, 61-68 and 154 kDa are present in all Legionella species (Sampson et al 1986). The 24 kDa antigen is unique to legionellae suggesting that it may have both genus- and species-specific determinants. Antigens of 44 and 97 kDa are present in some of the legionellae.

1.2.8.2 SPECIES-SPECIFIC ANTIGENS

The major component of the species-specific antigen has a molecular weight of 29 kDa, is heat-stable and is only partly exposed on the cell surface. Exposure can however be increased by treatment with detergent and EDTA (Gosting et al 1984). The 11, 19, 25 and 88 kDa antigens are also species-specific (Sampson et al 1986) [281] and the 19 kDa antigen is sensitive to formalin.

1.2.8.3 TYPE-SPECIFIC ANTIGENS

Type-specific antigens are high molecular-weight, protein-carbohydrate-lipid complexes containing the major branched-chain fatty acids and enzymes like hemolysins, proteases, esterases, phosphatases, aminopeptidases and endonucleases. The majority of type-specific antigens contain small amounts of KDO. They are situated in the lipopolysaccharide (LPS) part of the organism, consist of Lipid A and resemble the endotoxin of other gram negative bacteria like *S. minnesota, C. burnetti* and *C. psittaci* (Ciecielski et al 1986). Whether the LPS of all *L. pneumophila* strains is similar is not certain (Wilkinson et al 1979). The somatic (O) antigens are situated in the LPS.

1.2.8.4 CROSS-REACTIONS

Cross-reactions have been demonstrated between serogroups, species and with antigens of other gram negative bacteria (Winn 1984) for example between antigens of *L. pneumophila* SG1 and *L. bozemanii, L. dumoffii, L. gormanii* and *L. micdadei* (Collins et al 1987, Gosting et al 1984). Cross-reactive antigen fractions are composed almost entirely of protein (Pearlman et al 1985). Winn (1984) suggested that cross-reactions occur mainly between flagellar antigens. All motile strains have a common flagellar antigen and interspecies cross-reactions occur often (Brenner 1986). No cross-reactions have so far been demonstrated between the 24kD protein of *L. pneumophila* and Pseudomonas species, Flavobacterium, Pasteurella, Haemophilus, Acinetobacter and mycoplasmas. Boiling can decrease the number of cross-reacting antigens in serological tests but because some of the antigens are heat stable, cross-reactions may still occur (Bangsborg et al 1986).

1.2.9 GENETIC STRUCTURE

Legionellae are genetically heterogenous with DNA relatedness between the different species ranging from 0-67%. The G+C content of Legionellae is 39% and the genome size, 2.5×10^9 (Brenner et al 1979). The DNA from *L. pneumophila* is different from that of other gram negative bacteria, with the 3' half containing sequences that are homologous to the DNA from all the other legionellae and the 5' half showing homology to *L. pneumophila* strains only (Engleberg et al 1986).

Plasmids play an important role in the biology of pathogenic bacteria and have been demonstrated in most Legionella species (Aye et al 1981, Johnson et al 1982, Mikeshell et al 1981). The genetic information for producing toxins, surface antigens involved in adhesion, invasiveness, resistance to antibiotics, substrate utilization and the ability to colonize human tissue is often encoded on plasmids (Aye et al 1984). Plasmids are widely used in the identification of legionellae (Brown et al 1982, Chen et al 1984, Nolte et al 1984) but attempts to transfer plasmids either from or between the Legionellae have so far been unsuccessful (Chen et al 1984). Large plasmids, sized 60 - 80 daltons have been

demonstrated in most Legionella species (Johnson and Schalla 1982, Nolte et al 1984). These plasmids are large enough to contain several genes as well as unstable, transposable elements which may account for the differences in molecular weights among strains of the organism (Aye et al 1981).

Epidemiologic evidence suggests that certain plasmid-containing strains of *L. pneumophila* may be less virulent than those without plasmids isolated from the same environment (Chen et al 1984). Virulence of *L. pneumophila* to guinea pigs however does not appear to be plasmid-mediated (Aye et al 1981) and strains both with and without plasmids have been isolated from clinical specimens, suggesting that the presence of plasmids does not influence pathogenecity or antimicrobial resistance (Johnson et al 1982).

1.2.10 LEGIONELLA INFECTIONS

Infections caused by legionellae are collectively known as legionellosis and include Legionnaires' Disease and Pontiac Fever (Dowling et al 1984, MacFarlane 1989), although subclinical infections have also been reported (Girod et al 1982). Legionella infections occur worldwide, in people of all ages and race groups (Boldur et al 1986, Kaplan et al 1980) with no evidence of person-to-person spread of infection (Kurtz 1988). The mode of transmission, inoculum size, particle size and host susceptibility appear to influence the severity of the infection (Girod et al 1982, Yu 1990).

A seasonal distribution of Legionella infections has been reported, with more cases being identified during summer to autumn (Broome and Fraser 1979, Roig et al 1991, Tobiansky et al 1986). This seasonality can be explained by the fact that there may be more non-viable cells during winter months, or legionellae may undergo a period of 'nutrient-stress' during winter-spring, when they may remain viable but cannot be cultured (Tobiansky et al 1986). A similar viable but non-recoverable stage of existence has also been demonstrated in *V. cholerae* and *S. enteritidis*.

1.2.10.1 LEGIONNAIRES' DISEASE

Legionnaires' Disease refers to the pneumonia caused mainly by *L. pneumophila* and *L. micdadei* (Winn 1984). *L. micdadei* infection is also referred to as Pittsburgh pneumonia and accounts for approximately 6 - 20% of Legionella infections in the United States (Edelstein 1987, Schwebke et al 1990). Legionnaires' Disease is usually caused by inhalation of droplets containing viable legionellae small enough to enter the lungs. It has a low attack rate with ratio of 10-20% in sporadic cases (Ager et al 1983, Kurtz 1988). A mortality rate of 12% in Brittain and 15% in South Africa has been reported but may be as high as 50% without proper treatment (MacFarlane 1989, Zumla 1988). Legionnaires' Disease may have a sudden or a gradual onset (Kaplan et al 1980) and an incubation period of 2-10 days has been reported in epidemics (Blackmon et al 1981, Kurtz 1988).

1.2.10.1.1 SYMPTOMS

Legionnaires Disease is usually a severe multisystem disease with pneumonia as the most predominant clinical finding. Symptoms may range from a mild cough and slight fever to a coma wit' ldespread pulmonary infiltrates and multisystem failure. Clinical features of the disease are similar to those of other pneumonias and may lead to formation of lung abscesses (Shapiro 1986, Yu 1990). No symptomatic differences between community- and hospital-acquired cases have been reported to date (Roig et al 1991). Asymptomatic infections have been reported and occur more frequently in *L. micdadei* infections than in those caused by *L. pneumophila* (Finch 1988, MacFarlane 1989, Roig et al 1991). Survivors usually recover completely although lung fibrosis and some neurological abnormalities such as ataxia or amnesia occasionally persist. Spontaneous recovery has been reported in a few cases (Randall et al 1980).

During the first 24-48 hours of illness the symptoms are usually nonspecific and include fever, chills, malaise, myalgia, anorexia, dyspnoea and rales (Blackmon et al 1981, Broome et al 1979, Grady et al 1979, MacFarlane 1989, Roig et al 1991, Yu 1990). A mild, slightly productive cough may occur. In some patients chest pain is a prominent feature and may

be the only symptom besides fever in *L. micdadei* infections (Schwebke et al 1990). Nausea, vomiting, abdominal pain and severe diarrhea have been reported (Muraca et al 1988, Yu 1990).

Muscular, renal and cardiovascular symptoms, including pericarditis, pyelonephritis, peritonitis, pancreatitis and endocarditis may occur with hypotension and bradycardia also reported in a small percentage of patients. (Dournon et al 1882, Shapiro 1986, Winn 1984, Yu 1990). The spleen may be enlarged in some cases (Winn et al. 1981). No colonization of the oropharynx has been reported to date (Roig et al 1991). Neurological symptoms range from headache in 30-40% of patients and lethargy to encephalopathy with confusion and delirium in 20-30% of cases (Broome et al 1979, Davis et al 1985, Yu 1990). Specific organ damage in the absence of pneumonia has been reported (Shapiro 1986).

1.2.10.1.2 LABORATORY FINDINGS

Laboratory findings in Legionnaires' Disease include abnormal liver function, hypophosphataemia, haematuria, raised serum urea, hyponatraemia, hypoalbuminaemia and proteinuria (Macfarlane 1989, Broome et al 1979, Yu 1990). The CSF is usually normal (Kaplan et al 1980) but blood nitrogen, urea, erythrocyte sedimentation rate, serum glutamic oxalacetic transaminase and serum hepatic enzyme concentration are usually raised, and serum sodium and phosphorous are decreased in some cases (Broome et al 1979). Mild to moderate leukocytosis with a shift to the left is common and lymphopenia is sometimes present (Broome et al 1979, MacFarlane 1989). The concentration of selenium in the serum is considerably lower during the acute phase than during the convalescent phase of the disease (Chen and Anderson 1979). Organisms may be present, often in large numbers, both intra- and extracellularly in neutrophils and macrophages and can be demonstrated by silver impregnation stains. Although the clinical presentation of Legionnaires' Disease is nonspecific, factors like hyponatraemia, large numbers of neutrophils present in Gram stains with very few (if any) organisms, failure to respond to ß-lactam antibiotics (eg. penicillin and cephalosporins) and aminoglycosides, and contamination of the water supply by legionellae may be helpful in diagnosis.

1.2.10.1.3 RISK FACTORS

Legionnaires' Disease occurs in people of all ages but is more common in people over 50 years. The median age in most outbreaks is 55-60 years (Broome et al 1979). Men are more likely to be infected at a ratio of 3:1 in both outbreaks and sporadic cases, with most reported cases in the 40-70 years age group (Boldur et al 1986, Kurtz et al 1988). Although Storch et al (1979) reported a 14-fold lower risk of sporadic Legionnaires' Disease in caucasians than in other race groups, the racial distribution of the disease is generally consistent with that of the population involved (Broome et al 1979). According to Muldoon et al (1981), most reported cases however occur in white males.

Other risk factors for Legionnaires' Disease include smoking, alcohol abuse, malignancy, diabetes, chronic respiratory disease (eg. asthma), chronic kidney disease and immunosuppression (Ager and Tickner 1983, Berlin et al 1982, Boldur et al 1986, England and Fraser 1981, Grady and Gilfillan 1979). Immunosuppression is also a risk factor in children and asthmatic children have been found to be highly susceptible to Legionella infection, possibly due to their treatment with corticosteroids (Boldur et al 1986, Schwebke et al 1990). Legionnaires' Disease often occurs in heart, kidney or bone marrow transplant patients (Schwebke et al 1990, Winn 1984, Shapiro 1986).

1.2.10.1.4 CHEST RADIOGRAPHS

Abnormalities in chest radiographs usually occur from the third day, are seen in ≥90% of cases and are similar in *L. pneumophila* and *L. micdadei* infections (Schwebke et al 1990) although Roig et al (1991) reported differences in the radiological features of the two infections. In most patients initial involvement is unilateral, predominantly in the lower lobe (Kaplan et al 1980) but bilateral involvement has been described (MacFarlane 1989, Roig et al 1991). Initial densities are poorly marginated, homogenous, rounded, occur either on the periphery (Yu 1990) or in the centre of the lung (Kaplan et al 1980) and may be mistaken for pulmonary infarction. The peripheral densities enlarge during later stages of the disease with a typical ground glass appearance or dense consolidation. Total

opacification of the lung has also been described (Kaplan et al 1980). Pleural effusions are seen in 24-63% of cases caused by *L. pneumophila* but are uncommon in *L. micdadei* infections (Schwebke et al 1990). Cavitation occurs mostly in immunocompromised patients and is rarely seen in *L. micdadei* infections (Broome et al 1979, Kaplan et al 1980, Roig et al 1991, Schwebke et al 1990). Hilar adenopathy seldom occurs (Yu 1990). The extent of radiographic infiltration does not correlate well with the severity of the disease, but correlates with the presence of *L. pneumophila* in sputum. The time required to show clearing of infiltrates on radiographs is variable and may range from 1-4 months (MacFarlane 1989, Yu 1990). Some patients show diffuse alveolar damage.

1.2.10.1.5 HISTOLOGY

Pulmonary lesions usually consist of a mixture of neutrophils and macrophages, fibrin, proteinaceous material and red blood cells. Neutrophils and macrophages are frequently present in the centre of a lesion with mainly macrophages around the periphery. Intracellular bacteria are present in both neutrophils and macrophages. Further away from the site of acute inflammation, bacteria are mainly seen inside the macrophages (Winn et al 1981).

1.2.10.2 PONTIAC FEVER

Pontiac Fever is an acute, self-limiting, flu-like illness without symptoms of pneumonia. An outbreak caused by *L. feeleii* was described in 1981 (Herwaldt et al 1984). It occurs in all age groups, both sexes, and is more prevalent during summer months. Some authors suggest that it is a hypersensitivity pneumonitis caused either by infection with Acanthamoeba filled with legionellae (Vandenesch et al 1990) or as a result of a toxic reaction to the organism (Muder et al 1986). The incubation period in sporadic cases is unknown, but ranges from 5 hours to 3 days during outbreaks (Kaplan et al 1980, MacFarland 1989). The attack rate may be higher than 90% in some cases, but the fatality rate is low with no fatalities reported to date (MacFarlane 1989, Kurtz 1988).

Predominant symptoms resemble those of moderately severe influenza and include malaise, myalgia, fever, chills, cough, dizziness, chest and joint pain and headache. Neurological symptoms include confusion, photophobia and impaired coordination. A small percentage of patients also suffer from dry throat, sore eyes, abdominal pain, coryza, diarrhoea and anorexia (Blackmon et al 1981, Glick et al 1978). The symptoms usually resolve spontaneously within one week, only symptomatic treatment is needed and the chest radiograph is clear. Moderate leukocytosis is the only consistent laboratory finding (Broome et al 1979). There is no evidence of secondary spread of the organism in Pontiac Fever. Diagnosis can only be made by seroconversion which may be delayed for up to 6 weeks after onset of symptoms (Muder et al 1986).

1.2.11 IMMUNE RESPONSE TO LEGIONELLA INFECTIONS

Micro-organisms are normally cleared from the upper respiratory tract by the mucociliary process of cilia on the respiratory epithelial cells. The adherence of *L. pneumophila* to respiratory epithelial cells has not been studied to date, but the organisms possess fimbriae that are known to cause adherence of bacteria to epithelial cells. Symbiosis between normal flora of the oropharynx and legionellae has been suggested although colonization of the oropharynx has not been demonstrated to date (Yu 1990). Cell mediated immunity appears to be the primary host defense mechanism against Legionella infections (Horwitz 1983, Schwebke et al 1990).

1.2.11.1 CELL-MEDIATED IMMUNITY

L. pneumophila is phagocytosed by a mechanism called "coiling phagocytosis" in which the phagocytic pseudopodia are coiled around the bacterium as it is ingested. Either live, formalin-killed, gluteraldehyde-killed or heat-killed bacteria can be phagocytosed (Engleberg et at 1986, Horwitz 1984). The resident alveolar macrophage is the first and probably the most important phagocytic cell that legionellae will encounter. A vacuolar phagosome is formed containing the organisms. This phagosome is surrounded by smooth

vesicles, then mitochondria, and finally ribosomes. The phagosome however does not fuse with the ribosomes, allowing the organism to escape their microbiocidal effect and to continue multiplying until the cell ruptures. The freed bacteria are now phagocytosed by newly recruited cells and the process repeats itself. Phagocytosis of legionellae is enhanced by the presence of specific opsonising antibody (Yu 1990), but *L. pneumophila* is completely resistant to killing by human phagocytes and complement, even in the presence of high titres of specific antibody.

Polymorphonuclear leukocytes and monocytes are now recruited from the blood stream, probably by chemotactic factors derived from the alveolar macrophages or the infecting organisms. This recruitment starts with an influx of neutrophils but with the transformation of monocytes to macrophages, the numbers of neutrophils and macrophages become roughly equal. Legionellae are ingested sufficiently by neutrophils only in the presence of specific antibody or complement (Yu 1990). Phagocytosis is mediated by complement receptors on the monocytes. Legionella fixes complement C^3 via the alternative pathway: C^3 acts as a ligand on the bacterial surface that becomes available for binding to the monocyte receptor. The mononuclear cells proliferate after infection and monocyte-activating cytokines, including gamma-interferon and interleukin-1, are produced. Although activated monocytes and alveolar macrophages inhibit intracellular multiplication of legionellae, they do not stimulate killing of the organisms. Lymphokine-activated macrophages are more active against *L. pneumophila* than against *L. bozemanii* in vitro (Jaeger et al 1988).

The phagocytosis of legionellae by polymorphonuclear leukocytes is followed immediately by an increase in oxygen consumption and in glucose utilization through the hexose monophosphate shunt. The production of NADPH reflects the activity of a membrane associated superoxide-generating enzyme that is activated by, or during, membrane depolarization. This depolarization can be induced by either phagocytosis or by other chemical or physical interactions with the phagocyte membrane. Although legionellae are susceptible to oxygen-dependent microbiocidal systems in vitro, they appear to resist killing by polymorphonuclear leukocytes in vivo. No intracellular replication occurs within the polymorphonuclear leukocytes. Natural killer cells, triggered by interleukin-2, will however kill mononuclear cells infected by *L. pneumophila*.

1.2.11.2 HUMORAL IMMUNITY

The antibody response to Legionella infection is polyclonal and antibodies of the IgM, IgG and IgA classes are formed. Antibodies can be detected by techniques like indirect immunofluorescence and the ELISA test (Ciecielski et al 1986, Winn 1984). As discussed earlier, one of the major antigens in *L. pneumophila* is the LPS which contains carbohydrate as a major constituent. Specific antibodies are produced against the LPS in 98% of Legionella infections, but also to a variety of inner and outer membrane proteins (Gabay et al 1985). As is often the case with carbohydrate antigens, the immune response in Legionella infections is dominated by IgM. The clinical role of the antibodies produced against *L. pneumophila* is not clear, but they do not inhibit intracellular multiplication, or promote killing of the organism by phagocytosis. The presence of circulating antibodies does not always protect the host against *L. pneumophila* infections (Brown et al 1982) and may even be counterproductive to the immune response by increasing the uptake of legionellae by mononuclear phagocytes, where they can multiply further, in some cases (Horwitz 1983).

The amount and persistence of detectable antibody produced by individuals is variable (Fallon et al 1987, Nagington et al 1979). Patients may develop an antibody response within 1-2 weeks after onset of symptoms, but cases have been reported where an antibody response never occurred, even in culture-proven Legionnaires' Disease (Winn 1984). As many as 30% of patients with primary Legionella infections will produce only IgM (Zimmerman et al 1982), while in secondary infections IgM is more likely to be raised than the other classes and may persist for as long as a year after the infection has cleared. Most patients will however develop IgM followed by IgG during secondary infections. For the first 30 days after onset of symptoms, IgM is usually higher than IgG (Herbrinck et al 1983). Very few patients with active disease will develop only IgG or IgA (Winn 1984, Zimmerman et al 1982). The IgM response appears to be more specific than the IgG response in Legionnaires' Disease and may therefore be valuable to distinguish it from *C. psittaci* infections as they are clinically very similar. Studies have shown that patients with Legionnaires' Disease also produced IgG against *C. psittaci* but the IgM response was specific (Lattimer et al 1979). Seroconversion can usually be demonstrated after 21 days but antibodies may take as long as 6 weeks to develop. In some populations up to 26% of

individuals have antibodies to *L. pneumophila*, even in the absence of previous pneumonia (Winn 1984).

1.2.12 TREATMENT OF LEGIONELLA INFECTIONS

Legionellae are susceptible to a variety of antimicrobial agents in vitro but most of these drugs are clinically ineffective because legionellae are intracellular pathogens, and are therefore protected against drugs with poor cell penetration (Jaeger et al 1988, MacFarlane 1989, Winn 1984). Discrepancies between the in vitro and in vivo susceptibility of legionellae may also be related to the low pH of the media used for in vitro susceptibility tests, that may bind some antibiotics. The minimum inhibitory concentrations (MIC's) of most antibiotics are media dependent and are usually higher in charcoal yeast agar than in Mueller Hinton agar and may influence the results of susceptibility tests (Yu 1990).

Erythromycin and rifampicin can penetrate alveolar macrophages, thereby inhibiting intracellular *L. pneumophila* organisms at very low extracellular levels (Jaeger et al 1988). Erythromycin is therefore regarded by most authors as the drug of choice and can be used successfully in both immunocompetent and immunecompromised patients. Erythromycin plus second or third generation cephalosporins appear to be the most effective therapy in patients with severe pneumonia, especially those more than 60 years of age or suffering from debilitating diseases such as alcoholism or chronic obstructive pulmonary disease (Pachon et al 1990). The recommended dosage for erythromycin therapy is 2-4 g/day. A clinical response is usually seen in 3-5 days when the dose should be limited to 2 g/day for at least 10-14 days, or up to three weeks in some cases (Kaplan et al 1980). Side effects of erythromycin therapy at a dosage of 4 g/day include ototoxicity which is reversible with discontinuation or decrease in dosage (Yu 1990). It can be taken orally except by seriously ill patients, but the gastrointestinal dysfunction present in some patients may compromise absorption when taken orally. Rifampicin is recommended as an alternative in patients who do not respond to erythromycin treatment, but should not be used in sporadic, unconfirmed cases because the bacteria may become resistant (Kaplan et al 1980). Simultaneous use of the two antibiotics is recommended in confirmed cases (Yu 1990).

