Pathogenesis, pathology and chemotherapy of experimental Legionella pneumophila infection

Arthur O. Tzianabos

University of New Hampshire, Durham

Follow this and additional works at: https://scholars.unh.edu/dissertation

Recommended Citation
https://scholars.unh.edu/dissertation/1601

This Dissertation is brought to you for free and open access by the Student Scholarship at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in Doctoral Dissertations by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact nicole.hentz@unh.edu.
Pathogenesis, pathology and chemotherapy of experimental Legionella pneumophila infection

Abstract
Legionella pneumophila is the causative agent of a severe, often fatal pneumonic illness known as Legionnaires’ disease. The mechanisms by which L. pneumophila attaches to U937 (transformed human-like fibroblasts) were investigated. Experimental parameters for adherence assays were established prior to blocking studies designed to identify microbial adhesins and/or eukaryotic receptors that mediate bacterial attachment to target cells. Results from these studies indicated that a lectin-like component(s) associated with L. pneumophila may be responsible, at least in part, for microbial adherence to these eukaryotic host cells. Erythromycin is the drug of choice for the treatment of clinical legionellosis; however, difficulties with this antibiotic have been reported resulting in the need to seek alternative therapeutic regimens. In these studies, the effect of clinically relevant antibiotics that inhibited bacterial cell wall, protein and DNA synthesis of this pathogen was evaluated in vitro by growth and viability studies as well as morphologically by negative stain, scanning and thin-section electron microscopy. Of those tested, cefotaxime, an antibiotic of limited value in clinical trials, was most effective. The pathogenicity of L. pneumophila was assessed by LD$_{50}$ and bacterial growth estimations in the chick embryo animal system in ovo. In addition, histopathological and electron microscopic examination of the cellular and sub-cellular pathology induced in the organs of embryos previously infected with 100 times the yolk sac (YS)LD$_{50}$ of L. pneumophila was made as a prelude to chemotherapeutic treatment with a range of clinically putative antimicrobial agents. The promising new quinolone derivative, ciprofloxacin, was most effective in these trials. Results from these studies may be indicative of novel preventative and control measures for the therapy of human Legionnaires’ disease.

Keywords
Biology, Microbiology, Health Sciences, Pathology

This dissertation is available at University of New Hampshire Scholars' Repository: https://scholars.unh.edu/dissertation/1601
INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Pathogenesis, pathology and chemotherapy of experimental
*Legionella pneumophila* infection

Tzianabos, Arthur O., Ph.D.

University of New Hampshire, 1989
PATHOGENESIS, PATHOLOGY AND CHEMOTHERAPY OF
EXPERIMENTAL LEGIONELLA PNEUMOPHILA INFECTION

BY

ARTHUR O. TZIANABOS
B.S. BOSTON COLLEGE, 1985

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
Microbiology

December, 1989
This dissertation has been examined and approved.

Dissertation director, Dr. Frank G. Rodgers
Associate Professor of Microbiology

Dr. William R. Chesbro, Professor of Microbiology

Dr. Donald M. Green, Professor of Biochemistry

Dr. Joseph J. Moore III, Chief Veterinary Pathologist
Animal and Nutritional Sciences

Dr. Thomas G. Pistole, Professor of Microbiology

28 November 1989
Date
TO MY PARENTS
Acknowledgments

I wish to thank the Research Office and Graduate School of the University of New Hampshire for their support in the form of research funds, teaching and dissertation fellowships during my graduate studies.

These years have brought some of the most exhilarating and difficult times I have yet known. During this endeavor, I have had the pleasure of working with some great people: I express my deepest gratitude to my advisor, Dr. Frank Rodgers, for his instruction, advice and constant encouragement throughout the course of this investigation. I shall value what I have learned from him. I also thank the members of my doctoral committee, Dr. William Chesbro, Dr. Donald Green, Dr. Joe Moore and Dr. Tom Pistole, for their guidance and helpful suggestions.

I am indebted to Bob Mooney for his expertise and friendship during our many discussions of matters scientific and not so scientific. Thanks go to Linda Dibernardo and Alberta Moulton for their help and patience, members of Dr. Rodgers' lab for their useful discussion, and to fellow graduate students, Robin Ross, Bob Millham and Tod Gavron for their corrupting influence. I also thank Kirsten Quist for her love and forbearance during the last year.