Quinolone agents such as ciprofloxacin and perfloxacin are very active against legionellae in vitro and in vivo in some animal models (MacFarlane 1989, Yu 1990). They penetrate well into phagocytic cells and have the advantage of low toxicity. Tetracyclines (especially doxycycline), imipenem/cilastatin, ciprofloxacin, chloramphenicol have been used relatively successfully (Kaplan et al 1980). Trimethoprim-sulfamethoxazole has been used with success in patients with Pittsburgh pneumonia who did not respond to erythromycin therapy (Yu 1990).

Although cephalosporins and aminoglycosides are effective in vitro, they are clinically ineffective (Winn 1984). Legionellae are therefore resistant to tobramycin, gentamycin, penicillin and ampicillin (Pachon et al 1990, Randall et al 1980). Sparfloxacin appears to be effective against all organisms causing atypical pneumonia.

1.2.13 LABORATORY IDENTIFICATION OF LEGIONELLAE

Legionellae can be distinguished from other gram negative bacteria by techniques like cell wall fatty acid and quinolone analysis and by biochemical characters. Individual Legionella species can be identified by several methods including direct immunofluorescence and enzyme electrophoresis. The Gimenez stain is the most effective for visualizing legionellae. The Gram stain is not recommended, but by using basic fuchsin or carbolfuchsin as a counterstain, the visibility of the organisms can be improved. To visualize organisms in paraffin-fixed tissues, a silver stain such as the Dieterle or Warthin-Starry stains can be used (McDade et al 1977, Yu 1990).

Procedures for the identification of legionellae in environmental samples are similar to those used for clinical samples. For the detection of legionellae in water, samples are filtered or centrifuged to concentrate the bacteria. Legionellae can be detected in water samples by microscopy, culture and direct immunofluorescence. The high level of contamination in environmental samples can be reduced by acid treatment (Bopp et al 1981).

A soluble, heat-stable, trypsin-resistant antigen is excreted by *L. pneumophila* in the urine of most patients during the first few days of illness (except in the event of renal failure)

and can be detected for several weeks by methods like RIA, ELISA and agglutination tests (Bibb et al 1984, Grimont 1986). This antigen is excreted in the urine via the circulatory system and is therefore also present in the serum, but in much lower concentrations. Grimont et al (1986) found concentrations 30-100 fold lower in the serum than in urine samples taken on the same day. The antigen can also be detected in CSF and other body fluids, but the detection depends largely on the sensitivity of the test used (Berdal et al 1979, Bibb et al 1984). Heating of the specimen to 100°C for 5 minutes will increase the specificity of antigen detection in urine samples.

1.2.13.1 CULTURE

Legionellae can be isolated from environmental and clinical samples by inoculation onto artificial media, into guinea-pigs or into embryonated hen's eggs.

1.2.13.1.1 GUINEA-PIG INOCULATION

This technique involves inoculation of tissue suspensions intraperitoneally into adult, male guinea-pigs with subsequent culture of organisms from the liver, spleen and lungs and has been replaced by culture on artificial media (Fallon 1981, Fliermans et al 1981).

1.2.13.1.2 EGG YOLK INOCULATION

Embryonated hen's eggs are very sensitive for the culture of legionellae, but the method has also been replaced largely by culture on artificial media. It involves inoculation of body fluid or tissue specimens into 7 day old embryonated hen's eggs, harvesting the yolk sacs from eggs that die 4-10 days after inoculation and subsequent examination by direct or

indirect immunofluorescence or the Gimenez stain (Fallon et al 1981).

1.2.13.1.3 CULTURE ON ARTIFICIAL MEDIA

Culture of legionellae from clinical specimens is the most reliable method currently used for the diagnosis of Legionnaires' Disease. Transtracheal aspirates are best for culture, but wound swabs, pericardial fluids, swabs from perirectal abscesses, sputum, bronchial aspirates, pleural exudates, lung biopsies and autopsy material have been used successfully (Yu 1990). Culture of legionellae from clinical samples is highly specific, providing proper lower respiratory specimens are used (Wilkinson et al 1986). It is 50-70% sensitive for sputum and 90% for transtracheal aspirates (Yu 1990). Culture is about 1.5 - 3.0 times more sensitive than detection by direct immunofluorescence and 1.5 times as sensitive as antibody detection (Edelstein 1989). Water samples can also be cultured on selective media after acid treatment to reduce contaminants (Bopp et al 1981).

L. pneumophila has been isolated from blood using a biphasic medium and a radiometric blood culture system. A commercially available lysis-centrifugation system was successfully used for blood culture with seeded specimens prepared in the laboratory, but the success of the method with clinical samples is unknown (Winn 1984). Feeley and Gorman (1979) recommended a CYE biphasic medium for blood cultures.

The primary isolation medium for Legionella species is buffered charcoal yeast extract (BCYE) agar supplemented with α-ketoglutarate, iron and cysteine, on which grey-white colonies with a speckled ('ground-glass') appearance will be visible after 3-5 days at 35-37°C in a humid atmosphere (Grimont 1986, Jaeger et al 1988, Staneck et al 1988, Winn 1984). Other media that can be used include Feeley-Gorman agar, yeast extract tyrosine agar and Mueller-Hinton agar supplemented with hemoglobin and IsoVitaleX (Broome et al 1979). Broth media can be used for *L. pneumophila*, but a lag phase of 6 hours in chemically defined media and 2 hours in biphasic media with agitation has been reported (Winn 1984). Legionellae will not grow on blood or trypticase soy blood agar (Winn 1984).

Contamination by organisms like Bacillus species and *P. aeroginosa* can inhibit growth of

legionellae on solid media. Selectivity of the media can however be improved by addition of antimicrobial agents such as amphotericin B, colistin, cefamandole, polymyxin B, vancomycin and trimethoprim, antifungal agents like anisomycin and cycloheximide and inhibitors like glycine (Yu 1990). Glycine will also inhibit the growth of other gram negative bacteria (Jaeger et al 1988). To increase the selectivity further, environmental samples can be pretreated with acid, and sputum samples with heat or a cytolytic agent called digitonin (Bopp et al 1981, Zumla 1988). Addition of certain dyes to the media will make the colonies more visible (Vickers et al 1981).

The addition of 2.5-5% CO2 to the atmosphere is essential, except for *L. pneumophila* which will grow without the addition of CO2 on BCYE agar. Concentrations higher than 5% will however increase the pH of the medium, thereby inhibiting growth. An extracellular, water-soluble compound is produced when the organisms are grown on BCYE, resulting in yellow-green fluorescence of most Legionella colonies when exposed to long-wave ultraviolet light (Wood's lamp). *L. bozemanii, L. dumoffii, L gormanii* and *L. parisiensis* will however produce blue-white, and *L. rubrilucens*, red fluorescence under the Wood's lamp (Kurtz et al 1988, Thacker et al 1985). Dyes, added to the medium, produce various effects. Most species, except *L. birminghamensis*, produce a brown, melanin-like, water-soluble pigment on charcoal-free media containing tyrosine. *L. micdadei* and *L. maceachernii* form blue colonies on media containing bromcresol purple and bromothymol dyes. *L. pneumophila* will form pale green, and *L. bozemanii, L. dumoffii* and *L. gormanii*, bright green colonies on these media.

Growth is usually visible after 2-6 days (Bibb et al 1984, Grimont 1986), but may take longer to appear. Colony morphology varies under different culture conditions, but it is grey-white on BCYE with a speckled, 'ground-glass' appearance. Incubation temperatures may range between 25°C and 42°C (optimum 35°C) in a humid atmosphere with addition of 2,5-5% CO2 (Ager et al 1983, Grimont 1986). The optimum pH for growth is 6.9. Despite the high sensitivity and specificity of the culture techniqe, the slow delicate growth of the organism, contamination and the lack of suitable biochemical reactions makes it difficult to distinguish legionellae from other organisms.

1.2.13.2 DIRECT IMMUNOFLUORESCENCE (DFA)

Direct immunofluorescence is very useful to detect antigens in clinical samples when cultures cannot be obtained. It's value for environmental samples like water is controversial although it is routinely used (Tobiansky et al 1986). Direct immunofluorescence (DFA) involves conjugating the antibody to a fluorochrome and applying this directly to the specimen. Specimens are screened with polyvalent antisera and the serogroup determined by monovalent reagents (Ratshikhopha 1990). The sensitivity of the test ranges between 24% and 86% (Jaeger et al 1988, Grimont 1986, Wilkinson 1982, Edelstein 1987). Commercially available polyvalent antisera are highly specific for *L. pneumophila.* Background fluorescence can be decreased with the use of monovalent antisera (Yu 1990) or by adding rhodamine-stained normal globulin or dilute crystal violet to respiratory or tissue specimens (Winn 1984).

Cross-reactions that may lead to false positive results have been documented between Legionella species and *B. fragilis, P. alcaligenes, P. fluorescens* and *B. pertussis* in the DFA test (Grimont 1986, Roig et al 1991, Ruf et al 1990, Wilkinson 1982, Benson et al 1987, Fliermans et al 1987).

The DFA test should be interpreted carefully because a negative DFA does not rule out Legionella infection. More than 25 bacilli, morphologically compatible with Legionella species and reacting only with specific antiserum is regarded as positive and less than 5 bacilli per specimen is reported as suspicious (Winn 1984). Although conjugates without Freund's adjuvant is commercially available today, most antisera were raised in rabbits with the aid of Freund's complete adjuvant that contained mycobacteria in the past, resulting in the presence of antibodies against mycobacteria in the conjugate. To avoid cross-reactions between mycobacteria and legionellae in the DFA, an acid-fast stain can be used before the direct immunofluorescence test.

1.2.13.3 INDIRECT IMMUNOFLUORESCENCE (IFA)

The indirect immunofluorescence test was adapted from rickettsial techniques soon after the 1976 Legionnaires' Disease outbreak in Philadelphia and was introduced into Great Britain in 1978. It is today the standard, most commonly used and most available of the serological tests for legionellosis and is the most specific of the serological tests currently used in South Africa (Edelstein 1987, Lattimer and Cepil 1980, Pastoris et al 1984). The IFA can be used for either antigen or antibody detection, is reproducible and highly sensitive and specific for the diagnosis of *L. pneumophila* SG 1 infections (Harrison et al 1987, Winn 1984). The sensitivity will increase further when the IFA is used in combination with agglutination tests (Harrison et al 1987, Wilkinson and Brake 1982). The positive predictive value of the test for diagnosis of *L. pneumophila* SG 1 infections is also high (Harrison et al 1987).

Several factors such as the media used, methods of antigen preparation, culture conditions, method of fixation, class of immunoglobulin detected and strain differences can influence the reactivity of Legionella antigens in surface antigen-dependent serological tests such as the IFA and DFA (Harrison et al 1987, Pastoris et al 1984). Acetone is more effective for fixation of antigens to the microscope slides than ethanol (Wilkinson and Brake 1982). Addition of normal yolk sac (NYS) in both the antigen suspension and the serum diluent also enhances fixation of the antigen to the slide, makes subsequent fixation with acetone unnecessary and reduces background staining (Wilkinson et al 1982, Winn 1984). Bacteria from infected yolk sacs, grown on charcoal yeast extract (CYE) agar will maintain their antigenic stability for at least five sub-passages and are the best for antigen preparation (Wilkinson and Brake 1982). Samples can be screened with polyvalent antisera and subsequently tested for type-specific antibodies with monovalent antisera.

1.2.13.3.1 ANTIGENS

Legionella antigens can be inactivated in several ways, including treatment with phenol, ether, heat and formalin. As mentioned above, either polyvalent or monovalent antigens can

be used for indirect immunofluorescence (Winn 1984).

Ether-killed antigens were found to be unsatisfactory because ether apparently disrupts the antigenic integrity of the cell surface and extracts some of the *L. pneumophila* SG 1 (Philadelphia 1) and all the detectable SG 2 (Togus 1) antigen, leading to false positive results (Wilkinson et al 1982).

Bacteria inactivated by treatment with 0.5% phenol for 24-48 hours are used to prepare phenol-killed antigens. Results obtained with phenol-killed antigens are similar to those with heat-killed antigens. Although the sensitivity and specificity of the two methods is similar, phenol-killed antigens appear more homogenous and clear on IFA with hardly any background fluorescence (Edelstein 1987, Pastoris et al 1984). Phenol is lipid-soluble and is probably absorbed by the lipids of the cell wall which partially prevents the denaturing action upon the cell membrane proteins. By phenol treatment, a substance with lipid A activity, cross-reacting with the lipid A of some gram negative bacteria, can be extracted from *L. pneumophila* (Pastoris et al 1984). This phenol inactivation followed by washing probably eliminates a portion of this lipid A-like substance on the surface LPS, thereby decreasing the number of false positives at low serum dilutions. The antibody level to *L. pneumophila* SG 1 in a healthy population tested with phenol-killed antigen was shown to be extremely low (3.4% ≥1:16) which implied that those with titres of 1:16 actually had antibodies to the organism (Pastoris et al 1984). Results are therefore easier to read than with heat-killed antigens. Phenol-killed antigens are stable for more than 15 months when suspended in 0.5% normal yolk sac or phosphate buffered saline solution and kept at 4 °C.

Heat-killed antigens are prepared by inactivating the bacteria at 100°C for 15 minutes. Bacteria treated in this way were shown to retain their cross-reacting antigens (Pastoris et al 1984) and are more sensitive than those inactivated by formalin (Ratshikhopha et al 1990). Heat-killed antigens are used by most laboratories in the USA (Edelstein 1987), while in South Africa heat-killed antigens are used for prevalence studies and formalin-killed antigens for diagnostic studies. The specificity of seroconversion for heat-killed antigens is high enough for epidemiological purposes but not for diagnosis of low prevalence, sporadic disease. These antigens will bind antibodies with different specificities (Wilkinson et al 1982) and therefore have a higher potential for false positive results because they produce higher antibody titres but by adjusting the titres, the results are

nearly identical to those obtained by formalin-treated antigen (Winn 1984).

Formalin-killed antigens are the most frequently used antigens in Europe and are prepared by inactivating the bacteria in 1% formalin for 24-48 hours (Harrison et al 1988). The mechanism of this inactivation is unknown (Lattimer et al 1980, Wilkinson et al 1982). Although the exact specificity is not known, these antigens are more specific than heat-killed antigens and are mainly used when only one serum sample is available to reduce false positive results in patients with clinical symptoms of Legionnaires' Disease (Lattimer et al 1980). Antigens from a single serogroup can be used for identification of antibodies against all known serogroups of legionellae (Lattimer et al 1980). Although these antigens are less reactive immunologically, they stain more clearly and with fewer morphological aberrations than heat-killed antigens (Winn 1984). Background titres among healthy people can therefore be decreased by using formalin-killed antigens without decreasing the sensitivity of the test. Formalin (like diethyl ether) appears to disrupt the antigenic integrity of the cell surface but if the IFA titre is lowered by one twofold dilution factor, there is 96% agreement between results obtained with formalin- and heat-killed antigens (Wilkinson et al 1982).

1.2.13.3.2 INTERPRETATION

Experience of serological results in Legionella species other than *L. pneumophila* SG 1 is limited. Diagnosis of Legionnaires' Disease by serological methods such as the IFA is usually retrospective as serum samples from both the acute and convalescent stages of the disease are needed to demonstrate seroconversion.

The timing of the specimens is very important. Acute phase specimens should be collected within 7 days after onset of illness and convalescent sera after at least 22 days (Blackmon et al 1981, Edelstein 1987). Nagington et al (1979) however suggested that antibodies to *L. pneumophila* SG 1 never appear before 8 days after the onset of symptoms. Antibodies may take up to six weeks to develop, therefore a subsequent specimen after 42 days may increase the sensitivity of the test in cases where seroconversion cannot be demonstrated after 22

days (Blackmon et al 1981, Kaplan et al 1980). According to Ruf et al (1990) the convalescent serum should be collected more than 6 weeks after onset for the test to be 75% sensitive. For this reason it was suggested by Edelstein (1987) and others that serological methods are helpful in epidemiological studies but not for diagnosis in individual patients. According to Winn (1984) and Yu (1990) the IFA is most sensitive for diagnosis of acute, sporadic cases when both IgM and IgG are measured simultaneously. Diagnostic IgM titres alone will provide an earlier diagnosis than only raised IgG because it indicates a primary immune response (Zimmerman et al 1982).

In epidemiological studies, where the prevalence of antibodies to legionellae is studied, IgG should be measured (Yu 1990). Serologic criteria for diagnosis are different in epidemic and endemic times and results obtained by the IFA should always be interpreted in conjunction with the clinical presentation of disease (Harrison et al 1987). Titres below the diagnostic level together with clinical manifestations may be useful for early provisional diagnosis of Legionnaires' Disease although diagnosis by IFA is usually retrospective (Kaplan et al 1980).

The interpretation of the test should take into account the variation in the time of appearance of antibodies, the types of antibodies produced and the length of time the antibodies are detectable in sporadic cases as well as the prevalence of antibodies in the population studied (Nagington et al 1979, Wilkinson et al 1982). The high prevalence of antibody titres ≥1:128 in some communities makes the interpretation of the IFA difficult in sporadic cases when only one specimen is available (Blackmon et al 1981) and the test should be accompanied by culture and interpreted with regard to the clinical picture. Seroconversion can be missed easily as there are currently more than 35 antigenic types and non-homologous antigens may have been used in the test.

False negatives may result from the fact that a long time is needed for seroconversion to occur. Even if proper homologous antigens are used and serum samples are collected serially over a period of 6 - 9 weeks, up to 25% of culture-proven cases will still fail to develop significant antibodies (Edelstein 1987). A fourfold increase in titre to ≥1:128 or a single titre of ≥1:256 is considered as presumptive evidence of Legionella infection and diagnostic of Legionnaires' Disease (Blackmon et al 1981, Dowling et al 1980, McDade et al 1977, Zimmerman et al 1982). In communities where the prevalence is low, a single titre of 1:128 may be diagnostic, while in communities with high seroprevalence, a single titre of 1:256

may still provide only presumptive evidence of infection (Nagington et al 1979, Yu 1990). Low titres are usually indicative of past infections (Nagington et al 1979). When titres to multiple antigens are raised, the titre that is fourfold higher than the others is considered to be diagnostic. In epidemiological studies, diagnostic titres are usually one twofold dilution higher than for sporadic cases.

1.2.13.3.3 CROSS-REACTIONS

Cross-reactions occur frequently with *C. psittaci, B. fragilis, M. pneumoniae, P. aeroginosa, P. fluorescens, M. tuberculosis, P. pseudomallei, Y. pestis F. tularensis* and Campylobacter species, as well as with other Legionella species and serogroups (Anderson et al 1981, Berdal and Eng 1982, Lattimer et al 1979, Winn 1984). Although cross-reactions do not affect diagnosis of Legionnaires' Disease by IFA considerably, they may be important in the analysis of epidemiologic data. Many of the cross-reactions can be decreased or eliminated by absorption of sera with a heat-stable substance extracted from *E. coli* strain 013:K92:H4 (Kaplan et al 1980, Wilkinson et al 1981). This will however also decrease the level of antibodies to *L. pneumophila* in the serum and is not recommended for routine work (Winn 1984). Cross-reactions may cause false positive results in some cases, mainly in patients with cystic fibrosis (Yu 1990). The presence of rheumatoid factor in high titres may block the detection of IgG by combining with specific IgG, resulting in a positive IgM but a negative IgG (Zimmerman et al 1982).

1.2.13.4 ENZYME-LINKED IMMUNOASSAY (ELISA)

The ELISA test involves the detection of antigen or antibody by the binding of an enzyme coupled to either the antigen or an antibody specific for the antigen in the specimen. The technique is similar to immunofluorescence except that the fluorochrome is replaced by an enzyme. Four different antigen preparations have been described for antibody detection, namely boiled sonicated antigen, phenol-extracted antigen, DTA-extracted antigen and soluble antigen (Wreight et al 1982). The antigen or antibody is fixed to microtitre wells or

other solid phase surfaces by centrifugation and ethanol-methanol fixation. Serial dilutions of the specimen are added and the plate incubated and washed. Enzyme-labelled anti-human immunoglobulin is added. Horseradish peroxidase enzyme is most often used for this purpose. The mixture is washed and the substrate added resulting in a colour reaction which is measured either manually or by colorimeter or densitometer (Lennette et al 1979, Wreight et al 1982).