Finally, I thank my parents for all of their love and support. I am forever grateful.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td></td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td></td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td></td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td></td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF PLATES</td>
<td></td>
<td>xii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td></td>
<td>xv</td>
</tr>
<tr>
<td>FRONTISPICE</td>
<td></td>
<td>xvii</td>
</tr>
<tr>
<td>SECTION</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I.</td>
<td>NATURAL HISTORY of LEGIONNAIRES' DISEASE</td>
<td>1</td>
</tr>
<tr>
<td>1.1</td>
<td>Preface</td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>Historical perspective</td>
<td>1</td>
</tr>
<tr>
<td>1.3</td>
<td>The Organism</td>
<td>4</td>
</tr>
<tr>
<td>1.3.1</td>
<td>Taxonomy and nomenclature</td>
<td>4</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Morphological characteristics</td>
<td>7</td>
</tr>
<tr>
<td>1.3.3</td>
<td>Biochemical properties</td>
<td>10</td>
</tr>
<tr>
<td>1.3.4</td>
<td>Physico-chemical properties</td>
<td>13</td>
</tr>
<tr>
<td>1.3.5</td>
<td>Genetics</td>
<td>13</td>
</tr>
<tr>
<td>1.3.6</td>
<td>Environmental aspects</td>
<td>15</td>
</tr>
<tr>
<td>1.4</td>
<td>The Disease</td>
<td>16</td>
</tr>
<tr>
<td>1.4.1</td>
<td>Epidemiology</td>
<td>16</td>
</tr>
<tr>
<td>1.4.2</td>
<td>Clinical features</td>
<td>24</td>
</tr>
<tr>
<td>1.4.3</td>
<td>Pathology</td>
<td>25</td>
</tr>
<tr>
<td>1.4.4</td>
<td>Pathogenesis</td>
<td>27</td>
</tr>
<tr>
<td>1.4.5</td>
<td>Diagnosis</td>
<td>28</td>
</tr>
<tr>
<td>1.4.6</td>
<td>Immunity</td>
<td>31</td>
</tr>
<tr>
<td>1.4.7</td>
<td>Antimicrobial therapy</td>
<td>33</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>II.</td>
<td>HYPOTHESIS and SPECIFIC AIMS</td>
<td>35</td>
</tr>
<tr>
<td>III.</td>
<td>PARTIAL CHARACTERIZATION of LEGIONELLA PNEUMOPHILA ADHERENCE TO HOST</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>CELLS</td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>Abstract</td>
<td>37</td>
</tr>
<tr>
<td>3.2</td>
<td>Introduction</td>
<td>38</td>
</tr>
<tr>
<td>3.3</td>
<td>Materials and Methods</td>
<td>41</td>
</tr>
<tr>
<td>3.4</td>
<td>Results</td>
<td>47</td>
</tr>
<tr>
<td>3.5</td>
<td>Discussion</td>
<td>50</td>
</tr>
<tr>
<td>IV.</td>
<td>THE EFFECT OF ANTIBIOTICS THAT INHIBIT CELL WALL, PROTEIN AND DNA</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>SYNTHESIS on the GROWTH and MORPHOLOGY OF LEGIONELLA PNEUMOPHILA</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>Abstract</td>
<td>77</td>
</tr>
<tr>
<td>4.2</td>
<td>Introduction</td>
<td>78</td>
</tr>
<tr>
<td>4.3</td>
<td>Materials and Methods</td>
<td>79</td>
</tr>
<tr>
<td>4.4</td>
<td>Results</td>
<td>81</td>
</tr>
<tr>
<td>4.5</td>
<td>Discussion</td>
<td>85</td>
</tr>
<tr>
<td>V.</td>
<td>PATHOGENESIS, PATHOLOGY and CHEMOTHERAPY of EXPERIMENTAL LEGIONELLA</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>PNEUMOPHILA INFECTION in the CHICK EMBRYO</td>
<td></td>
</tr>
<tr>
<td>5.1</td>
<td>Abstract</td>
<td>118</td>
</tr>
<tr>
<td>5.2</td>
<td>Introduction</td>
<td>120</td>
</tr>
<tr>
<td>5.3</td>
<td>Materials and Methods</td>
<td>123</td>
</tr>
<tr>
<td>5.4</td>
<td>Results</td>
<td>126</td>
</tr>
<tr>
<td>5.5</td>
<td>Discussion</td>
<td>131</td>
</tr>
<tr>
<td>VI.</td>
<td>GENERAL DISCUSSION</td>
<td>167</td>
</tr>
<tr>
<td>VII.</td>
<td>POSSIBLE FUTURE STUDIES</td>
<td>169</td>
</tr>
<tr>
<td>VIII.</td>
<td>LIST of REFERENCES</td>
<td>171</td>
</tr>
<tr>
<td>IX.</td>
<td>APPENDICES</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Appendix 1 Media used in biological studies</td>
<td>186</td>
</tr>
<tr>
<td></td>
<td>Appendix 2 Reagents and materials used in histological studies</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>Appendix 3 Reagents and materials used in electron microscopy studies</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td>Appendix 4 Production of polyclonal antisera against</td>
<td>201</td>
</tr>
<tr>
<td></td>
<td>L. pneumophila</td>
<td></td>
</tr>
<tr>
<td>X.</td>
<td>REPRINTS OF PUBLICATIONS</td>
<td>204</td>
</tr>
</tbody>
</table>
LIST of TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Taxonomy and classification of the genus <em>Legionella</em></td>
<td>5</td>
</tr>
<tr>
<td>1.2</td>
<td>Morphological properties of <em>L. pneumophila</em></td>
<td>8</td>
</tr>
<tr>
<td>1.3</td>
<td>Biochemical properties of <em>L. pneumophila</em></td>
<td>14</td>
</tr>
<tr>
<td>1.4</td>
<td>Comparison of patients with Legionnaires' disease or Pontiac Fever</td>
<td>17</td>
</tr>
<tr>
<td>1.5</td>
<td>Clinical presentation of legionellosis</td>
<td>20</td>
</tr>
<tr>
<td>1.6</td>
<td>Diagnosis of Legionnaires' disease</td>
<td>29</td>
</tr>
<tr>
<td>3.1</td>
<td>Bacterial treatments prior to adherence assays</td>
<td>57</td>
</tr>
<tr>
<td>3.2</td>
<td>Eukaryotic cell treatments prior to adherence assays</td>
<td>58</td>
</tr>
<tr>
<td>3.3</td>
<td>Competitive binding experiments</td>
<td>59</td>
</tr>
<tr>
<td>4.1</td>
<td>Susceptibility of <em>L. pneumophila</em> to selected antibiotics</td>
<td>90</td>
</tr>
<tr>
<td>4.2</td>
<td>Morphological response of <em>L. pneumophila</em> following exposure to antibiotics for 6 and 24 hours</td>
<td>91</td>
</tr>
<tr>
<td>5.1</td>
<td>LD$_{50}$ values for <em>L. pneumophila</em> in the embryonated hen's egg</td>
<td>135</td>
</tr>
<tr>
<td>5.2</td>
<td>Histopathological observations on organs of embryos inoculated with 1, 10, 100 or 1000 times the 100 YSLD$_{50}$ of <em>L. pneumophila</em></td>
<td>136</td>
</tr>
<tr>
<td>5.3</td>
<td>Ultrastructural observations on cells of embryo organs inoculated with 100 times the 100 YSLD$_{50}$ of <em>L. pneumophila</em></td>
<td>137</td>
</tr>
</tbody>
</table>
TABLE 5.4 Appearance of inflammatory cells in chick embryo organs infected with 100 times the 100 YSLD$_{50}$ of *L. pneumophila* and protected with 10 times the MIC of each antibiotic............ 138