Antibodies of IgG, IgM and IgA classes can be detected in serum (Wilkinson et al 1981), but virtually any specimen can be used for antigen detection (Berdal et al 1979, Fehrenbach et al 1986, Bibb et al 1984). ELISAs will not be accurate for antigen detection in convalescent stage serum due to antibody production (Ruf et al 1990). The sensitivity and specificity of the test for antibody detection compares well to the IFA and agglutination tests, although it is not as reproducible as the IFA (Edelstein 1987, Wilkinson et al 1979, Winn 1984). Non-specific reactions, probably due to rheumatoid factor, have been reported in urine, CSF, stool and serum samples. Cross-reactions have also been demonstrated in urine samples, but these can be decreased by boiling the samples prior to testing (Ruf et al 1990).

Advantages over the IFA include the objective determination of the endpoint, automation of the procedure, the large numbers of specimens that can be tested simultaneously, the quantitative results and the simplicity of the test (Barka et al 1986). Advantages of the ELISA for antigen detection over radioimmunoassays include the prolonged shelf-life of the reagents compared to ^{125}I labelled reagents, the absence of a radiation hazard, the relatively cheap reagents and the simplicity of the test.

1.2.13.5 RADIO-IMMUNOASSAY (RIA)

Radio-immunoassays can be used to detect either antigens or antibodies. The technique is similar to the ELISA but involves radioactively labelled reactants. It is less sensitive than the ELISA (Fehrenbach et al 1986) but is highly specific (Boonmee et al 1982). Diagnostic kits are commercially available but can only be used to detect *L. pneumophila* SG 1 antigens (Wilkinson et al 1986). Cross-reactions have not been demonstrated so far. The RIA

becomes positive early in the infection and remains positive for long periods of time. Advantages of the RIA over the ELISA include the fact that the RIA requires less time to do and is more specific.

1.2.13.6 AGGLUTINATION TESTS

Agglutination tests involve the clumping of antigens by reaction with specific antibodies that form bridges between antigenic determinants. Either heat- or formalin-killed antigens can be used (Wilkinson and Fikes 1980. Farshy et al 1978). Agglutination tests offer a cheap alternative for the DFA, and are considered better for antibody detection than the ELISA. A combination of agglutination tests with ELISA is considered best for screening of specimens. The sensitivity and specificity of the test compares well to the IFA and it is easy to perform, rapid and reliable.

Polyvalent pools are used for screening purposes. Monovalent antisera are then used to identify serogroups (Thacker et al 1983). Cross-reactions have been demonstrated with *B. pertussis* and *C. jejuni* but can be removed by absorption (Boswell et al 1992). Agglutination tests include micro-agglutination (MAT), slide-agglutination (SAT), reverse passive hemagglutination (RPHA), indirect hemagglutination, immune adherence agglutination and slide-coagglutination (coSAT) (Joly and Winn 1984, Yonke et al 1991). The MAT will detect mainly IgM while the RPHA test for urinary antigen detection is highly sensitive but cannot be used on serum samples (Mangiafico et al 1981). An adaptation of the MAT, the rapid MAT (RMAT), has recently been described (Constantine et al 1991).

The upper limit of normal in human sera is a titre of 1:8. A titre of 1:32 in a single specimen is suggestive of prior infection, titres of ≥1:32 indicate a bactericidal effect against the organism and titres of ≥1:64 is suggestive of active disease (Collins et al 1982).

1.2.13.7 NUCLEIC ACID PROBES

Family-, genus- and species-specific nucleic acid probes (eg. the commercially available Gen-Probe DNA probe) can be used for identification of legionellae (Edelstein et al 1986, Grimont 1986). Nucleic acids are made accessible through lysis of the cell and react with a radioactive or chemically labelled nucleic acid probe. This technique cannot replace culture but is useful when used in addition to other tests. Although false positive results have been reported in some studies, the sensitivity is very high (Wilkinson et al 1986). The test is rapid and does not depend on phenotypic characteristics other than character of the cell envelope (Wilkinson et al 1986). It may therefore overcome the problem of slow growth and the antigenic diversity of Legionella species. A disadvantage is that it has a relatively short shelf-life.

1.2.13.8 MONOCLONAL ANTIBODIES

Monoclonal antibodies can be either group-, species- or serogroup-specific and are currently available for identification of *L. pneumophila*, *L. dumoffii* and *L. micdadei* (Cercenado et al 1987). The technique is sensitive and specific with no cross-reactions reported so far except with *S. aureus* because of the high protein A content of this organism.

1.2.13.9 OTHER IDENTIFICATION TECHNIQUES

Flow cytometry is used for detection of legionellae in water samples and involves concentration of sample, treatment with RNAse and staining with propidium iodide. The specimen is then analysed with a cytofluorograph (Grimont 1986). Flow cytometry is also effective for detecting legionellae in unconcentrated water samples.

Rings of precipitated antibodies surround and define *L. pneumoniae* colonies grown on

filtered yeast extract agar, supplemented with polyvalent anti-*L. pneumophila* antibodies. *L. pneumophila* can also be detected on BCYE plates by obtaining colony imprints on nitrocellulose filters. Dry filters are reacted with specific antisera, then treated with ^{125}I labelled with protein A and autoradiographed. This method allows location of colonies of a given serotype on agar plates and is useful to quantitate Legionella serotypes in water samples. Species-specific DNA probes are effective for detection of *L. pneumophila* colonies on plates by colony hybridization. Counter immunoelectrophoresis is less sensitive than the RPHA, ELISA and RIA for antigen detection but may be useful in serogrouping Legionella isolates. The FIAX is a semi-automated fluorescence immunoassay recently developed for diagnosis of legionellae. It is easy to perform and highly reproducible.

1.2.14 SOURCES OF LEGIONELLA INFECTION

Legionellae are freshwater organisms that occur worldwide in natural sources like rivers, lakes, fountains and streams (Kurtz et al 1982, Watkins et al 1985), as well as in man-made systems like cooling towers, evaporative condensers, air-conditioning and water distribution systems and humidifyers. Legionellae have also been cultured from soil and dust samples taken at building sites and from industrial cutting-grinding fluid (Brundett 1989, Anrahan et al 1987, Muraca et al 1988, Schofield et al 1985). They are very prevalent in the South African environment (Tobiansky et al 1986).

Factors known to predispose a water distribution system to the presence of *legionellae* include the age of the system, the tank configuration, water temperatures below 60°C, the presence of scale, sediment and commensal bacteria and stagnant or intermittently flowing water (Muraca et al 1988). The presence of slime, amoebae and flavobacteriae on surfaces provide growth factors for legionellae and may aid attachment to surfaces like rubber tubing (Barbaree et al 86, Fields et al 1989). Legionellae become dormant in water temperatures below 60°C and have to be present in large numbers, be disseminated and inoculated into a susceptible host to be a health problem. Legionellae are frequently present in water systems of hotels, hospitals and domestic systems in Britain (Brundett 1989, Kurtz 1988). Species so far isolated from water distribution systems include *L. pneumophila, L. micdadei, L. bozemanii, L. dumoffii, L. feelei, L. wadsworthii, L. hackeliae, L.*

birminghamensis, L. jordanis, L. gormanii and *L. oakridgensis.* Of these, *L. pneumophila* causes 60%, *L. bozemanii* 15%, *L. dumoffii* 10% and *L. longbeacheae* 5% and the rest of the Legionella species together 10% of infections (Yu 1990).

1.2.14 MECHANISMS OF TRANSMISSION

Mechanisms of transmission include aerosolization, aspiration and ingestion. The first evidence of aerosolization as a mechanism of transmission was provided in 1978 during an outbreak in Memphis, Tenessee (Muder et al 1986). Several other studies have also supported the aerosolization theory (Brundett 1989, Davis et al 1985, Muraca et al 1988). Aerosols usually enter buildings via ventilation and dehumidification systems and are disseminated to susceptible persons through water distribution systems (Muder et al 1986). Aerosolization may also be caused by drift from cooling towers and faults in ventilation systems and humidifiers (MacFarlane 1989, Muraca et al 1988). Although only small numbers of legionellae are aerosolized by eg. shower heads, the particles are small enough to penetrate into the lower respiratory tract. Respiratory therapy devices like nebulizers may also disseminate organisms by creating and delivering gases at high pressures and volumes, overwhelming the normal defence mechanisms.

Bacteria associated with aspiration usually possess pili that allow them to adhere to pharyngeal epithelial cells. Symbiosis between the normal flora of the oropharynx and legionellae have been demonstrated (Muder et al 1986, Roig et al 1991). Pneumonias associated with aspiration have a low attack rate (Muraca et al 1988). The fact that legionellae possess fimbriae, have a low attack rate and Legionnaires' Disease occurs more often in heavy smokers and patients with lung disease, who are more susceptible to disease. than Pontiac Fever, may provide evidence for the aspiration theory. According to Roig et al (1991), aspiration is a common way of infection with legionellae.

Although evider for the ingestion theory is scanty, the fact that diarrhoea is a common symptom of Legionella infection may be a result of the organism penetrating the gastro-intestinal tract and spreading into extrathoracic organs. Legionellae can often be demonstrated in the blood of patients (Muder et al 1986).

Dissemination of legionellae via water distribution systems can be prevented by correct design and maintenance. Guidelines include the following (Finch 1988, Kurtz 1988, Sykes et al 1988): Pipe runs should be as short as possible and hot and cold pipes should not run close to each other. When designing a water distribution system, areas where water can become stagnant, and non-metallic components that will increase bacterial growth should be avoided and the system should be easily cleaned, drained and flushed. Wetted surfaces should be protected from direct sunlight to avoid growth of algae. To reduce particle formation, drift eliminators should be fitted into cooling towers and evaporative condensers. Cooling towers should be sited down wind from fresh air inlets of buildings, cleaned and treated with biocides regularly and cold water storage tanks heat insulated and covered, the temperature of the water kept under 20°C, and adequate concentrations of chlorine maintained at all times, adjusting regularly for variations in pH. Hot water systems should use calorifiers that can achieve storage temperatures higher than 55°C and draw off temperatures of above 46°C. Regular maintenance of water systems is important and a written policy regarding safety regulations for water systems and operative records should be kept. Routine monitoring of water distribution systems should include regular checking of pH, total dissolved solids and/or conductivity, bacterial counts and concentration of disinfectant or biocide used. The total number of bacteria, algae and other organisms should be kept low and a proper chemical treatment program followed.

1.2.15 WATER TREATMENT SYSTEMS

Methods mostly used to treat contaminated water systems include hyperchlorination, superheating, biocides, irradiation and ozone treatment. Legionellae are relative chlorine tolerant and viable *L. pneumophila* cells can still be recovered after exposure to the standard chlorine concentration of 0.2 mg/l for as long as one hour (Muraca et al 1988). The efficiency of chlorine treatment is therefore controversial. According to Muraca et al (1988), neither continuous or shock chlorination is effective in cooling towers, while Snyder et al (1990) and others found continuous chlorination effective, although legionellae may persist for 4 months after the treatment is started. A disadvantage of continuous chlorination of potable water systems is the production of trihalomethanes which may increase the risk of carcinogenesis of the bladder, rectum and colon in humans (Helms et

al 1988, Yu 1990). Hyperchlorination may also cause corrosion of plumbing systems, which can be decreased by addition of silicate to the water system (Snyder et al 1990).

Legionellae are killed by water temperatures exceeding 60°C (Muraca et al 1988). The hot water temperature is raised to 60-80°C for several days, and then each distal water site is flushed with warm water for 30 minutes. The water temperature is then maintained at 65°C (Yu 1990). Snyder et al (1990) reported a 66% reduction of *L. pneumophila* cultures after heat flushing, although the growth recurred after four months. Continuous supplemental chlorination then significantly decreased the number of positive cultures. The main disadvantage of superheating of potable water systems is the possibility of burn injuries.

The susceptibility of legionellae to biocides is controversial. According to (Muraca et al 1988) biocides are not effective against *L. pneumophila* in cooling towers and will only decrease the numbers slightly. Legionellae are highly susceptible to a variety of biocides in vitro (Grace et al 1981) but they may not be effective in outbreak situations (Winn 1984) and discrepancies exist between laboratory results and field studies with some of these biocides (Elsmore 1986). BNPD was the only biocide that was effective both in the laboratory and in the field. The organisms are relatively resistant to treatment with acids (Winn 1984). Biocides so far tested against legionellae include phenolic compound, idophors, quaternary ammonium compound, alcohol, aldehydes, hypochloride, dichlorophene, sodium hypochlorite (chlorine), dibromonitrilo propionamide, sodium pentachlorophenate, potassium orthobenzyl parachlorophenate, alkyl-propylenediamine monopropionate, alkyl-ammonium chloride and isothiazolon (Grace et al 1981, Muraca et al 1988). Biocides that are effective against both legionellae and algae all include bis(tri-n-butyltin-oxide (TBTO) (Yu 1990). Continuous ultraviolet irradiation is effective against water-borne *L. pneumophila*. It is often used in potable water systems as it leaves no bad odours, tastes or barmful chemical byproducts (Muraca et al 1988).

Ozone, a gaseous oxidant generated by electrically exciting O2 to the tri-atomic state and was shown to be effective against *L. pneumophila* in vitro. The concentration of ozone is needed to kill the bacteria is uncertain. Some authors believe a concentration of <1 mg/l will kill legionellae while others could only detect a 5 log decrease in numbers with a concentration of 1-2 mg/l, and then only after a period of 6 hours (Muraca et al 1988).

CHAPTER 2

MATERIALS AND METHODS

2.1 STUDY TYPE : Cross-sectional

2.2 SAMPLING METHOD : Workers were asked to volunteer. All volunteers were included, which comprised more than 90% of the recruits.

2.3 HUMAN ETHICS APPROVAL : Protocol Number 18/01/91

2.4 STUDY POPULATIONS : Mine workers, factory workers and pneumonia patients

2.4.1 MINE WORKERS

A total of 155 black, male workers were tested to determine their baseline antibody titres to *L. pneumophila*. Baseline titres to *C. pneumoniae* were determined on 133 of them. Paired samples were obtained six months apart from 95 workers. The rate of seroconversion to *L. pneumophila* was determined in all 95, and 81 were tested for conversion to *C. pneumoniae*. The workers included in the study all had previous experience of mine work and lived in hostels. They were migrant workers mainly from Mocambique (67%), while the rest were from different areas within the Republic of South Africa. Their ages ranged from

25 to 62 years (mean and standard deviation = 41.8 ± 7.9). Every employee in any of the South African mines who works underground or on the surface in specified high risk occupations as defined by law, is legally required to undergo an initial medical examination to determine his fitness for work. At the East Rand Proprietary Mines contracts of twelve months are served, alternating with compulsory leave periods ranging from three to six months. On returning to work, the workers undergo medical examinations before each new contract starts. Blood samples were taken and questionnaires administered on the first day of a new contract. The study was explained by black employees of the mine and the National Centre for Occupational Health (NCOH) and only volunteers were included in the study. Information sheets and informed consent forms were available. Workers were assured of the confidentiality of the results and that their decision to participate or not would in no way affect their conditions of employment.

2.4.2 FACTORY WORKERS

During 1988 a survey of a cotton factory situated in Mooiriver, Natal, was carried out by the NCOH. Requirements of the Human Ethics Committee were adhered to and consent had been obtained to use specimens for research purposes. Blood was taken and questionnaires administered from a random sample of black, male factory workers. From the total sample of 148, 145 workers were tested for the presence of antibodies to *L. pneumophila* and 143 for antibodies to C. pneumoniae. Paired samples were not obtained. The workers lived either in hostels, in the township or in the nearby town of Mooiriver, and were aged between 21 and 61 years (mean and standard deviation = 36.2 ± 8.9).

2.4.3 PNEUMONIA PATIENTS

A total of 86 serum samples from pneumonia patients from Groote Schuur Hospital, Cape Town were examined at the request of Dr. Gary Maartens for diagnostic purposes, and permission was obtained to include the results in this study. The patients were all hospitalised between July 1987 and July 1988 with symptoms of community acquired

pneumonia, which was defined as an acute respiratory illness with compatible shadowing on chest radiographs. Patients with pulmonary tuberculosis and severe immunosuppression were excluded. This study population consisted of 49 male and 38 female patients of all races. Of the females, 7 were black and 31 coloured. The males included 11 black, 32 coloured and 6 white patients. Their ages ranged from 12-77 years (mean and standard deviation = 37,3 ± 20,5).

2.5 SPECIMEN COLLECTION

10 cc venous blood was collected from each of the mine- and factory workers. Follow-up samples were obtained from the mine workers after a period of six months. All the samples were centrifuged on the day of collection and stored at -20°C. Acute and convalescent stage sera from the pneumonia patients were received frozen and were stored at -20°C until use. Care was taken not to repeatedly thaw and refreeze specimens. The determination of antibody titres in the paired sera from each worker were carried out on the same day to standardize test conditions.

2.6 QUESTIONNAIRES

Standard respiratory questionnaires, with special reference to past and present exposure to high levels of dust, were administered to the mine- and factory workers in a familiar language by trained black interviewers employed by the NCOH. The questionnaire used for the mine workers also included a section on previous exposure to humid working conditions.

2.7 INDIRECT IMMUNOFLUORESCENCE TEST (IFA)

2.7.1 EQUIPMENT

2.7.1.1 MICROSCOPY

An Olympus Model BH2 standard fluorescence microscope, equipped with a HBO-100 mercury incident light source was used for immunofluorescence. The primary filters are composed of a heat absorbing filter, a blue light passage filter with secondary filters for selective UV protection and a yellow filter. Observations were made under a dark field using 10 x occular and 50 x objective oil immersion lenses.

Heavy teflon coated, autoclavable microscope slides with 12 x 4 mm wells per slide were obtained from Sterilab Services, Johannesburg, RSA. These were used for immunofluorescence of both organisms throughout the study. Standard 24 mm x 50 mm cover slips were used to mount the slides.

2.7.1.2 MICROTITRE PLATES

Standard U-bottomed microtitre plates (Bibby Sterilin Ltd, Stone, Staffs, UK) were used for dilution of sera throughout the study.

2.7.1.3 INCUBATION

A standard incubator, with no added CO2 was used. Microscope slides were incubated in a moist chamber at 37°C.

2.7.2 REAGENTS

2.7.2.1 ANTIGENS

The antigen for the detection of antibodies to *C. pneumoniae* consisted of whole organisms with intact surfaces kindly provided by Professor C.C. Kuo from the School of Public Health and Community Medicine, Department of Pathobiology, University of Washington (Seattle, Washington). The antigen is prepared from HeLa-229 cells, highly infected with the organism, that are homogenized and purified. The cells are then treated with 0.02% formalin, which does not alter the quality of the surface antigens. This concentrated antigen can be stored for up to two years at 4°C. Before use, equal volumes (usually 20 µl of each) of the concentrated antigen and normal yolk sac are mixed. This mixture can be stored at 4°C for up to two weeks. The normal yolk sac was added to enhance the adhesion of the organisms to the slides and to facilitate location of the antigen spots during microscopic examination.

L. pneumophila polyvalent antigen A (serogroups 1-4) (heat-killed) was obtained from Zeus Scientific, Rowitan, New Jersey. The antigen is prepared by suspending the growth from a charcoal yeast agar plate in distilled water and placing this suspension in boiling water for 15 minutes to kill the cells. The cells are then centrifuged and the sediment resuspended in sterile distilled water.

Antigen slides were prepared by placing a small amount of antigen inside the wells, aspirating the excess, allowing them to air dry and fixing them in acetone for 15 minutes at room temperature. Legionella slides were kept at -20°C until needed. Chlamydia slides were prepared fresh before each test.

2.7.2.2 NORMAL YOLK SAC

Normal yolk sac (NYS) at a concentration of 3-5%, provided by Professor Kuo was used for the preparation of Chlamydia slides. Commercially available NYS at a concentration of 3% was used for the preparation of Legionella slides and was obtained from Diagnostic and Technical Service, Randburg, RSA.

2.7.2.3 CONTROLS

The positive control serum for Chlamydia was provided by Professor Kuo and was stored at 4°C. The positive control serum (monkey) for Legionella was obtained from Zeus Scientific, Rowitan, New Jersey. It was reconstituted as recommended by the manufacturers and was stored at 4°C.

2.7.2.4 WASHING SOLUTIONS

Phosphate buffered saline (PBS) (Zymed, California, USA) at pH 7.6 and distilled water were used throughout the study.

2.7.2.5 FLUORESCEIN

Fluorescein conjugated sheep anti-human immunoglobulin (IgA, IgM and IgG) was obtained from Wellcome Diagnostics, Johannesburg, RSA and was used throughout the study. The stain was reconstituted with sterile distilled water, aliquotted in sterile plastic tubes and stored at -20°C until needed. Final dilutions were made with PBS just before use. For the detection of antibodies to *C. pneumoniae*, Evan's Blue was added as a counterstain to a final concentration of 0.05% to reduce background fluorescence.

2.7.2.6 MOUNTING FLUID

Buffered glycerol (pH 9) was prepared by mixing one part PBS with 9 parts glycerol, and was used as mounting fluid and as immersion medium for microscopy of both organisms.