TABLE 5.5 Ultrastructural cell damage in embryo organs previously inoculated with 100 times the 100 YSLD$_{50}$ of *L. pneumophila* and protected with antibiotic............................. 139
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIGURE 1.1</td>
<td>Incidence of Legionnaires' disease cases reported to the CDC</td>
<td>19</td>
</tr>
<tr>
<td>FIGURE 1.2</td>
<td>Reported sporadic cases of legionellosis to the CDC</td>
<td>23</td>
</tr>
<tr>
<td>FIGURE 1.3</td>
<td>Flow scheme for the identification of <em>L. pneumophila</em></td>
<td>30</td>
</tr>
<tr>
<td>FIGURE 3.1</td>
<td>Schematic representation of potential <em>L. pneumophila</em> adhesins</td>
<td>56</td>
</tr>
<tr>
<td>FIGURE 3.2</td>
<td>Bacterial binding curve of <em>L. pneumophila</em> to U937 cells as measured by viable count assays</td>
<td>60</td>
</tr>
<tr>
<td>FIGURE 3.3</td>
<td>Bacterial binding curve of <em>L. pneumophila</em> to U937 cells as measured by IFA</td>
<td>61</td>
</tr>
<tr>
<td>FIGURE 3.4</td>
<td>Bacterial binding curve of <em>L. pneumophila</em> to MRC-5 cells as measured by viable count assays</td>
<td>62</td>
</tr>
<tr>
<td>FIGURE 3.5</td>
<td>Bacterial binding curve of <em>L. pneumophila</em> to MRC-5 cells as measured by IFA</td>
<td>63</td>
</tr>
<tr>
<td>FIGURE 3.6</td>
<td>Reduction of <em>L. pneumophila</em> adherence to U937 cells as measured by viable counts following bacterial treatments</td>
<td>64</td>
</tr>
<tr>
<td>FIGURE 3.7</td>
<td>Reduction of <em>L. pneumophila</em> adherence to U937 cells as measured by IFA following bacterial treatments</td>
<td>65</td>
</tr>
<tr>
<td>FIGURE 3.8</td>
<td>Reduction of <em>L. pneumophila</em> adherence to MRC-5 cells as measured by viable counts following bacterial treatments</td>
<td>66</td>
</tr>
<tr>
<td>FIGURE 3.9</td>
<td>Reduction of <em>L. pneumophila</em> adherence to MRC-5 cells as measured by IFA following bacterial treatments</td>
<td>67</td>
</tr>
</tbody>
</table>
FIGURE 3.10 Reduction of \textit{L. pneumophila} adherence to U937 cells as measured by viable counts following eukaryotic cell treatments........................................... 68

FIGURE 3.11 Reduction of \textit{L. pneumophila} adherence to U937 cells as measured by IFA following eukaryotic cell treatments........................................................................ 69

FIGURE 3.12 Reduction of \textit{L. pneumophila} adherence to MRC-5 cells as measured by viable counts following eukaryotic cell treatments........................................... 70

FIGURE 3.13 Reduction of \textit{L. pneumophila} adherence to MRC-5 cells as measured by IFA following eukaryotic cell treatments........................................................................ 71

FIGURE 3.14 Reduction of \textit{L. pneumophila} adherence to U937 cells as measured by viable counts following addition of monosaccharide sugars........................................... 72

FIGURE 3.15 Reduction of \textit{L. pneumophila} adherence to U937 cells as measured by IFA following addition of monosaccharide sugars........................................................................ 73

FIGURE 3.16 Reduction of \textit{L. pneumophila} adherence to MRC-5 cells as measured by viable counts following addition of monosaccharide sugars........................................... 74

FIGURE 3.17 Reduction of \textit{L. pneumophila} adherence to MRC-5 cells as measured by IFA following addition of monosaccharide sugars........................................... 75

FIGURE 4.1 The effect of cefotaxime on the growth of \textit{L. pneumophila}....... 92

FIGURE 4.2 The effect of methicillin on the growth of \textit{L. pneumophila}....... 93

FIGURE 4.3 The effect of ampicillin on the growth of \textit{L. pneumophila}....... 94

FIGURE 4.4 The effect of erythromycin on the growth of \textit{L. pneumophila}................................................................. 95

FIGURE 4.5 The effect of rifampicin on the growth of \textit{L. pneumophila}....... 96
FIGURE 4.6 The effect of ciprofloxacin on the growth of *L. pneumophila* ................................................................. 97

FIGURE 4.7 Proportion of *L. pneumophila* exhibiting morphological changes following exposure to selected antibiotics........... 98