2.7.3 TEST PROCEDURE

The following technique recommended by Professor CC Kuo (University of Washington) was used for determination of antibody titres to *C. pneumoniae*. Dilutions of the sera were made in PBS. All sera were screened at a dilution of 1:8. Subsequent doubling dilutions of positive sera were made up to a dilution of 1:1024 or further if necessary. Ten microliters of serum dilution was placed onto each well of a microscope slide, starting from the highest to the lowest dilution to avoid transfer of antibodies. The slides were incubated in a moist chamber at 37°C for 30 minutes. After incubation, the slides were rinsed gently by dips and drains in four changes of PBS and three changes of distilled water and were then stood on their sides to dry at room temperature. Fluorescein conjugate was then diluted as explained earlier and the counterstain added. FITC-labelled antihuman immunoglobulin was then applied to each well and the slides were incubated again at 37°C for 30 minutes (in a moist chamber). The process of rinsing and air drying was repeated, the slides were mounted with buffered glycerol and were read immediately.

For detection of antibodies to *L. pneumophila*, sera were diluted 1:16 in 3% NYS. Subsequent doubling dilutions were made up to a dilution of 1:2048 in PBS. Ten microliters of serum dilution was placed onto each well of the microscope slide, starting from the highest to the lowest dilution to avoid transfer of antibodies. The slides were incubated in a moist chamber at 37°C for 30 minutes, rinsed quickly with PBS and placed in a PBS bath for 10 minutes. The slides were then rinsed quickly with distilled water and air dried. FITC-labelled antihuman immunoglobulin was applied to each well. The process of incubation, washing and drying was repeated. The slides were mounted with buffered glycerol and read immediately.

2.7.4 INTERPRETATION

In the case of *C. pneumoniae*, the highest serum dilution resulting in definite fluorescence of the elementary bodies was considered the antibody titre of that specimen. Only fluorescence associated with evenly distributed elementary bodies was considered positive.

The highest serum dilution that resulted in definite fluorescence of approximately 50% of the bacteria per microscope field studied was considered the antibody titre to *L. pneumophila*.

2.8 WATER SAMPLES

2.8.1 EQUIPMENT

Water samples were filtered through sterile Millipore Type HA filters (pore size 0.45 µm), available from Millipore Corporation, Bedford, using a 3-place PVC manifold (Millipore South Africa, Johannesburg, RSA).

2.8.2 MEDIA AND REAGENTS

2.8.2.1 DIRECT IMMUNOFLUORESCENCE (DFA)

L. pneumophila serogroups 1-6 and *L. micdadei* polyvalent conjugate A for direct immunofluorescence was obtained from Zeus Scientific, Raritan, New Jersey and was used undiluted.

2.8.2.2 CULTURE

Sheep Blood agar, Buffered Charcoal Yeast Extract agar plates (BCYE) and BCYE agar plates supplemented with glycine, vancomycin, polymyxin B and primaricin were obtained from the Media Department of the South African Institute for Medical Research, Johannesburg. Acid buffer was prepared using the method by Bopp et al (1981). This involved mixing 3.9 ml of 0.2 M HCl with 25 ml of 0.2 M KCl to yield a buffer solution with a pH of 2.2.

2.8.3 TEST PROCEDURE

Water samples (500 milliliters) were collected in plastic bottles with screw caps and filtered through Millipore filters. The filters were transferred into 10 ml of sterile distilled water and shaken vigorously. The water was poured off and centrifuged at 3000 rpm for 15 minutes. Agar plates were inoculated with the sediment as follows: For each specimen, one drop of sediment was placed on a blood agar plate, half of a normal BCYE and half of a BCYE plate with antibiotics. A 1:10 dilution was made of the sediment in acid buffer, which was left on the bench for 5 minutes. This was then inoculated onto the other half of the normal BCYE and BCYE agar with antibiotics. The plates were all incubated at 35°C without addition of CO^2. Blood agar plates were examined for growth after 24 hours incubation. All colonies on the blood agar plates were stained by DFA for comparison to organisms seen in the original DFA. This was done as follows: microscope slides were kept in 95% alcohol until use, when they were flamed. A drop of sediment was placed onto a slide and allowed to air dry and heat fixed. One drop of polyvalent conjugate was placed on the slide using a sterile pasteur pipette. The slides were left in a moist chamber at room temperature for 20 minutes, rinsed with PBS, and left for another 5 minutes before the PBS was poured off. The slides were then rinsed with distilled water, air dried and mounted in buffered glycerol. The slides were checked for fluorescence of bacteria resembling legionellae, and reported as scanty, one-, two or three plus positive. Any growth on the blood agar plates were suspended in sterile distilled water, and DFA stained as above. The original DFA was then compared with the DFA of colonies on blood agar: if the organisms

resembled legionellae and appeared to be the same on the two DFA's, the water sample was considered to be negative for legionellae, as the organism does not grow on blood agar. The DFA slides were kept at 4°C for comparison purposes until the BCYE plates were discarded. The BCYE (with and without antibiotics) were incubated for approximately 7-10 days before being discarded as negative. The plates were checked every day for growth. Colonies resembling legionellae were stained for DFA and compared with the original stains.

2.9 STATISTICAL ANALYSIS

Comparisons were made using the Chi-squared (X^2) test with Fisher's Exact modification where necessary for smaller numbers. The multivariate linear regression analysis was performed by using the Epi-Info software package. A P value of less than 0.05 was considered statistically significant.

CHAPTER 3

ANTIBODIES TO CHLAMYDIA PNEUMONIAE

Antibody titers ≥1:8 to *C. pneumoniae* were regarded as positive. Baseline titres ranged from 1:8 to 1:256 in the mine workers, from 1:8 to 1:2048 in the factory workers and from 1:8 to 1:128 in the pneumonia patients. The positive baseline titres were grouped by antibody titre into three groups, namely <1:64, 1:64 and >1:64 to simplify statistical analysis. Percentages given in the tables were calculated from the total population (n) in each category except where otherwise indicated in the text. The data were grouped in two ways for the statistical analysis. One way was to compare positive versus negative titres and the other was the comparison of positive titre groups. A fourfold or greater increase in antibody titre over a period of time (6 months in the mine workers and 1-6 weeks in the pneumonia patients) was considered as evidence of seroconversion, regardless of the titre levels of either the first or the second samples.

3.1 MINE WORKERS

The following potential risk factors were examined in relation to baseline titres and seroconversion : age, smoking habits, past underground (mining) experience, past exposure to high levels of dust and/or humidity in the work environment and respiratory symptoms.

3.1.1 BASELINE TITRES

Of the 133 mine workers tested to determine their baseline titres to *C. pneumoniae*, 66%

were positive, with the majority of positives (41%) of all the specimens tested at the level <1:64 and 25% at the level ≥1:64 (Figure 3.1).

Baseline titres associated with age are presented in Table 3.1. The ages of 129 of the mine workers were known and ranged from 25 to 60 years (mean and standard deviation=40.19 x 10.38), with the majority (76%) aged between 31 and 50. The prevalence of antibodies to *C. pneumoniae* ranged from 90% in the <31 years category to 56% in the category above 50 years of age. The majority of positive baseline titres were below 1:64 except in the youngest age category (<31 years) where 50% of the positive titres were at the level ≥1:64. None of these differences were statistically significant.

Information regarding previous underground experience of 114 workers was obtained from staff records at the mine. Previous experience ranged from 1-37 years with the majority (68%) of workers falling in the categories between 6 and 15 years (mean and standard deviation=10.85 ± 5.72). Of the workers with past underground experience, the majority (87%) were still working underground at the time of the study. Baseline titres associated with underground experience are presented in Table 3.2. The prevalence of antibodies to *C. pneumoniae* ranged from 59% in the workers with 1-5 years to 72% in those with 11-15 years experience. These differences were not statistically significant. A predictable observation was the increase in mean age with years experience (Table 3.3).

The influence of smoking on baseline titres to *C. pneumoniae* was studied and is presented in Table 3.4. Workers who were smoking at the time of the study, or have smoked in the past, were included in the category for smokers. The prevalence of antibodies and the number of workers with titres ≥1:64 were slightly higher in smokers than in non-smokers but the differences were not statistically significant.

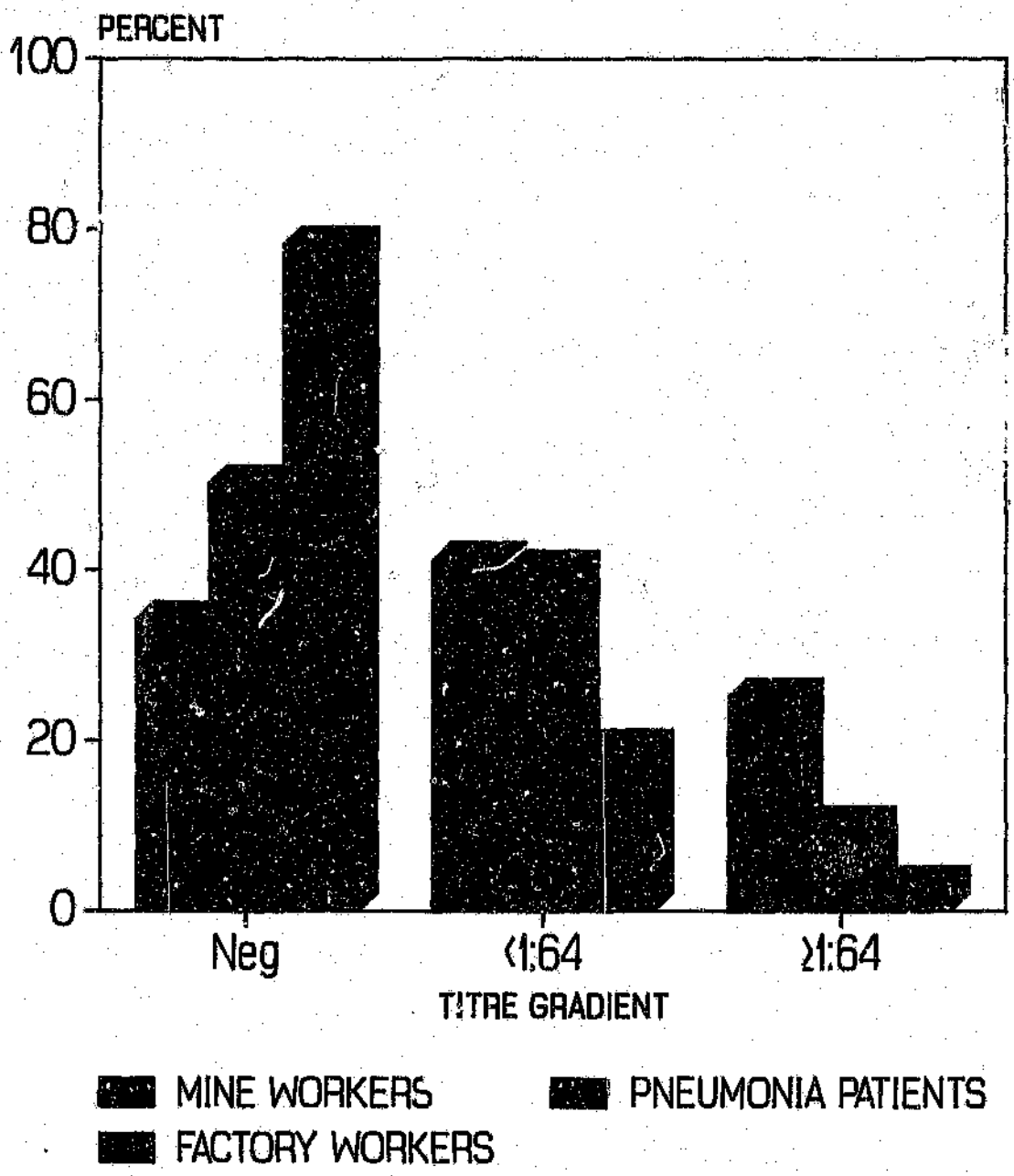


FIGURE 3.1
Antibodies to C. pneumoniae

TABLE 3.1

BASELINE TITRES TO *C. PNEUMONIAE* IN MINE WORKERS

ASSOCIATED WITH AGE

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
< 31 years	10	9 (90)	1 (10)	4 (40)	2 (20)	3 (30)
31-40 years	50	33 (66)	17 (34)	22 (44)	10 (20)	1 (2)
41-50 years	51	34 (67)	17 (33)	22 (43)	10 (20)	2 (4)
> 50 years	18	10 (56)	8 (44)	6 (33)	2 (11)	2 (11)
Unknown	4	2 (50)	2 (50)	1 (25)	1 (25)	-
TOTAL	133	88 (66)	45 (34)	55 (41)	25 (19)	8 (6)

Percentages are in parentheses

TABLE 3.2

BASELINE TITRES TO *C. PNEUMONIAE* IN MINE WORKERS

ASSOCIATED WITH UNDERGROUND EXPERIENCE

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
1- 5 years	17	10 (59)	7 (41)	8 (47)	1 (6)	1 (6)
6-10 years	46	29 (63)	17 (37)	18 (39)	9 (20)	2 (4)
11-15 years	32	23 (72)	9 (28)	17 (53)	4 (13)	2 (6)
> 15 years	19	12 (63)	7 (37)	6 (32)	5 (26)	1 (5)
Unknown	19	14 (73)	5 (26)	6 (32)	6 (32)	2 (11)
TOTAL	133	88 (66)	45 (34)	55 (41)	25 (19)	8 (6)

Percentages are in parentheses

TABLE 3.3

MEAN AGE ASSOCIATED WITH PREVIOUS UNDERGROUND EXPERIENCE IN MINE WORKERS

	n	MEAN AGE	SD
1- 5 years	17	36.12	8.01
6-10 years	45 (1)	40.20	9.41
11-15 years	32	41.66	7.35
> 15 years	19	47.53	5.32
Unknown	16 (3)	42.88	6.65
TOTAL	129 (4)		

() : Age unknown
SD : Standard deviation

TABLE 3.4

BASELINE TITRES TO *C. PNEUMONIAE* IN MINE WORKERS ASSOCIATED WITH SMOKING

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
Smokers	35	27 (77)	8 (23)	15 (43)	8 (23)	4 (11)
Non-smokers	77	50 (65)	27 (35)	33 (43)	15 (20)	2 (3)
Unknown	21	11 (52)	10 (48)	7 (33)	2 (10)	2 (10)
TOTAL	133	88 (66)	45 (34)	55 (41)	25 (19)	8 (6)

Percentages are in parentheses

Information regarding respiratory symptoms of 114 workers was obtained from questionnaires and medical records kept at the mine hospital. The majority of workers (81%) did not complain of any respiratory symptoms, while 11% had tuberculosis and pneumonia was reported in 6%. Only two workers complained of upper respiratory symptoms, both with negative baseline titres. The baseline titres associated with respiratory symptoms are presented in Table 3.5. The prevalence of antibodies was similar in the workers with and without respiratory symptoms.

Information regarding past exposure to high levels of dust and/or humidity in the work environment was obtained from 114 workers. Their baseline titres to *C. pneumoniae* are presented in Table 3.6. The prevalence of antibodies ranged from 76% in the workers with exposure to water only to 68% each in wor kers with exposure to dust only and to dust plus water, and 63% in workers without exposure to either. None of these differences were statistically significant.

TABLE 3.5

BASELINE TITRES TO *C. PNEUMONIAE* IN MINE WORKERS ASSOCIATED WITH RESPIRATORY SYMPTOMS

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
WITH SYMPTOMS	21	14 (67)	7 (33)	9 (43)	3 (14)	2 (10)
Pneumonia	7	3 (43)	4 (57)	2 (29)	1 (14)	-
Tuberculosis	12	9 (75)	3 (25)	5 (42)	2 (17)	2 (17)
UR	2	2 (100)	-	2 (100)	-	-
WITHOUT SYMPTOMS	93	64 (69)	29 (31)	39 (42)	21 (22)	4 (4)
UNKNOWN	19	10 (53)	9 (48)	7 (37)	1 (5)	2 (11)
TOTAL	133	88 (66)	45 (34)	55 (41)	25 (19)	8 (6)

Percentages are in parentheses
UR : Upper respiratory symptoms

TABLE 3.6

BASELINE TITRES TO *C. PNEUMONIAE* IN MINE WORKERS ASSOCIATED WITH DUSTY AND HUMID ATMOSPHERES

	n	BASELINE TITRES		POSITIVE TITRE LEVEL		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
Humidity only	25	19 (76)	6 (24)	12 (48)	6 (24)	1 (4)
Dust only	15	10 (67)	5 (33)	4 (27)	6 (40)	-
Humidity and dust	47	32 (68)	15 (32)	20 (43)	8 (17)	4 (9)
Neither	27	17 (63)	10 (37)	13 (48)	3 (11)	1 (4)
Unknown exposure	19	10 (53)	9 (47)	6 (32)	2 (11)	2 (11)
TOTAL	133	88 (66)	45 (34)	55 (41)	25 (19)	8 (6)

Percentages are in parentheses

3.2 SEROCONVERSION

Paired samples from 81 mine workers were tested to determine whether seroconversion occurred within a period of six months while working at the mine. The influence of age on seroconversion to *C. pneumoniae* is presented in Table 3.7. From these results it was evident that age did not influence seroconversion significantly. It should be noted however that 14 of the adult mine workers (17%) experienced a significant increase in *C. pneumoniae* antibodies during a six month period.

Paired samples from 69 workers with known previous underground experience were tested for seroconversion. Of these, 62 (90%) were still working underground at the time of the study (Table 3.8). A total of 74 workers with known smoking habits were tested to determine the rate of seroconversion (Table 3.9). The differences between the groups were not statistically significant.

To study the association between respiratory symptoms and the rate of seroconversion, paired samples from 73 workers were tested, and the results are presented in Table 3.10. Of these workers, 14 had respiratory symptoms: four had pneumonia, eight were diagnosed as having tuberculosis and two had upper respiratory symptoms. Seroconversion was demonstrated in 12% of workers without symptoms compared to the 43% of workers with symptoms, a statistically significant difference (X^2=5.46, DF=1, P<0.05).

Past exposure to high levels of dust and/or humidity in the working environment did not influence the rate of seroconversion significantly (Table 3.11). Seroconversion was demonstrated in 25% of workers without previous exposure to either of these risk factors in the environment, in 21% in workers with exposure to humid atmospheres, 18% with exposure to dust only and 10% in workers with previoius exposure to both environments.

TABLE 3.7

SEROCONVERSION TO *C. PNEUMONIAE* IN MINE WORKERS ASSOCIATED WITH AGE

	n	SEROCONVERSION
< 31 years	2	-
31-40 years	34	5 (15)
41-50 years	32	6 (19)
> 50 years	12	2 (17)
Unknown	1	1 (100)
TOTAL	81	14 (17)

Percentages are in parentheses

TABLE 3.8

SEROCONVERSION TO *C. PNEUMONIAE* IN MINE WORKERS ASSOCIATED WITH UNDERGROUND EXPERIENCE

GROUP	n	CONVERSION	MEAN AGE	SD
1- 5 years	10	3 (30)	39.40	8.71
6-10 years	27	3 (11)	40.07	5.86
11-15 years	23	3 (13)	41.30	7.17
> 15 years	9	1 (11)	47.33	6.56
Unknown	12	4 (33)	43.45	6.41
TOTAL	81	14 (17)		

Percentages are in parentheses
SD : Standard deviation

TABLE 3.9

SEROCONVERSION TO *C. PNEUMONIAE* IN MINE WORKERS ASSOCIATED WITH SMOKING

GROUP	n	SEROCONVERSION
Smokers	21	2 (10)
Non-smokers	53	11 (21)
Unknown	7	1 (14)
TOTAL	81	14 (17)

Percentages are in parentheses

TABLE 3.10

SEROCONVERSION TO *C. PNEUMONIAE* IN MINE WORKERS ASSOCIATED WITH RESPIRATORY SYMPTOMS

	n	SEROCONVERSION
TOTAL WITH SYMPTOMS:	14	6 (43)
Pneumonia	4	2 (50)
Tuberculosis	8	3 (38)
Upper respiratory	2	1 (50)
TOTAL WITHOUT SYMPTOMS:	59	7 (12)
UNKNOWN:	8	1 (25)
TOTAL	81	14 (17)

Percentages are in parentheses

TABLE 3.11

SEROCONVERSION TO *C. PNEUMONIAE* IN MINE WORKERS ASSOCIATED WITH DUST AND HUMIDITY IN THE ENVIRONMENT

	n	SEROCONVERSION
Humidity only	14	3 (21)
Dust only	11	2 (18)
Dust and humidity	30	3 (10)
Neither	20	5 (25)
Unknown exposure	6	1 (17)
TOTAL	81	14 (17)

Percentages are in parentheses

3.2 PNEUMONIA PATIENTS

A total of 86 pneumonia patients were tested to determine their baseline titres to *C. pneumoniae* and paired samples were obtained from 85 of them. The influence of the age, race and gender of the patients on baseline titres as well as seroconversion was studied.