FIGURE 5.1 Schematic illustration of various routes of inoculation of the chick embryo......................................................... 140

FIGURE 5.2 Schematic illustration of the CAM route of inoculation........ 141

FIGURE 5.3 Bacterial viable counts of embryo organs following inoculation with the YSLD$_{50}$ of *L. pneumophila*.................. 142

FIGURE 5.4 Bacterial viable counts of embryo organs following inoculation with 10 times the YSLD$_{50}$ of *L. pneumophila*..... 143

FIGURE 5.5 Bacterial viable counts of embryo organs following inoculation with 100 times the YSLD$_{50}$ of *L. pneumophila*.... 144

FIGURE 5.6 Bacterial viable counts of embryo organs following inoculation with 1000 times the YSLD$_{50}$ of *L. pneumophila*. 145

FIGURE 5.7 Bacterial viable counts of embryo organs previously inoculated with 100 times the YSLD$_{50}$ of *L. pneumophila* and treated one day later with selected antibiotics......................... 146

FIGURE 5.8 Viability of embryos previously infected with 100 times the YSLD$_{50}$ of *L. pneumophila* and treated one day later with selected antibiotics......................................................... 147
LIST of PLATES

PLATE 3.1  L. pneumophila binding to U937 and MRC-5 cells .................. 76
PLATE 4.1  Untreated L. pneumophila organisms ................................... 99
PLATE 4.2  L. pneumophila exposed to cefotaxime for 6 h ....................... 100
PLATE 4.3  L. pneumophila exposed to cefotaxime for 24 h ...................... 101
PLATE 4.4  L. pneumophila exposed to methicillin for 6 h ..................... 102
PLATE 4.5  L. pneumophila exposed to methicillin for 6 h ..................... 103
PLATE 4.6  L. pneumophila exposed to methicillin for 6 h ..................... 104
PLATE 4.7  L. pneumophila exposed to methicillin for 24 h ................... 105
PLATE 4.8  L. pneumophila exposed to methicillin for 24 h ................... 106
PLATE 4.9  L. pneumophila exposed to rifampicin for 6 h ..................... 107
PLATE 4.10 L. pneumophila exposed to rifampicin for 24 h .................... 108
PLATE 4.11 L. pneumophila exposed to rifampicin for 24h ..................... 109
PLATE 4.12 L. pneumophila exposed to rifampicin for 24 h ................... 110
PLATE 4.13 L. pneumophila exposed to ciprofloxacin for 6 h ................. 111
PLATE 4.14 L. pneumophila exposed to ciprofloxacin for 6 h ................. 112
PLATE 4.15 L. pneumophila exposed to ciprofloxacin for 6 h ................. 113
PLATE 4.16 L. pneumophila exposed to ciprofloxacin for 6 h ................. 114
PLATE 4.17 L. pneumophila exposed to ciprofloxacin for 6 h ................. 115
PLATE 4.18 L. pneumophila exposed to ciprofloxacin for 24 h ............... 116
PLATE 4.19  L. pneumophila exposed to ciprofloxacin for 24 h....................... 117

PLATE 5.1  Histological sections of liver from normal and infected embryos................................................................................................ 148

PLATE 5.2  Histological sections of heart from normal and infected embryos................................................................................................ 149

PLATE 5.3  Histological sections of spleen from normal and infected embryos................................................................................................ 150

PLATE 5.4  Histological sections of kidney from normal and infected embryos................................................................................................ 151

PLATE 5.5  High magnification of inflammatory cells in liver of infected embryo................................................................................................. 152

PLATE 5.6  Ultrastructural changes in kidney of infected embryo three days post-inoculation........................................................................ 153

PLATE 5.7  Ultrastructural changes in heart of infected embryo three days post-inoculation........................................................................ 154

PLATE 5.8  Ultrastructural changes in spleen of infected embryo four days post-inoculation........................................................................ 155

PLATE 5.9  Hemolytic process in kidney of infected embryo four days post-inoculation.............................................................................. 156

PLATE 5.10 Advanced stages of infection in liver of embryo five days post-inoculation................................................................................... 157