3.2.1 BASELINE TITRES

A total of 86 pneumonia patients were tested to determine their baseline titres to *C. pneumoniae*. Of these, 43 (50%) had positive baselines: 40% of the patients had titres <1:64 and 10% had titres ≥1:64.

The patients were aged between 12 and 77 years (mean and standard deviation=42.26 ± 16.51) and were grouped into categories <31 years, 31-50 years and >50 years of age. Baseline titres associated with age are presented in Table 3.12. The age of the patients did not influence the prevalence of antibodies significantly, although the number of patients with baselines ≥1:64 was slightly higher in the category aged between 31 and 50 years.

A total of 18 black, 60 coloured and five white patients were tested to determine their baseline titres. Table 3.13 illustrates the prevalence of antibodies in the different race groups. Although the prevalence was highest in the black patients (56%), compared to the 50% in coloured and 20% in white patients, the difference was not statistically significant. Titres ≥1:64 were only observed in the coloured patients.

A total of 47 male and 36 female patients were studied to determine their baseline titres. The results are presented in Table 3.14 and indicated a slightly higher prevalence in the female patients (53%) than in the males (47%). Of the female patients, 17% had titres ≥1:64 compared to the 6% of male patients with titres at this level. The differences were however not statistically significant.

TABLE 3.12

BASELINE TITRES TO *C. PNEUMONIAE* IN PNEUMONIA PATIENTS ASSOCIATED WITH AGE

	n	BASELINE TITRES		POSITIVE TITRE LEVELS	
		POSITIVE	NEGATIVE	<1:64	≥1:64
< 31 years	23	11 (48)	12 (52)	10 (43)	1 (4)
31-50 years	28	15 (54)	13 (46)	11 (39)	4 (14)
> 50 years	26	12 (46)	14 (54)	10 (38)	2 (8)
Unknown	9	5 (56)	4 (44)	3 (33)	2 (22)
TOTAL	86	43 (50)	43 (50)	34 (40)	9 (10)

Percentages are in parentheses

TABLE 3.13

BASELINE TITRES TO *C. PNEUMONIAE* IN PNEUMONIA PATIENTS ASSOCIATED WITH RACE

	n	BASELINE TITRES		POSITIVE TITRE LEVELS	
		POSITIVE	NEGATIVE	< 1:64	≥ 1:64
Black	18	10 (56)	8 (44)	10 (56)	-
Coloured	60	30 (50)	30 (50)	21 (35)	9 (15)
White	5	1 (20)	4 (80)	1 (20)	-
Unknown	3	2 (67)	1 (33)	2 (67)	-
TOTAL	86	43 (50)	43 (50)	34 (40)	9 (10)

Percentages are in parentheses

TABLE 3.14

BASELINE TITRES TO *C. PNEUMONIAE* IN PNEUMONIA PATIENTS ASSOCIATED WITH GENDER

	n	BASELINE TITRES		POSITIVE TITRE LEVELS	
		POSITIVE	NEGATIVE	< 1:64	≥ 1:64
Male	47	22 (47)	25 (53)	19 (40)	3 (6)
Female	36	19 (53)	17 (47)	13 (36)	6 (17)
Unknown	3	2 (67)	1 (33)	2 (67)	-
TOTAL	86	43 (50)	43 (50)	34 (40)	9 (10)

Percentages are in parentheses

3.2.2 SEROCONVERSION

Seroconversion was demonstrated in a total of 19 (22%) of the 85 paired samples obtained from the pneumonia patients. The paired samples were obtained from 23 patients were under the age of 31 years, 28 between 31 and 50 years and 26 over the age of 50. Although not significantly different, the rate of conversion increased with age, from 13% in the youngest to 31% in the oldest category (Table 3.15). Stratification of results by ethnic groups in Table 3.16 and gender in Table 3.17 showed that neither factor is significant in seroconversion.

TABLE 3.15

SEROCONVERSION TO *C. PNEUMONIAE* IN PNEUMONIA PATIENTS ASSOCIATED WITH AGE

	n	SEROCONVERSION
< 31 years	23	3 (13)
31-50 years	28	6 (21)
≥ 50 years	26	8 (31)
Unknown	8	2 (25)
TOTAL	85	19 (22)

Percentages are in parentheses

TABLE 3.16

SEROCONVERSION TO *C. PNEUMONIAE* IN PNEUMONIA PATIENTS ASSOCIATED WITH RACE

	n	SEROCONVERSION
Black	18	5 (28)
Coloured	60	14 (23)
White	5	-
Unknown	2	-
TOTAL	85	19 (22)

Percentages are in parentheses

TABLE 3.17

SEROCONVERSION TO *C. PNEUMONIAE* IN PNEUMONIA PATIENTS ASSOCIATED WITH GENDER

	n	SEROCONVERSION
Male	47	11 (23)
Female	36	8 (22)
Unknown	2	-
TOTAL	85	19 (22)

Percentages are in parentheses

3.3 FACTORY WORKERS

Risk factors studied in the factory workers were age, smoking, respiratory symptoms and exposure (past and present) to dust in their working environment. None of the workers tested had previous experience of mine work of any kind, either underground or on the surface. Paired samples were not obtained from any of the factory workers.

3.3.1 BASELINE TITRES

A total of 147 factory workers were tested to determine their baseline titres to *C. pneumoniae*. Of these, 22% had positive baseline titres, indicating a significantly lower prevalence than in the other study populations (X^2=55.79, DF=2, P<0.0001). One of the workers had a titre of 1:2048, indicating an acute infection at the time of the study. The influence of age on baseline titres in the factory workers is presented in Table 3.18. Age groups studied were <31, 31-40, 41-50 and >50 years. The age of the workers did not influence the prevalence of antibodies to *C. pneumoniae* significantly. The worker with a titre of 1:2048 was aged between 31 and 40. A comparison of baseline titres between smokers vs. non-smokers did not indicate a statistically significant difference (Table 3.19).

Information regarding the respiratory symptoms of the factory workers was obtained from questionnaires. The workers complained mainly of pneumonia, upper respiratory symptoms, bronchitis, asthma, high blood pressure and tuberculosis. The only symptoms of interest in the present study were pneumonia, upper respiratory symptoms and tuberculosis and the majority of workers with symptoms (83%) complained of upper respiratory symptoms only. No statistically significant differences were noted (Table 3.20). The worker with a titre of 1:2048 had no respiratory symptoms. None of the factory workers interviewed had any previous experience of mine work (i.e. underground work) and only one of them had experience of work in a dusty environment other than his present job at the factory. This worker had a baseline titre of 1:8. The influence of present dust exposure at the factory on baseline titres was studied, and the results are presented in Table 3.21. There was no significant difference in baseline titres in those exposed versus those not exposed to dust.

TABLE 3.18

BASELINE TITRES TO *C. PNEUMONIAE* IN FACTORY WORKERS
ASSOCIATED WITH AGE

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
< 31 years	36	8 (22)	28 (78)	8 (22)	-	
31-40 years	69	13 (19)	56 (81)	11 (16)	1 (1)	1 (1)
41-50 years	30	7 (23)	23 (77)	6 (20)	-	1 (3)
> 50 years	10	4 (40)	6 (60)	2 (20)	1 (10)	1 (10)
TOTAL	145	32 (22)	113 (78)	27 (18)	2 (1)	3 (2)

Percentages are in parentheses

TABLE 3.19

BASELINE TITRES TO *C. PNEUMONIAE* IN FACTURY WORKERS
ASSOCIATED WITH SMOKING

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
Smokers	99	17 (17)	82 (83)	14 (14)	1 (1)	2 (2)
Non-smokers	45	15 (33)	30 (67)	13 (29)	1 (2)	1 (2)
Unknown	1	-	1 (100)	-	-	-
TOTAL	145	32 (22)	113 (78)	27 (19)	2 (1)	3 (2)

Percentages are in parentheses

TABLE 3.20

BASELINE TITRES TO *C. PNEUMONIAE* IN FACTORY WORKERS ASSOCIATED WITH RESPIRATORY SYMPTOMS

	n	BASELINE TITRES		POSITIVE TITRES	
		POSITIVE	NEGATIVE	<1:64	≥1:64
WITH SYMPTOMS:	86	16 (19)	70 (81)	15 (17)	1 (1)
PN and UR	5	2 (40)	3 (60)	2 (40)	-
TB and UR	1	-	1 (100)	-	-
PN only	1	1 (100)	-	1 (100)	-
UR only	71	12 (17)	59 (83)	11 (16)	1 (1)
TB only	2	-	2 (100)	-	-
*** Other	6	1 (17)	5 (83)	1 (17)	-
WITHOUT SYMPTOMS:	57	16 (28)	41 (72)	12 (21)	*4 (7)
UNKNOWN SYMPTOMS:	2	-	2 (100)	-	-
TOTAL	145	32 (22)	113 (78)	27 (19)	5 (3)

* : One titer was 1:2048
TB : Tuberculosis
PN : Pneumonia
** : Neither TB, UR or PN
UR : Upper respiratory
() : Percentage

TABLE 3.21

BASELINE TITRES TO *C. PNEUMONIAE* IN FACTORY WORKERS ASSOCIATED WITH DUST EXPOSURE

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
With exposure	78	19 (27)	59 (76)	16 (21)	1 (1)	2 (3)
Without exposure	67	13 (. ,	54 (81)	11 (16)	1 (2)	1 (2)
TOTAL	145	32 (22)	113 (78)	27 (19)	2 (1)	3 (2)

Percentages are in parentheses

3.4 COMPARISON OF STUDY POPULATIONS

The comparison of baseline titres observed in the three study populations is presented in Table 3.22 and indicates a statistically significant difference in the prevalence of antibodies to *C. pneumoniae* ($P<0.0001$), with positive baselines seen in 66% of mine workers, 50% of pneumonia patients and 22% of factory workers. The number of mine workers with baseline titres at the level ≥1:64 was also significantly higher than the other two populations ($P<0.05$), where 38% of mine workers had titres at this level compared to the 21% of pneumonia patients and the 16% of factory workers. These data are all illustrated in Figure 3.1 (page 79). A logistic regression analysis was performed on the data from the mine workers and the factory workers. There was no significant association between any of the risk factors or symptoms studied and baseline titres to *C. pneumoniae* in the mine workers or the factory workers.

TABLE 3.22

COMPARISON OF THREE STUDY POPULATIONS

	STUDY POPULATION			x^2	D	P-VALUE
	M	F	P			
n	133	145	86			
Negative:	45 (34)	113 (78)	43 (50)			
Positive:	88 (66)	32 (22)	43 (50)			
Titre <1:64	55 (41)	27 (19)	34 (40)			
Titre ≥1:64	33 (25)	5 (3)	9 (11)			
<1:64 vs. ≥1:64	55 /33	27 / 5	34 / 9	7.25	2	0.0266 +
Pos vs. Neg	88 /45	32 /113	43 /43	55.79	2	< 0.001 +
Paired samples	81	-	85			
Seroconversion	14 (17)	-	19 (22)	0.39	1	0.5330

Percentages are in parentheses
M : Mine workers
P : Pneumonia patients
F : Factory workers
X^2 : Chi-squared
D : Degrees of freedom
+ : Statistically significant

CHAPTER 4

ANTIBODIES TO LEGIONELLA PNEUMOPHILA

Antibody titres ≥1:16 to *L. pneumophila* were regarded as positive. Positive baseline titres ranged from 1:16 to 1:1024 in mine workers and from 1:16 to 1:128 in factory workers and pneumonia patients, and were grouped by antibody titre into three groups namely <1:64, 1:64 and >1:64 to simplify statistical analysis and to distinguish past contact with the organism (titres <1:64) from possible infection (titres ≥1:64). Percentages given in the tables were calculated from the total population (n) in each category except where otherwise indicated in the text. The data were grouped in two ways for the statistical analysis: firstly for comparison of positive versus negative baseline titres, and secondly for comparison of the titre groups. A fourfold or greater increase in antibody titre over a period of time (6 months in the mine workers and 1-6 weeks in the pneumonia patients) was considered as evidence of seroconversion, regardless of the titre levels of either the first or the second samples.

4.1 MINE WORKERS

Risk factors studied in relation to baseline titres and seroconversion in the mine workers included age, smoking, past underground experience, past exposure to environments with high levels of dust and/or humidity, and respiratory symptoms.

4.1.1 BASELINE TITRES

A total of 155 mine workers were tested to determine their baseline titres to *L.*

pneumophila. Of these, 36% were positive, 10% with titres <1:64, 16% with titres of 1:64 and 10% with titres >1:64. One worker had a titre of 1:256, two had titres of 1:512 and one had a titre of 1:1024 (Figure 4.1).

The ages of the mine workers ranged from 25 to 62 years (mean and standard deviation=41.81 ± 7.97). They were grouped into categories <31 years, 31-40, 41-50 and >50 years, with the majority (73%) aged between 31 and 50 years. The baseline titres associated with the different age groups are presented in Table 4.1. Age, within the range of ages of the mine workers, did not influence the prevalence of antibodies to *L. pneumophila* significantly.

Information regarding smoking was obtained from 112 workers of whom 35 (31%) were smokers and 77 (69%) were non-smokers. The workers were classified as smokers if they were smoking at the time of the study or if they have smoked in the past. Antibodies to *L. pneumophila* were slightly more prevalent in smokers (40%) than in non-smokers (34%) but the difference was not statistically significant (Table 4.2). The positive titre groups for the two categories were not significantly different, with titres ≥1:64 observed in 20% of smokers and 24% of non-smokers. No information regarding smoking could be obtained from any of the workers with titres ≥1:256.

Staff records were used to obtain information regarding previous underground (mining) experience of 129 workers who were grouped into categories 1-5, 6-10, 11-15 and >15 years experience. The majority (67%) had between 6 and 15 years experience. Baseline titres associated with previous underground experience are presented in Table 4.3. Previous underground experience did not influence the prevalence of antibodies to *L. pneumophila* significantly.

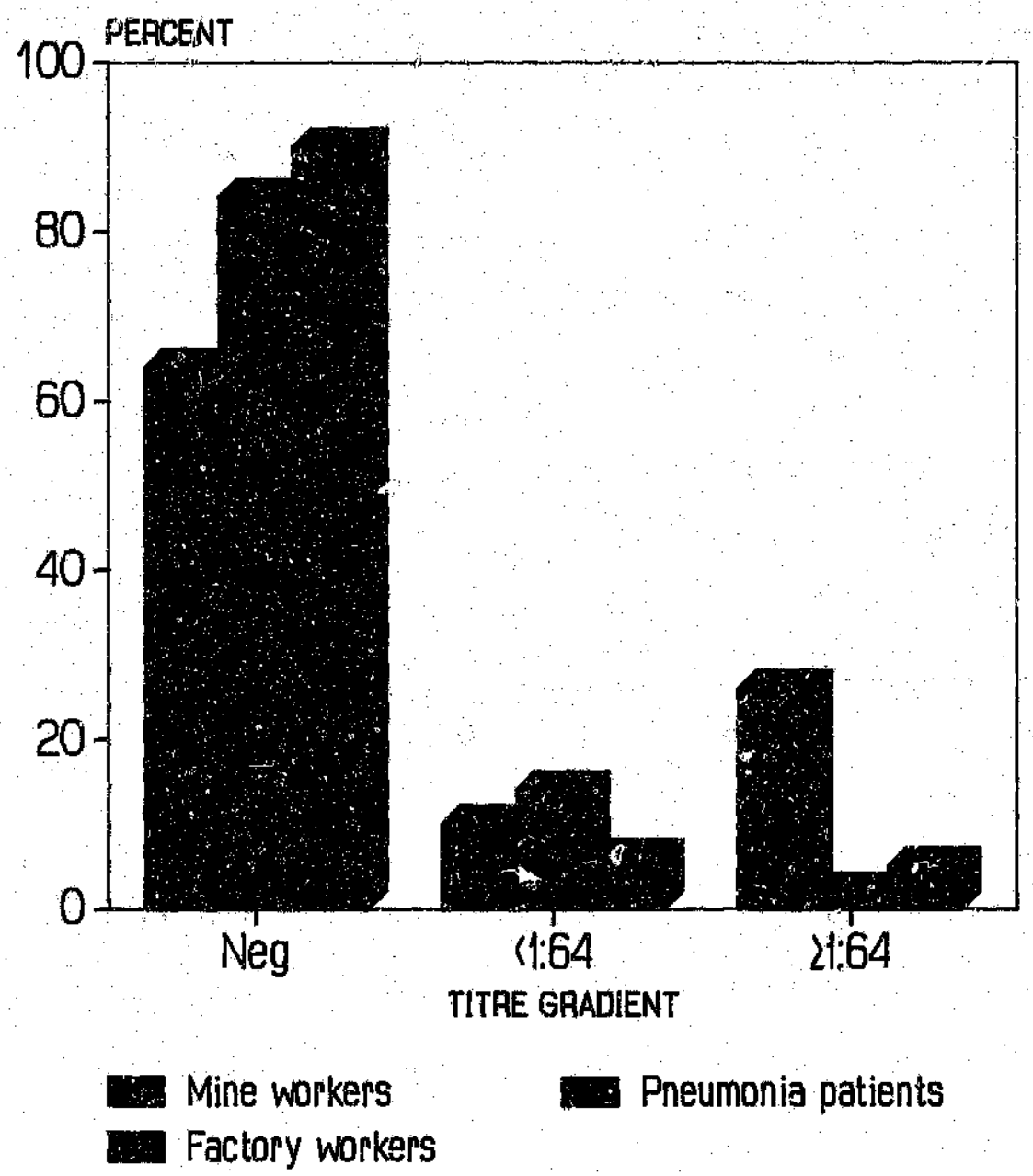


FIGURE 4.1
Antibodies to L. pneumophila

TABLE 4.1

BASELINE TITRES TO *L. PNEUMOPHILA* IN MINE WORKERS

ASSOCIATED WITH AGE

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
< 31 years	10	2 (20)	8 (80)	1 (10)	1 (10)	-
31-40 years	55	19 (35)	36 (66)	4 (7)	10 (18)	5 (9)
41-50 years	58	24 (41)	34 (59)	8 (14)	8 (14)	8 (14)
> 50 years	23	5 (22)	18 (78)	2 (9)	2 (9)	1 (4)
Unknown	9	6 (67)	3 (33)	-	4 (44)	2 (22)
TOTAL	155	56 (36)	99 (64)	15 (10)	25 (16)	16 (10)

Percentages are in parentheses

TABLE 4.2

BASELINE TITRES TO *L. PNEUMOPHILA* IN MINE WORKERS

ASSOCIATED WITH SMOKING

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
Smokers	35	14 (40)	21 (60)	7 (20)	6 (17)	1 (3)
Non-smokers	77	26 (34)	51 (66)	8 (10)	8 (10)	10 (13)
Unknown	43	16 (37)	27 (63)	-	11 (26)	5 (12)
TOTAL	155	56 (36)	99 (64)	15 (10)	25 (16)	16 (10)

Percentages are in parentheses

TABLE 4.1

BASELINE TITRES TO *L. PNEUMOPHILA* IN MINE WORKERS

ASSOCIATED WITH AGE

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
< 31 years	10	2 (20)	8 (80)	1 (10)	1 (10)	-
31-40 years	55	19 (35)	36 (66)	4 (7)	10 (18)	5 (9)
41-50 years	58	24 (41)	34 (59)	8 (14)	8 (14)	8 (14)
> 50 years	23	5 (22)	18 (78)	2 (9)	2 (9)	1 (4)
Unknown	9	6 (67)	3 (33)	-	4 (44)	2 (22)
TOTAL	155	56 (36)	99 (64)	15 (10)	25 (16)	16 (10)

Percentages are in parentheses

TABLE 4.2

BASELINE TITRES TO *L. PNEUMOPHILA* IN MINE WORKERS

ASSOCIATED WITH SMOKING

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
Smokers	35	14 (40)	21 (60)	7 (20)	6 (17)	1 (3)
Non-smokers	77	26 (34)	51 (66)	8 (10)	8 (10)	10 (13)
Unknown	43	16 (37)	27 (63)	-	11 (26)	5 (12)
TOTAL	155	56 (36)	99 (64)	15 (10)	25 (16)	16 (10)

Percentages are in parentheses

TABLE 4.3

BASELINE TITRES TO *L. PNEUMOPHILA* IN MINE WORKERS ASSOCIATED WITH UNDERGROUND EXPERIENCE

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
1- 5 years	17	8 (47)	9 (53)	3 (18)	3 (18)	2 (12)
6-10 years	45	12 (27)	33 (73)	4 (9)	6 (13)	2 (4)
11-15 years	40	14 (35)	26 (65)	4 (10)	5 (13)	5 (13)
> 15 years	24	9 (38)	15 (63)	2 (8)	4 (17)	3 (13)
Unknown	29	13 (45)	16 (55)	2 (7)	7 (24)	4 (14)
TOTAL	155	56 (36)	99 (64)	15 (10)	25 (16)	16 (10)

Percentages are in parentheses

Legionnaires' Disease (pneumonia) and Pontiac Fever (a flu-like illness) are both clinical manifestations of Legionella infections. The two main respiratory symptoms studied were therefore pneumonia and upper respiratory symptoms. *M. tuberculosis* has been implicated in cross-reactions with *L. pneumophila* in the indirect immunofluorescence test, therefore the association between tuberculosis and baseline titres was also investigated. Baseline titres associated with respiratory symptoms are presented in Table 4.4. There was no significant difference in the prevalence of antibodies between workers with and without respiratory symptoms, or between the various symptoms studied (i.e. pneumonia, tuberculosis and upper respiratory symptoms). All the workers with baselines ≥1:256 had pneumonia symptoms.

Information regarding previous exposure to dust and/or humidity in the work environment of 87 mine workers was obtained from questionnaires. Their baseline titres are presented in Table 4.5. No significant associations were found between baseline titres and dust or humidity exposure.

TABLE 4.4

BASELINE TITRES TO *L. PNEUMOPHILA* IN MINE WORKERS
ASSOCIATED WITH RESPIRATORY SYMPTOMS

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
UNKNOWN SYMPTOMS	10	3 (30)	7 (70)	-	2 (20)	1 (10)
WITHOUT SYMPTOMS	93	35 (38)	58 (62)	15 (16)	10 (11)	10 (11)
WITH SYMPTOMS	52	18 (35)	34 (65)	-	13 (25)	5 (10)
PN	38	15 (40)	23 (61)	-	10 (26)	5 (13)
TB	12	3 (25)	9 (75)	-	3 (25)	-
UR	2	-	2 (100)	-	-	-
TOTAL	155	56 (36)	99 (64)	15 (10)	25 (16)	16 (10)

Percentages are in parentheses
PN : Pneumonia
TB : Tuberculosis
UR : Upper respiratory symptoms

TABLE 4.5

BASELINE TITRES TO *L. PNEUMOPHILA* IN MINE WORKERS
ASSOCIATED WITH EXPOSURE TO DUST AND HUMIDITY IN THE ENVIRONMENT

	n	BASELINE TITRES		POSITIVE TITRE LEVEL		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
Humidity only	25	7 (28)	18 (72)	2 (8)	4 (16)	1 (4)
Dust only	15	6 (40)	9 (60)	4 (27)	-	2 (13)
Dust and humidity	47	19 (40)	28 (60)	7 (15)	6 (13)	6 (13)
Neither	27	9 (33)	18 (67)	2 (7)	5 (19)	2 (7)
Unknown exposure	41	15 (37)	26 (63)	-	10 (24)	5 (12)
TOTAL	155	56 (36)	99 (64)	15 (10)	25 (16)	16 (10)

Percentages are in parentheses

4.1.2 SEROCONVERSION

Paired samples from 95 mine workers were tested to determine the rate of seroconversion within a period of six months. Seroconversion was demonstrated in 17 (18%) of the workers. The age of the workers did not influence the rate of seroconversion significantly (Table 4.6).

Paired samples were obtained from 21 smokers and 57 non-smokers. Seroconversion was demonstrated in only one (5%) of the smokers compared to the 11 (19%) of non-smokers (Table 4.7). The difference was not statistically significant.

The influence of previous underground (mining) experience on seroconversion is presented in Table 4.8. Underground experience did not influence the rate of seroconversion significantly.

Paired samples from 25 workers with, and 62 without respiratory symptoms were tested for seroconversion. The workers with symptoms consisted of 14 (56%) with pneumonia, nine (36%) with tuberculosis and two (8%) with upper respiratory symptoms. Seroconversion could only be demonstrated in 14% of the workers with pneumonia and in none of the other symptoms (Table 4.9). The difference was not statistically significant.

Paired samples from 79 workers with known past exposure to high levels of dust and/or humidity in the work environment were tested to determine the rate of seroconversion. Of these, the majority of seroconversions were demonstrated in the workers without past exposure to either of the environments (25%). The results are presented in Table 4.10 and indicated no statistically significant influence.

TABLE 4.6

SEROCONVERSION TO *L. PNEUMOPHILA* IN MINE WORKERS
ASSOCIATED WITH AGE

	n	SEROCONVERSION
< 31 years	6	1 (17)
31-40 years	37	5 (14)
41-50 years	37	9 (24)
> 50 years	15	2 (13)
TOTAL	95	17 (18)

Percentages are in parentheses

TABLE 4.7

SEROCONVERSION TO *L. PNEUMOPHILA* IN MINE WORKERS
ASSOCIATED WITH SMOKING

	n	SEROCONVERSION
Smokers	21	1 (5)
Non-smokers	57	11 (19)
Unknown	17	5 (29)
TOTAL	95	17 (18)

Percentages are in parentheses

TABLE 4.8

SEROCONVERSION TO *L. PNEUMOPHILA* IN MINE WORKERS ASSOCIATED WITH UNDERGROUND EXPERIENCE

	n	SEROCONVERSION
1- 5 years	10	-
6-10 years	29	4 (14)
11-15 years	27	7 (26)
> 15 years	12	-
Unknown	17	6 (35)
TOTAL	95	17 (18)

Percentages are in parentheses

TABLE 4.9

SEROCONVERSION TO *L. PNEUMOPHILA* IN MINE WORKERS ASSOCIATED WITH RESPIRATORY SYMPTOMS

	n	SEROCONVERSION
WITH SYMPTOMS	25	2 (8)
Pneumonia	14	2 (14)
Tuberculosis	9	-
Upper respiratory	2	-
WITHOUT SYMPTOMS	62	12 (19)
UNKNOWN	8	3 (38)
TOTAL	95	17 (18)

Percentages are in parentheses

TABLE 4.10

SEROCONVERSION TO *L. PNEUMOPHILA* IN MINE WORKERS ASSOCIATED WITH HIGH LEVELS OF DUST AND HUMIDITY IN THE ENVIRONMENT

	n	SEROCONVERSION
Humidity only	15	2 (13)
Dust only	11	-
Dust and humidity	33	6 (18)
Neither	20	5 (25)
Unknown exposure	16	4 (25)
TOTAL	95	17 (18)

Percentages are in parentheses

4.2 PNEUMONIA PATIENTS

The influence of age, gender and race on baseline titres and seroconversion rate in the pneumonia patients was studied.

4.2.1 BASELINE TITRES

A total of 81 patients were studied to determine their baseline titres to *L. pneumophila*. Of these, 16% had positive baselines, 14% had titres <1:64 and only 2% had titers ≥1:64 (Figure 4.1, page 101).

The ages of 72 patients were known and ranged from 12-77 years (mean and standard deviation=42.92 ± 16.72). The age groups studied were <31 years, 31-50 and >50 years and the results are presented in Table 4.11. Age did not influence the prevalence of antibodies significantly.

A total of 18 black, 55 coloured and five white patients were tested to determine their baseline titres to *L. pneumophila*. Although the prevalence was highest in the black patients, the difference was not statistically significant (Table 4.12). The race of the patients also did not influence the grouping of the positive titres.

A total of 44 male and 34 female patients were studied for prevalence of antibodies to *L. pneumophila*. No significant differences were noted (Table 4.13).

TABLE 4.11

BASELINE TITERS TO *L. PNEUMOPHILA* IN PNEUMONIA PATIENTS ASSOCIATED WITH AGE

	n	BASELINE TITERS		POSITIVE TITER LEVEL	
		POSITIVE	NEGATIVE	< 1:64	≥ 1:64
< 31 years	21	2 (10)	19 (90)	2 (10)	-
31-50 years	25	6 (24)	19 (76)	5 (20)	1 (4)
> 50 years	26	3 (12)	23 (88)	2 (8)	1 (4)
Unknown	9	2 (22)	7 (78)	2 (22)	-
TOTAL	81	13 (16)	68 (84)	11 (14)	2 (2)

Percentages are in parentheses

TABLE 4.12

BASELINE TITERS TO *L. PNEUMOPHILA* IN PNEUMONIA PATIENTS ASSOCIATED WITH RACE

	n	BASELINE TITERS		POSITIVE TITER LEVEL	
		POSITIVE	NEGATIVE	< 1:64	≥ 1:64
Black	18	4 (22)	14 (78)	3 (17)	1 (6)
Coloured	55	8 (15)	47 (86)	7 (13)	1 (2)
White	5	-	5 (100)	-	-
Unknown	3	1 (33)	2 (67)	1 (33)	-
TOTAL	81	13 (16)	68 (84)	11 (14)	2 (2)

Percentages are in parentheses

TABLE 4.13

BASELINE TITERS TO *L. PNEUMOPHILA* IN PNEUMONIA PATIENTS ASSOCIATED WITH GENDER

	n	BASELINE TITERS		POSITIVE TITER LEVEL	
		POSITIVE	NEGATIVE	< 1:64	≥ 1:64
Male	44	4 (9)	40 (91)	3 (7)	1 (2)
Female	34	8 (24)	26 (77)	7 (21)	1 (3)
Unknown	3	1 (33)	2 (67)	1 (33)	-
TOTAL	81	13 (16)	68 (84)	11 (14)	2 (2)

Percentages are in parentheses

4.2.2 SEROCONVERSION

Paired samples from 79 pneumonia patients were tested to determine the rate of seroconversion to *L. pneumophila* during the convalescent stage of illness. Seroconversion was demonstrated in 11 (14%) of the patients.

The patients were grouped into those <31 years, 31-50 years and >50 years old to determine the rate of seroconversion. The differences were not statistically significant. The results are presented in Table 4.14.

Paired samples were obtained from 17 black, 55 coloured and 5 white patients to determine the rate of seroconversion. The results (Table 4.15) indicated no race-related difference in the rate of seroconversion in the pneumonia patients.

Paired samples from 43 male and 34 female patients were tested for seroconversion. Although seroconversion occurred more frequently in the males (19%) than in the females (9%), the results presented in Table 4.16 were not significantly different from each other.

TABLE 4.14

SEROCONVERSION TO *L. PNEUMOPHILA* IN PNEUMONIA PATIENTS ASSOCIATED WITH AGE

	n	SEROCONVERSION
< 31 years	21	5 (24)
31-50 years	25	3 (12)
> 50 years	25	2 (8)
Unknown	8	1 (13)
TOTAL	79	11 (14)

Percentages are in parentheses

TABLE 4.15

SEROCONVERSION TO *L. PNEUMOPHILA* IN PNEUMONIA PATIENTS ASSOCIATED WITH RACE

	n	SEROCONVERSION
Black	17	2 (12)
Coloured	55	8 (15)
White	5	1 (20
Unknown	2	-
TOTAL	79	11 (14)

Percentages are in parentheses

TABLE 4.16

SEROCONVERSION TO *L. PNEUMOPHILA* IN PNEUMONIA PATIENTS ASSOCIATED WITH GENDER

	n	SEROCONVERSION
Male	43	8 (19)
Female	34	3 (9)
Unknown	2	-
TOTAL	79	11 (14)

Percentages are in parentheses

4.3 FACTORY WORKERS

The influence of several risk factors on the baseline titres of factory workers were studied and included age, smoking, respiratory symptoms and present exposure to dust in the work environment.

4.3.1 BASELINE TITRES

A total of 143 factory workers were tested to determine their baseline titres to *L. pneumophila*. Of these, 10% had positive baselines: 6% with titres <1:64 and 4% with titres ≥1:64.

The workers were grouped into categories <31 years, 31-40, 41-50 and >50 years of age. The influence of age on the baseline titres of the factory workers is presented in Table 4.17 and indicated no age-related difference in prevalence of antibodies or positive titre groups.

Sera from 100 smokers and 42 non-smokers were tested and the results are presented in Table 4.18. Antibodies were slightly more prevalent in smokers (10%) than in non-smokers (7%), but the difference was not statistically significant. Titres ≥1:64 were observed in 4% of smokers and 2% of non-smokers.

TABLE 4.17

BASELINE TITRES TO *L. PNEUMOPHILA* IN FACTORY WORKERS

ASSOCIATED WITH AGE

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
< 31 years	34	3 (9)	31 (91)	2 (6)	1 (3)	-
31-40 years	70	7 (10)	63 (90)	3 (4)	3 (4)	1 (1)
41-50 years	29	3 (10)	26 (90)	2 (7)	1 (3)	-
> 50 years	10	1 (10)	9 (90)	1 (10)	-	-
TOTAL	143	14 (10)	129 (90)	8 (6)	5 (4)	1 (1)

Percentages are in parentheses

TABLE 4.18

BASELINE TITRES TO *L. PNEUMOPHILA* IN FACTORY WORKERS

ASSOCIATED WITH SMOKING

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
Smokers	100	10 (10)	90 (90)	6 (6)	3 (3)	1 (1)
Non-smokers	42	3 (7)	39 (93)	2 (5)	1 (2)	-
Unknown	1	1 (100)	-	-	1 (100)	-
TOTAL	143	14 (9)	129 (90)	8 (6)	5 (4)	1 (1)

Percentages are in parentheses

Information regarding the respiratory symptoms of the factory workers was obtained from questionnaires. Sera from 80 workers with and 62 workers without symptoms were tested to determine their baseline titres. The symptoms included were pneumonia (n=1), tuberculosis (n=2) and upper respiratory symptoms (n=71). Five of the workers with upper respiratory symptoms also had pneumonia symptoms and one had tuberculosis (Table 4.19). Respiratory symptoms were not significantly associated with baseline titres to *L. pneumophila*.

A total of 76 workers were exposed to high levels of dust in the work environment, while 67 were not exposed. The influence of this dust exposure on the baseline titres of factory workers is presented in Table 4.20. There were no statistically significant differences between the groups.

TABLE 4.19

BASELINE TITRES TO *L. PNEUMOPHILA* IN FACTORY WORKERS ASSOCIATED WITH RESPIRATORY SYMPTOMS

	n	BASELINE TITRES		POSITIVE TITRES	
		POSITIVE	NEGATIVE	<1:64	≥1:64
WITH SYMPTOMS	80	9 (11)	71 (89)	5 (6)	4 (5)
PN only	1	-	1 (100)	-	-
UR only	71	7 (10)	64 (90)	5 (7)	2 (2)
TB only	2	-	2 (100)	-	-
Other	6	2 (33)	4 (67)	-	2 (33)
WITHOUT SYMPTOMS	62	4 (7)	58 (94)	2 (3)	2 (3)
UNKNOWN SYMPTOMS	1	1 (100)	-	1 (100)	
TOTAL	143	14 (10)	129 (90)	8 (6)	6 (4)

Percentages are in parentheses
PN : Pneumonia
TB : Tuberculosis
UR : Upper respiratory

TABLE 4.20

INFLUENCE OF DUST EXPOSURE ON BASELINE TITRES TO *L. PNEUMOPHILA* IN FACTORY WORKERS

	n	BASELINE TITRES		POSITIVE TITRE LEVELS		
		POSITIVE	NEGATIVE	<1:64	1:64	>1:64
With exposure	76	6 (8)	70 (92)	5 (7)	1 (1)	-
Without exposure	67	8 (12)	59 (88)	3 (5)	4 (6)	1 (2)
TOTAL	143	14 (10)	129 (90)	8 (5)	5 (4)	1 (1)

Percentages are in parentheses

4.4 COMPARISON OF STUDY POPULATIONS

The comparison of baseline titres in the three study populations is presented in Table 4.21 and indicates a statistically significant difference in the prevalence of antibodies to *L. pneumophila* with positive baseline titres in 36% of mine workers, versus the 16% of pneumonia patients and 10% of factory workers ($P<0.001$). The positive titre levels in the mine workers were also significantly higher than in the other populations with titres ≥1:64 observed in 26% of mine workers compared to the 3% in factory workers and 2% in pneumonia patients (Figure 4.1). The rate of seroconversion was similar in the mine workers and pneumonia patients. It should however be kept in mind that the period between first and second samples were different in the two populations. A logistic regression analysis was performed on the data from the mine workers and the factory workers. There was no significant association between any of the risk factors or symptoms studied and baseline titres in the mine workers.

TABLE 4.21

COMPARISON OF THREE STUDY POPULATIONS

	STUDY POPULATION			X^2	D	P-VALUE
	M	F	P			
n	155	143	81			
Negative:	99 (64)	129 (90)	68 (84)			
Positive:	56 (36)	14 (10)	13 (16)			
Titre <1:64	15 (10)	8 (6)	11 (14)			
Titre ≥1:64	41 (26)	5 (3)	2 (2)			
<1:64 vs. ≥1:64	15 /41	8 / 5	11 / 2	32.23	2	<0.001 ←
Pos vs. Neg	56 /99	14 /129	13 /84	17.10	2	0.0002←
Paired samples	95	-	79			
Seroconversion	17 (18)	-	11 (14)	0.25	1	0.6153

Percentages are in parentheses
M : Mine workers
F : Factory workers
P : Pneumonia patients
X^2 : Chi-squared
D : Degrees of freedom
← : Statistically significant

CHAPTER 5

PRESENCE OF LEGIONELLAE IN WATER SAMPLES

A total of 127 water samples, comprising of 63 surface and 63 underground samples, were tested for the presence of *L. pneumophila* serogroups 1-6 and *L. micdadei* by culture and direct immunofluorescence.

5.1 SURFACE WATER

The surface samples consisted of 49 samples collected at the showers in the ablution blocks at Cinderella hostel: 7 were collected in the showers on the north, 8 on the east, 18 on the south and 16 on the west side of the hostel. Five samples were collected in showers at the mine, consisting of 2 in the showers at the Far East Vertical (FEV) shaft, 2 at the South East Vertical (SEV) shaft and one in a shower designated 'surface change-house'. The areas sampled are illustrated schematically in Figure 5.1. Nine additional samples were collected on the surface: 8 from potable water and one from service water. Except for one sample collected at the Hercules shaft dam, all the surface samples were from clear, running, potable water. Temperatures of the potable water samples (n=8) ranged from 17°C to 40°C (mean and standard deviation=36.29 ± 6.02) and from showers (n=54) ranged from 18°C to 44°C (mean and standard deviation=36.24 ± 5.98). Results indicating scanty, one plus, two plus or three plus organisms resembling Legionella on direct immunofluorescence (DFA) were regarded as positive. No legionellae were cultured from any of the surface samples, therefore the organisms observed in the DFA were reported as *organisms resembling Legionella*.

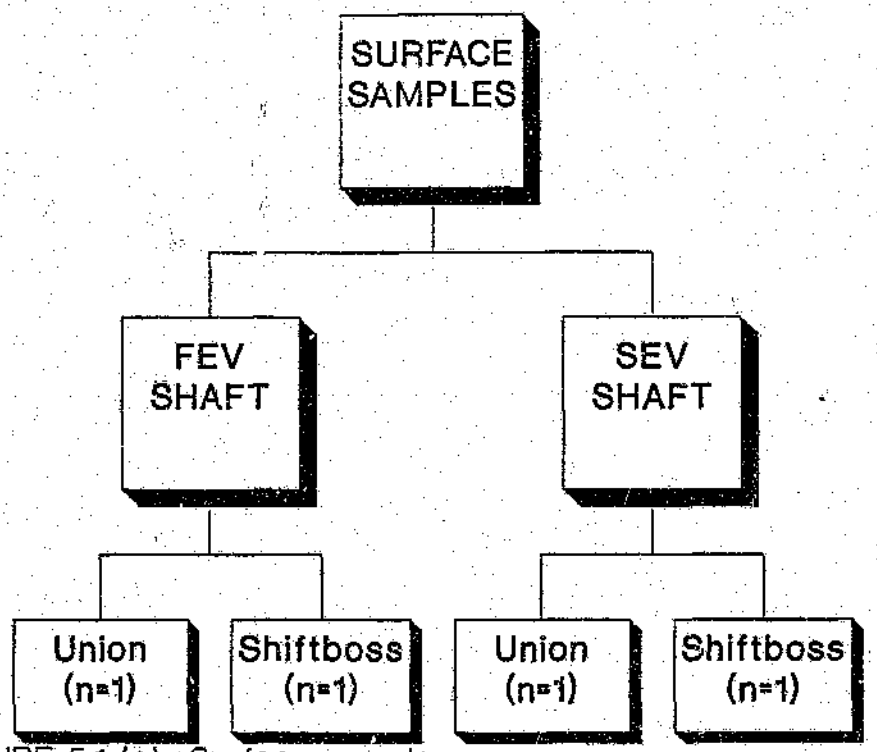


FIGURE 5.1 (a) Surface samples

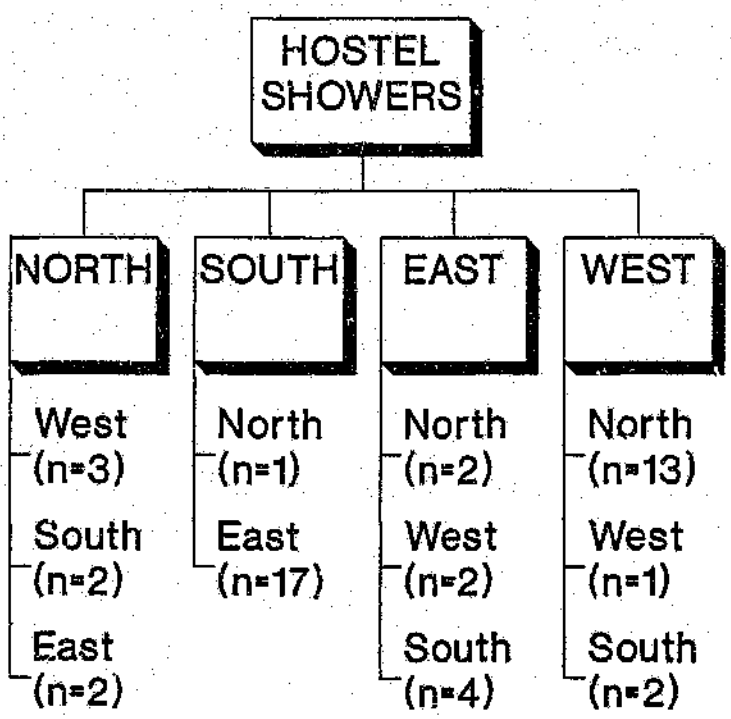


FIGURE 5.1 (b) Hostel showers

5.1.1 SHOWERS

The layout of the showers is illustrated schematically in Figure 5.1. There are ablution blocks situated on the north, south, east and west sides of the Cinderella hostel and samples were collected at each of these ablution blocks, from taps as illustrated. The change houses at the SEV and FEV shafts were also sampled as well as the shiftboss- and union change houses at both shafts. The results are presented in Tables 5.1-5.5.

5.1.1.1 CINDERELLA HOSTEL

Seven samples were tested from the showers on the north side of the hostel. In four of these (57%) scanty to one plus organisms resembling legionellae were observed by direct immunofluorescence. The positives were from shower 3 on the west side, shower 7 on the south side and shower 15 on the east side (Table 5.1).

Of the eighteen samples collected in the ablution blocks on the south side of the hostel, eight (44%) were positive on the DFA, the degree of positivity ranging from one- to three plus. The positives were from shower 15 on the north, and showers 1, 3, 10, 11, 12 and 14 on the south side (Table 5.2).

A total of eight samples from showers on the east side of the hostel were tested. Four of these (50%) were positive with the degree of positivity ranging from one to three plus. The positive samples were collected from showers 5 and 8 on the south side, shower 8 on north side and shower 6 on the west side (Table 5.3).

Of the sixteen samples collected in the showers on the west side of the hostel, only two (13%) were weak positive: one from shower 9 on the north side and one from shower 2 on the south side. Results are presented in Table 5.4.

TABLE 5.1

SHOWERS ON THE NORTH SIDE

	n	DFA	CULTURE
WEST SIDE:	3		
Shower 3 tap 1		-	-
Shower 3 tap 2		-	-
Shower 3 tap 3		sc	-
SOUTH SIDE:	2		
Shower 7 tap 1		+	-
Shower 7 tap 3		sc	-
EAST SIDE:	2		
Shower 15 tap 1		-	-
Shower 15 tap 3		sc	-
TOTAL	7		

sc : scanty

TABLE 5.2

SHOWERS ON SOUTH SIDE OF HOSTEL

	n	DFA	CULTURE
NORTH SIDE:	1		
Shower 15 tap 1		+	-
EAST SIDE:	17		
Shower 1 tap 1		++	-
Shower 2 tap 1		-	-
Shower 3 tap 1		-	-
Shower 3 tap 2		+++	-
Shower 3 tap 3		+++	-
Shower 3 tap 4		-	-
Shower 4 tap 1		-	-
Shower 5 tap 1		-	-
Shower 6 tap 1		-	-
Shower 7 tap 1		-	-
Shower 8 tap 1		-	-
Shower 9 tap 1		-	-
Shower 10 tap 1		+	-
Shower 11 tap 1		+	-
Shower 12 tap 1		+	-
Shower 13 tap 1		-	-
Shower 14 tap 1		+	-
TOTAL	18		

sc : scanty

TABLE 5.3

SHOWERS ON THE EAST SIDE

	n	DFA	CULTURE
SOUTH SIDE:	4		
Shower 5 tap 1		-	-
Shower 5 tap 2		++	-
Shower 5 tap 3		-	-
Shower 8 tap 1		++	-
NORTH SIDE:	2		
Shower 5 tap 1		-	-
Shower 8 tap 1		+++	-
WEST SIDE:	2		
Shower 6 tap 1		-	-
Shower 6 tap 3		+	-
TOTAL	8		

TABLE 5.4

SHOWERS ON THE WEST SIDE

	n	DFA	CULTURE
NORTH SIDE:	13		
Shower 1 tap 1		-	-
Shower 2 tap 1		-	-
Shower 5 tap 1		-	-
Shower 6 tap 1		-	-
Shower 7 tap 1		-	-
Shower 8 tap 1		-	-
Shower 9 tap 1		++	-
Shower 10 tap 1		-	-
Shower 11 tap 1		-	-
Shower 12 tap 1		-	-
Shower 13 tap 1		-	-
Shower 14 tap 1		-	-
Shower 15 tap 1		-	+
WEST SIDE:	1	-	-
SOUTH SIDE:	2		
Shower 2 tap 1		+	-
Shower 2 tap 2		-	-
TOTAL	16		

5.1.1.2 SHIFTBOSS CHANGE HOUSE

One sample was collected from each of the FEV and SEV shaft change houses. These were both negative on the DFA and culture (Table 5.5).

5.1.1.3 SURFACE CHANGE HOUSE

One sample was tested and was negative on both DFA and culture (Table 5.5).

5.1.1.4 UNION CHANGE HOUSE

Two samples were tested, one from the change house at the FEV, and one at the SEV shaft. The sample from shower 5 north from east to west gave a strong positive result on the DFA, while the other was negative. These samples were not marked clearly, therefore it was not certain from which shaft each of them was collected. No legionellae were cultured.

5.1.2 SERVICE WATER

The one sample of service water collected was negative on both DFA and culture (Table 5.6).

5.1.3 DRINKING WATER TAPS

Samples were collected at the acclimatization centre, the reduction works office and sandplant, drinking crushers, the surface taps at Hercules and FEV shafts and from two taps at wash-basins in the surface change house. Of the eight samples tested, scanty organisms were observed only in the sample from the surface tap at FEV shaft (13%). The results are presented in Table 5.6.

TABLE 5.5

CHANGE HOUSE SHOWERS

	n	DFA	CULTURE
UNION:	2		
Shower 2 south		-	-
Shower 5 north		+++	-
SHIFTBOSS:	2		
Shower 4 north		-	-
Shower 3 north		-	-
SURFACE CHANGE HOUSE:	1	-	-
TOTAL:	5		

TABLE 5.6

SURFACE DRINKING WATER AND SERVICE WATER

	n	DFA	CULTURE
DRINK WATER TAPS:	8		
Acclimitization		-	-
Reduction works			
Office		-	-
Sandplant		-	-
Drinking crushers		-	-
H-shaft tap		-	-
FEV-shaft tap		sc	-
Change house tap		-	-
H-shaft change house tap		-	-
SERVICE WATER:	1	-	-
TOTAL	9		

sc : scanty

5.2 UNDERGROUND WATER

A total of sixty-three samples, consisting of forty-one potable and twenty-two machine water samples were tested. The samples were collected from three main underground areas: the Hercules (H), the South East Vertical (SEV) and the Far East Vertical (FEV) shaft. Positions of the underground areas are indicated on Figures 5.2 and 5.3. The FEV shaft includes the O, and the SEV shaft includes the K and L shafts. Legionellae were not cultured from any of the samples, but organisms resembling legionellae were observed in the DFA. The drink water temperature ranged between 5°C and 31°C (mean and standard deviation=20.28 ± 7.15) and the machine water between 20°C to 34°C (mean and standard deviation=27.99 ± 4.59).

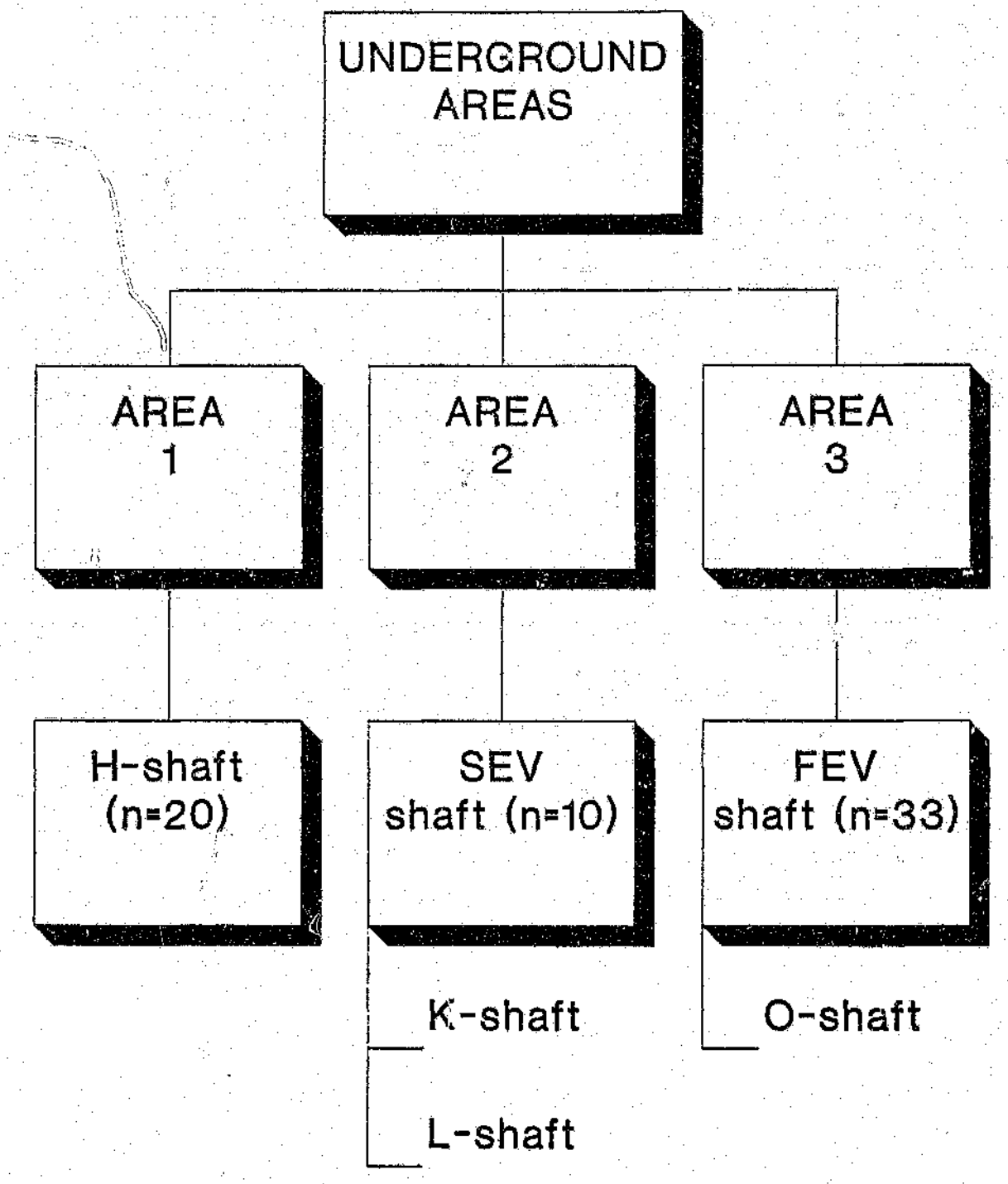


FIGURE 5.2 Underground samples

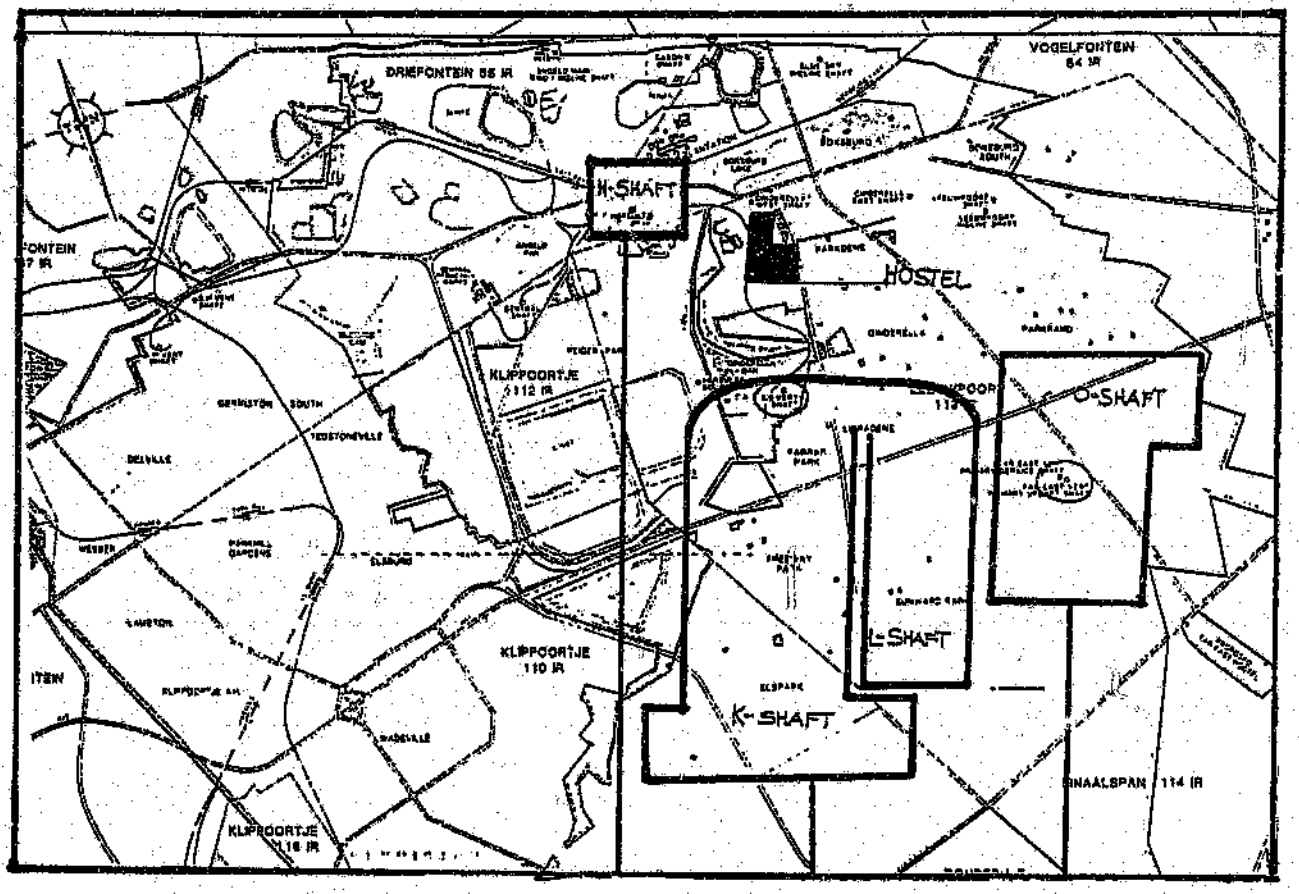


Figure 5.3 AREAS SAMPLED

5.2.1 AREA 1 (H SHAFT)

A total of nine potable water and eleven machine water samples were tested and were all negative on DFA and culture. The results are presented in Table 5.7.

5.2.2 AREA 2 (SEV SHAFT)

Eight drink water and two machine water samples were tested. Of the drink water samples, four were collected in the K, two in the L and two in the SEV shaft. Scanty organisms resembling Legionella were observed in one of the eight drink water samples (13%) and in one of the two drink water samples (50%). The two positive samples were both from K shaft. Results are presented in Table 5.8.

5.2.3 AREA 3 (FEV SHAFT)

A total of 24 potable water samples, consisting of 3 from E, 11 from FE, 4 from FEV and 6 from O shaft were tested. Eight of these were positive on the DFA. Two (67%) of the positive samples were from E, 3 (27%) were from FE and 3 (50%) from O shaft. All nine machine water samples tested were negative on both DFA and culture. Results are presented in Table 5.9.

TABLE 5.7

RESULTS AREA 1

	n	DFA	CULTURE
H SHAFT DRINK WATER:	9		
West refuge bay tap		-	-
23 level station (a)		-	-
23 level station (b)		-	-
44 level tertiary hoist drive		-	-
50 level tertiary station		-	-
58 level tertiary pump chamber		-	-
25 level station		-	-
30 level secondary station		-	-
27 level station		-	-
H SHAFT MACHINE WATER:	11		
Fissure water 21 west drive		-	-
27 east at tip		-	-
23 west stope		-	-
27 west x-cut		-	-
21 west		-	-
58 tertiary pump chamber dam		-	-
25 west		-	-
27 west angelo drain		-	-
27 west machine 4		-	-
27 west old dam x-cut		-	-
29 west drive		-	-
TOTAL	20		

TABLE 5.8

RESULTS AREA 2

	n	DFA	CULTURE
K SHAFT DRINK WATER:	4		
58 west bank		-	-
68 west bank		sc	-
75 level cooling plant		-	-
77 level		-	-
K SHAFT MACHINE WATER:	1		
77 level service water		sc	-
L SHAFT POTABLE WATER:	2		
58 level		-	-
74 level		-	-
L SHAFT MACHINE WATER:	1		
58 level bulk sprays dam		-	-
SEV POTABLE WATER:	2		
Change house		-	-
42 level dam		-	-
TOTAL	10		

sc : scanty

TABLE 5.9

RESULTS AREA 3

	n	DFA	CULTURE
7 SHAFT DRINK WATER:	6		
58 bank		-	-
East stope		-	-
East foot wall drive		sc	-
East main tip		sc	-
Shaft bank		-	-
Shaft pillar removal		sc	-
E SHAFT DRINK WATER:	3		
X-cut north		+	-
Heat exchanger		sc	-
64 level heat exchanger		-	-
FE SHAFT DRINK WATER:	11		
58 level ice dam (a)		-	-
58 level ice dam (b)		-	-
68 level		-	-
64 station dam		sc	-
64 west drive		-	-
58 station ice dam		-	-
58 station		sc	-
62 west drive		-	-
64 wast reef drive		sc	-
66 level station dam		-	-
62 level dam		-	-
FE STATION MACHINE WATER:	3		
68 level drain west		-	-
42 level hoist coolers		-	-
64 level station		-	-
FEV SHAFT DRINK WATER:	4		
64 level station dam		-	-
68 level station tap		-	-
41,5 level main dam		-	-
58,5 level ice dam		-	-
FEV SHAFT MACHINE WATER:	6		
66 level drain hole		-	-
68 level discharge pipe		-	-
62 level station drain		-	-
58 level station drain		-	-
42 level drain at air/c		-	-
68 level drain		-	-
TOTAL	33		

sc : scanty

5.3 COMPARISON OF RESULTS

A comparison of results from all the areas tested, both on the surface and underground, is listed in Table 5.10.

5.3.1 COMPARISON OF SURFACE SAMPLES

Scanty organisms resembling legionellae were observed only in the sample collected from the drinking water tap on the surface at SEV shaft, i.e. 13% of surface drink water samples. The number of DFA positive samples was significantly higher in the showers on the north side of the ablution blocks ($P<0.05$) than in the other samples collected at the hostel.
A comparison of the results from the drinking water taps with the showers on the surface indicated no statistically significant difference.

5.3.2 COMPARISON OF UNDERGROUND SAMPLES

A comparison of results from the three areas sampled underground indicated a significantly higher number of DFA positive results in Area 3 than in Area 1 ($P<0.05$). The difference between Area 1 and Area 2, and between Area 2 and Area 3 were not statistically significant.

5.3.3 COMPARISON OF SURFACE WITH UNDERGROUND SAMPLES

The number of DFA positive samples were significantly higher in the surface than in the underground samples ($P<0.05$). The mean temperature of the surface samples was however significantly higher than that of the underground samples ($P<0.001$), which may have influenced the results.

TABLE 5.10

COMPARISON OF RESULTS: SURFACE AND UNDERGROUND

COMPARISON	P-VALUE
SURFACE SAMPLES:	
Showers south to west	0.0630
Showers south to north	0.6728
Showers south to east	1.0000
Showers west to east	0.1288
Showers west to north	0.0450 ←
Showers east to north	1.0000
Drink water to Hostal showers	0.2468
Drink water to Union showers	0.3778
Drink water to Shiftboss showers	1.0000
UNDERGROUND SAMPLES:	
Area 1 to Area 2	0.1034
Area 1 to Area 3	0.0187 ←
Area 2 to Area 3	1.0000
SURFACE TO UNDERGROUND SAMPLES:	0.0332 ←

← : statistically significant

CHAPTER 6

DISCUSSION

6.1 ANTIBODIES TO CHLAMYDIA PNEUMONIAE

Sero-epidemiologic studies have shown that *C. pneumoniae* has a high prevalence in adults worldwide. Antibodies have been demonstrated in 25% of adults in some parts, while as many as 60% of adults have antibodies to the organism in other areas of the world. The prevalence is higher in tropical countries (eg. Panama and Taiwan) than in the northern countries (eg. Canada and Scandinavia) and has not been studied in South Africa to date (Grayston et al 1986, Grayston 1989, Marrie 1993, Torres et al 1993).

Antibodies to *C. pneumoniae* were present in 66% of the mine workers, 50% of the pneumonia patients and 22% of factory workers. The prevalence of antibodies was significantly different in the three populations studied ($P<0.001$), and ranged from the lowest to the highest percentage reported from other parts of the world. The reason for this variation in prevalence is unclear. The majority of mine workers were from Mocambique, a sub-tropical area, with previous experience of mine work and were all living in hostels. This may explain the high prevalence compared to the factory workers who were all from a rural area in Natal, did not all live in hostels and had no previous experience of mine work. The possibility of an increased risk of infection in factory workers living in hostels (n=80) as opposed to those living with their families in the township (n=65) however indicated no increased prevalence in the workers living in hostels. It is therefore possible that some other aspect of the mining environment is a risk factor for exposure to *C. pneumoniae*.

Although most authors consider IgG titres between 1:16 and 1:256 as either pre-existing or chronic antibody, and titres ≥1:512 as presumptive evidence of a current or recent *C.*

pneumoniae infection, the classification of Thom et al (1992), who grouped positive titres into low (≤1:8), medium (>1:8, <1:64) and high (≥1:64), was used as a guideline in the present study, and titres ≥1:256 were considered as indicative of a current or recent *C. pneumoniae* infection. Two of the mine workers had titres of 1:256 and one of the factory workers had a titre of 1:2048, indicating acute antibody at the time of the study. This worker was 36 years old, lived in the hostel at the factory, worked in a dusty environment, and was a past smoker. He did not complain of respiratory symptoms other than wheezing and chest tightness. The titre gradients for the three populations were similar, with the majority of positive titres at the lowest level (<1:64), indicating previous exposure to *C. pneumoniae*. The prevalence of titres ≥1:64 was however significantly higher in the mine workers (25%) when compared to the pneumonia patients (10%) and the factory workers (3%) ($P<0.05$), indicating a possible increased risk of infection in the mines.

A fourfold or greater increase in IgG to ≥1:16 between paired sera is usually considered as seroconversion, indicating a current *C. pneumoniae* infection. Some authors however also consider a fourfold or greater decrease in antibody titre as seroconversion (Puolakkainen et al 1993, Saikku et al 1985). In the present study only a fourfold or greater increase was considered as seroconversion; any decrease in titre was ignored. The incubation period of *C. pneumoniae* infection is long, and convalescent samples taken before three weeks after onset of symptoms may fail to show antibodies. An increase in antibodies (IgG) often reach diagnostic levels only after 3-6 months (Grayston et al 1989). The timing of collection of the samples is therefore very important in the interpretation of results in paired sera. The period between collection of acute and convalescent stage sera for pneumonia patients was between one and four weeks, and the period between the first and follow-up samples of the mine workers, six months. The rate of seroconversion in the pneumonia patients may therefore be higher than that indicated by the results of this study, which indicated seroconversion in 22% of pneumonia patients. If a fourfold decrease in titre was taken into account (and considered as seroconversion), these figures would have increased to 32% in the pneumonia patients: a much higher rate of seroconversion than the 6-12% normally reported in community acquired pneumonia cases (Marrie 1993). The possible risk factors for infection, that may have influenced the prevalence of antibodies and the rate of seroconversion, will be discussed below. This is the first study in South Africa to document the importance of *C. pneumoniae* as a cause of community acquired pneumonia.

In most previous studies, the prevalence of antibodies to *C. pneumoniae* was very low in children, and increased sharply until it reached a peak in the age group between 30 and 40 years, with another peak in the 70's (Grayston et al 1990, Marrie 1993). The prevalence of antibodies in mine workers under the age of 31 years was very high compared to other studies with the majority of positive titres at the level ≥1:64. The reason for the high prevalence in this age group is unclear, but the small number of workers studied in this category may have influenced the results. Another possible explanation may be that the workers are infected with *C. pneumoniae* when they start working underground and that the titre levels are therefore high in comparison with those of the 31-50 age group, who have more experience of underground work. The prevalence in the workers over 30 years of age correlated well with other studies, and the slight decrease with age to 56% in the workers over the age of 50, was similar to that reported by Grayston et al (1989). Age did not appear to influence the rate of seroconversion in the mine workers. In the factory workers there was no significant difference in prevalence between any of the age groups, although the number of positive baseline titres as well as the positive titre level was highest in the workers over 50. A possible explanation is that the factory workers, a rural population, are not exposed to the organism at an early age.

Very little is known about the transmission of *C. pneumoniae* apart from the fact that it is transmitted from human to human with no bird or animal reservoir (Grayston et al 1990). Transmission in schools and other institutions, where people are in close contact, has been suggested (Grayston 1989). South African gold miners are continuously exposed to high levels of humidity and dust in the atmosphere, that may increase the risk of respiratory infections (Wyndham et al 1986). A possible increased risk of infection with previous underground experience in the mine, where people work in groups and in dusty and humid, warm atmospheres, and previous exposure to high levels of dust and/or humidity in the working environment, was therefore studied. Although it was not certain if the mine records reflected only previous underground experience at this particular mine, and how often these records are updated, the information was used as an indication of previous experience. We were unable however to associate the prevalence of antibodies with duration of underground work. The high rate of seroconversion in the workers with 1-5 years experience suggested that the workers may be infected when they first start working underground. None of the factory workers had previous underground experience, therefore the level of dust in their present working environment was studied as a possible risk factor

for infection. This association could not be proven.

It is generally accepted that smokers are at high risk of respiratory infections (Ager et al 1983, Berlin et al 1982). In the mine workers, the prevalence of antibodies to *C. pneumoniae* as well as the positive titre level were higher in smokers than in non-smokers, although the rate of seroconversion was higher in the non-smokers. In the factory workers, antibodies were more prevalent in the non-smokers, with no difference in positive titre gradient between the two categories. These differences were not statistically significant. The small number of non-smokers studied in the factory workers may however have influenced the results.

Pneumonia and upper respiratory symptoms are common manifestations of *C. pneumoniae* infections. The symptoms are mostly mild, and asymptomatic infections have been documented (Grayston et al 1990, Marrie et al 1987). The prevalence of antibodies as well as the positive titre gradients were similar in mine workers with and without respiratory symptoms, while both the prevalence and titre levels were higher in factory workers without symptoms, correlating with the results from Grayston et al (1990). The worker with a baseline titre of 1:2048 did not complain of any respiratory symptoms apart from tight chest and wheezing. This worker may however have suffered from mild flu-like symptoms, and not reported it. The high prevalence of antibodies in the mine workers with tuberculosis was surprising, although chronic respiratory infections (like tuberculosis) are generally considered as risk factors for pneumonia. The number of workers tested in each individual category (pneumonia, upper respiratory symptoms and tuberculosis) was however small. The three factory workers with tuberculosis all had negative baseline titres and could therefore not be used for comparison. A higher rate of seroconversion was demonstrated in the mine workers with symptoms than in those without, supporting the fact that *C. pneumoniae* causes respiratory infections, although the seroconversions that occurred in the workers without symptoms proofs that it also causes asymptomatic infections.

According to Maartens et al (1994), *M. pneumoniae* is usually the most common aetiological agent of atypical pneumonia, causing between 2% and 18% of cases in the United Kingdom, Spain and Australia. Other organisms causing atypical community acquired pneumonia include Legionella species (2-15%) and *C. psittaci* (<1-5%). Table 6.1 illustrates the aetiology of community-acquired pneumonia in 301 hospitalized patients in Washington

during the period between 1981-1884. Convalescent stage sera were collected 2-4 weeks after admission to hospital in this study. The rate of seroconversion in the pneumonia patients of the present study was much higher than that reported elsewhere. The race and gender of these patients did not appear to influence the prevalence of antibodies or the rate of seroconversion significantly. It should be kept in mind that an increase in IgG often reach diagnostic levels only after 2-6 months (Grayston et al 1989), and that the rate of seroconversion to *C. pneumoniae* may therefore be even higher in community acquired pneumonia cases.

TABLE 6.1

AETIOLOGICAL AGENT	PATIENTS (%)
Streptococcus pneumoniae	8.6
Haemophilus influenzae	6.3
Chlamydia pneumoniae	5.9
Influenza A virus	5
Staphylococcus aureus	4
Cytomegalovirus	3.9
Parainfluenza viruses	3.9
Legionella pneumophila	3.9
Mycoplasma pneumoniae	3.3
Streptococcal species other than S. pneumoniae	3.3
Aerobic gram negative rods	2.3
Mycobacterium tuberculosis	1.9
Coxiella burnetti	1.6
Influenza B virus	1.6
Respiratory syncytial virus	1.3
Branhamella catarrhalis	0.6
Chlamydia trachomatis	0.3

Marrie et al (1987)

6.2 ANTIBODIES TO LEGIONELLA PNEUMOPHILA

Legionella infections occur worldwide, in people of all ages and race groups (Boldur et al 1988, Kaplan et al 1980). The prevalence of antibodies to Legionellae is very low in some populations (eg. 1.5% in Nottingham, England) (Macrae et al 1983), while in other populations it may be as high as 26% (Winn 1984) [129]. The only other study conducted in mine workers to date reported a prevalence of 5% in coal mine workers (Davies et al 1985). This variation in prevalence may be explained by the different methods used for antigen preparation for indirect immunofluorescence. In Europe, formalin-killed antigens, that are highly specific, are routinely used, which may explain the low prevalence compared to other parts of the world where heat-killed antigens, that are not as specific but more sensitive, are used. According to Edelstein (1987), the specificity of heat-killed antigens is 96-99%, which is too low for diagnostic purposes, but is acceptable for epidemiological studies. In South Africa, formalin-killed antigens are normally used for diagnosis of sporadic cases of legionellosis, while heat-killed antigens are used for epidemiologic studies (Ratshikhopha et al 1990). Mauff et al (1984) demonstrated low titre antibodies in a large number of people from the Johannesburg area, and Ratshikhopha et al (1990) reported antibodies at titre level ≥1:16 to *L. pneumophila* serogroups 1-6 in 65% of healthy adult blood donors in Johannesburg, using heat-killed antigens. These results suggested widespread exposure to the organism in the PWV area, but the prevalence in other parts of the country has not been studied to date.

In the present study, antibodies to *L. pneumophila* serogroups 1-4 were demonstrated in 36% of mine workers using heat-killed antigens. This figure was significantly higher than in the acute sera of the pneumonia patients from Groote Schuur hospital in Cape Town (16%) and the factory workers (10%) from Mooiriver in Natal ($P<0.001$). The prevalence of antibodies in all three study populations was lower than that reported by Ratshikhopha et al in 1990, but correlated well with studies from other parts of the world, although the prevalence in the mine workers in this study was slightly higher. The difference between results from the present study and that reported by Ratshikhopha may have been due to the fact that they tested for antibodies to serogroups 1-6, while in the present study only the presence of antibodies to serogroups 1-4 was determined.

Cross-reactions occur frequently between *L. pneumophila* and other organsims such as *C. psittaci, B. fragilis, P. alcaligenes, M. tuberculosis, P. aeroginosa* and other Gram negative organisms the person may have been exposed to previously. Ratshikhopha et al (1990) demonstrated antibodies to *L. pneumophila* in 20-30% of tuberculosis patients, but suggested that these cross-reactions can be decreased by absorption with *M. tuberculosis.* Twelve of the mine workers were diagnosed as having tuberculosis, and the high background prevalence in these workers may therefore have been due to cross-reacting antigens. Although cross-reactions between *L. pneumophila* and *C. pneumoniae* have not been demonsti. ed to date, the possibility of cross-reactions should be kept in mind, as *C. pneumoniae* is antigenically similar to *C. psittaci,* that has been reported to cross-react with *L. pneumophila* in the indirect immunofluorescence test in some studies (Winn 1984). The high prevalence in the mine workers may therefore have been influenced by cross-reactions between the two organisms.

Legionellae are aquatic bacteria that are spread through the aerosolization of droplets (Muder et al 1986), although dust had also been implicated in transmission of the organism (Brundett et al 1989, Morris et al 1979). The higher prevulence of antibodies in the mine workers compared to the factory workers and pneumonia patients may therefore have been a result of their previous experience in mines, where they work in groups, in warm, dusty and humid atmospheres and live in communal hostels.

A single titre of ≥1;256 is generally accepted as evidence of a current or recent *L. pneumophila* infection. The classification by Shands and Fraser (1980), who regarded single titres ≥1:128 as indicative of a current or recent infection, was used as a guideline in the present study, and baseline titres were grouped into low (≤1:64), medium (1:64) and high (>1:64). The positive titre gradient of the factory workers and pneumonia patients correlated well with that reported by Ratshikhopha, with the majority at the level <1:64, while in the mine workers, the majority of positive titres were at the level 1:64. Positive titres were on the whole slightly higher in the mine workers than in the other populations, with significantly more positives above 1:64 than in the other populations ($P<0.001$). Our results correlated well with studies from other parts of the world, which reported the highest prevalence at the titre level ≤1:64 (Boldur et al 1986, Bornstein et al 1986).

A fourfold or greater increase in antibody titre to a level ≥1:128 is generally accepted as

evidence of seroconversion (Koornhof et al 1980, Shands et al 1980, Wilkinson 1982), although Winn (1984) considered a fourfold or greater increase to ≥1:64 as seroconversion. In the present study, any fourfold or greater increase in titre was considered as evidence of seroconversion, regardless of the titre level of either of the first or the second samples. A high seroconversion rate was reported in this study. The rate of seroconversion was similar in the mine workers over 6 months compared to the pneumonia patients with sera taken 1-4 weeks after the acute stage of disease. The fact that antibodies to *L. pneumophila* may take a long time to reach diagnostic levels should be taken into account, and the period between the acute and convalescent stage sera may have been to short to detect all the seroconversions in the pneumonia patients. Nagington et al (1979) and Harrison et al (1988) suggested that the second sample should be taken at least 28 days after onset of illness, while Blackmon et al (1981) recommended that the acute stage sample should be taken within seven days after onset of illness and the convalescent stage sample at least 22 days after onset, with an additional sample after 42 days. According to Edelstein (1987), up to 25% of culture-proven cases of legionellosis may be missed by the IFA, even if regular serum samples are tested over a 6-9 week period, and only 20-40% of Legionnaires' Disease patients will develop diagnostic levels of antibody within a week after onset of illness. It is therefore possible that the rate of seroconversion was higher than that reported in this study. The risk factors for Legionella infection, that may have influenced the baseline titres and rate of seroconversion, will be discussed below.

The prevalence of antibodies in the mine workers was already high in the age groups studied, and did not increase further with increasing age. Similarly, age did not appear to influence the prevalence of Legionella antibodies in the factory workers within the age groups studied. The positive titre gradients were similar in the pneumonia patients of all ages, while titres above 1:64 were more prevalent in the mine workers aged between 41 and 50 years. An interesting observation was that, while the rate of seroconversion increased with age in the mine workers, the opposite was true for the pneumonia patients. This was unexpected, because the risk of Legionella infections and pneumonia increases with age (MacFarlane 1989).

Smoking is a known risk factor for symptomatic Legionella infections (Ager et al 1983), and the amount a person smokes apparently influences the relative risk of infection (Broome et al 1979). Wyndham et al (1986) reported an increased risk of chronic respiratory disease in

mine workers who smoke. The prevalence of antibodies to *L. pneumophila* was slightly higher in smokers than non-smokers in both the mine- and factory workers, but our data suggest that smoking does not affect the background exposure to Legionella organisms.

South African gold miners work under stressful conditions of heat and humidity and are exposed to varying dust levels, containing numerous atmospheric pollutants (Wyndham et al 1986). The period of past mining experience (mainly underground) and previous exposure to high levels of dust and/or humidity in the working environment was therefore regarded as possible risk factors for increased prevalence and rate of seroconversion in the mine workers. As the mining workforce is now stable and the population tested has a long previous exposure to the mining environment, it was not possible to study the impact of exposure to Legionella organisms in new recruits. Nonetheless our data show a very high level of seroconversion (18%) in established miners over a 6 month period.

Infections caused by Legionella species include Legionnaires' Disease and Pontiac Fever, and subclinical infections have been documented (Girod et al 1982). The incidence of community acquired pneumonia due to Legionella infections may be as high as 32%. Edelstein (1983) suggested that 7% of community acquired and 14% of nosocomial pneumonias in the United Kingdom are caused by *L. pneumophila* and *L. micdadei*. Legionella infections account for about 4% of all pneumonias in South Africa (Koornhof 1979). The pneumonia patients in the present study had a seroconversion rate of 14% to *L. pneumophila* serogroups 1-4. The seroconversion rate in the same patients to *L. pneumophila* serogroup 1 alone was 9% (Maartens et al 1994). The figures may however have been higher if the period between collection of the acute and convalescent stage sera was longer, as discussed above. In the mine workers, the prevalence of antibodies and the positive titre gradients were similar in workers with respiratory symptoms (i.e. either tuberculosis, upper respiratory symptoms or pneumonia) and in those without symptoms. This correlated with data published by Winn (1984) and suggests widespread asymptomatic infections.

Legionella infections occur in all race groups and the race groups of people infected during outbreaks are usually reflective of the general population of the area (Boldur et al 1986, Kaplan et al 1980). Although the prevalence of antibodies was slightly higher in black pneumonia patients than in the coloured patients, the difference was not statistically

significant. The rate of seroconversion was also similar in pneumonia patients of all races.

6.3 ANTIBODIES TO BOTH C. PNEUMONIAE AND L. PNEUMOPHILA

Cross-reactions between *L. pneumophila* and *C. psittaci* have been demonstrated in the past (Wilkinson et al 1979), but it is not known if cross-reactions will also occur between *L. pneumophila* and *C. pneumoniae*. Six of the mine workers had baseline titres ≥1:64 to both organisms, and seroconversion to both organisms was demonstrated in one of the workers, who had a negative baseline, rising to 1:16 to *C. pneumoniae* and a baseline titre of 1:128, increasing to 1:1024 to *L. pneumophila*. None of the pneumonia patients had baseline titres ≥1:64 to both organisms, but seroconversion to both organisms was demonstrated in three of the patients. In all three cases, the baseline titres to both organisms were negative, increasing to 1:16, 1:32 and 1:64 respectively to *C. pneumoniae* and to 1:256 to *L. pneumophila*. This would suggest pneumonia caused by *L. pneumophila*, rather than *C. pneumoniae*. The possibility of cross-reactions between the two organisms should be kept in mind, although dual infections cannot be excluded in these patients.

6.4 THE PRESENCE OF LEGIONELLAE IN WATER SAMPLES

Legionellae are freshwater organisms that occur worldwide in natural as well as man-made sources like cooling towers, air-conditioning and water distribution systems and have been cultured from shower heads and nebulizers (Anrahan et al 1987, Brundett 1989, Muder et al 1986, Muraca et al 1988). The organisms are very prevalent in the South African environment (Tobiansky et al 1986) and are disseminated by aerosolization of droplets. Transmission through dust particles has also been suggested. The working consitions in South African gold mines and the living conditions in the communal hostels are therefore very favourable for the transmission of legionellae and subsequent infection of the workers.

The inability to culture legionellae from any of the water samples may be due to a 'nutrient-stress' period as described by Tobiansky et al (1986), at the time of collection of

the samples. The results from the surface samples indicated a higher prevalence of organisms in the showers at the ablution blocks on the south and east sides of the hostel than in any of the other surface samples. The presence of organisms resembling legionellae in the showers correlated with results from several studies worldwide (Cordes et al 1981). The fact that only scanty organisms were observed from the underground samples may be because these samples were taken from clear, chlorine treated water sources. Machine water samples should be investigated in the future. The positive results in the direct immunofluorescence test may have been caused by cross-reactions with organisms like *B. pertussis*, *P. alcaligenes* and *P. fluorescens* and our data cannot confirm the presence of legionellae in these sources.

CHAPTER 7

CONCLUSION

In general the prevalence of antibodies and seroconversion to both *C. pneumoniae* and *L. pneumophila* were higher than previously reported. Workers in the mine studied would appear to suffer widespread exposure to both organisms, with frequent symptomatic or asymptomatic infections. Seroconversion to *C. pneumoniae* was associated with respiratory tract infections, and occurred in a significant number of workers, despite probable previous exposure to similar working conditions. The high rate of seroconversion to *L. pneumophila* indicated this organism as a possible significant cause of pneumonia in mine workers.

The study of hospitalised pneumonia patients confirmed the importance of both *C. pneumoniae* and *L. pneumophila* as common causes of community acquired pneumonia. Treatment strategies should therefore consider the possibility of infections with these pathogens, especially if treatment with conventional beta lactam drugs fail.

Further studies indicated would be a prospective investigation of new recruits to the mine, with no previous experience. Periodic clinical examinations of the workers, including sampling in working areas, may identify risk factors associated with antibody production and seroconversion to the organisms. Additional populations are presently studied to determine the prevalence of antibodies to *C. pneumoniae* in South Africa. These populations include babies, teenagers, white miners and stroke patients and the results will be published shortly.

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