PLATE 5.11 Degenerative changes in heart of infected embryo five days post-inoculation............................................................................. 158

PLATE 5.12 Pyknosis and karyolysis of nuclei of cells five days post-inoculation........................................................................................ 159

PLATE 5.13 Intracellular organism within spleen of infected embryo....... 160

PLATE 5.14 Thin-sectioned kidney at four days post-inoculation from infected embryo treated with erythromycin three days after bacterial infection........................................................................... 161
PLATE 5.15  Thin-sectioned kidney at four days post-inoculation from infected embryo treated with doxycycline three days after bacterial infection.................................................. 162

PLATE 5.16  Thin-sectioned kidney at four days post-inoculation from infected embryo treated with rifampicin three days after bacterial infection.................................................. 163

PLATE 5.17  Thin-sectioned kidney at eight days post-inoculation from infected embryo treated with rifampicin three days after bacterial infection.................................................. 164

PLATE 5.18  Thin-sectioned liver at four days post-inoculation from infected embryo treated with ciprofloxacin three days after bacterial infection.................................................. 165

PLATE 5.19  Thin-sectioned liver at eight days post-inoculation from infected embryo treated with ciprofloxacin three days after bacterial infection.................................................. 166
Abstract

PATHOGENESIS, PATHOLOGY AND CHEMOTHERAPY OF EXPERIMENTAL LEGIONELLA PNEUMOPHILA INFECTION

by

Arthur O. Tzianabos
University of New Hampshire, December, 1989

*Legionella pneumophila* is the causative agent of a severe, often fatal pneumatic illness known as Legionnaires' disease. The mechanisms by which *L. pneumophila* attaches to U937 (transformed human macrophage-like cells) and to MRC-5 cells (semi-continuous human lung fibroblasts) were investigated. Experimental parameters for adherence assays were established prior to blocking studies designed to identify microbial adhesins and/or eukaryotic receptors that mediate bacterial attachment to target cells. Results from these studies indicated that a lectin-like component (s) associated with *L. pneumophila* may be responsible, at least in part, for microbial adherence to these eukaryotic host cells. Erythromycin is the drug of choice for the treatment of clinical legionellosis; however, difficulties with this antibiotic have been reported resulting in the need to seek alternative therapeutic regimens. In these studies, the effect of clinically relevant antibiotics that inhibited bacterial cell wall, protein and DNA synthesis of this pathogen was evaluated *in vitro* by growth and viability studies as well as morphologically by negative stain.
scanning and thin-section electron microscopy. Of those tested, cefotaxime, an antibiotic of limited value in clinical trials, was most effective. The pathogenicity of \textit{L. pneumophila} was assessed by \textit{LD}_{50} and bacterial growth estimations in the chick embryo animal system \textit{in ovo}. In addition, histopathological and electron microscopic examination of the cellular and sub-cellular pathology induced in the organs of embryos previously infected with 100 times the yolk sac (YS)\textit{LD}_{50} of \textit{L. pneumophila} was made as a prelude to chemotherapeutic treatment with a range of clinically putative antimicrobial agents. The promising new quinolone derivative, ciprofloxacin, was most effective in these trials. Results from these studies may be indicative of novel preventative and control measures for the therapy of human Legionnaires' disease.
*Legionella pneumophila*, serogroup 1, strain Nottingham N7. Scanning electron microscopy (SEM) preparation. Bar=0.5μm.
Section I

The Natural History of Legionnaires’ Disease

1.1 Preface. Legionnaires’ disease is a severe illness of humans which often occurs in spectacular outbreaks. Although an extensive data base concerning all aspects of legionellosis has been established since the outbreak in Philadelphia 13 years ago, the mechanisms by which the subsequently described causative agent, *Legionella pneumophila*, induces disease are not fully understood. This study examines the pathogenesis of this organism at the host, cellular and sub-cellular levels and defines the role of clinically relevant antibiotics in the disease process.

1.2 Historical perspective. The outbreak of a severe form of acute lobar pneumonia during an American Legion Convention held at the Bellevue Stratford Hotel in Philadelphia in July 1976 was the precipitating factor which led to the isolation and identification of this previously unrecognized human pathogen. During this epidemic, 29 of the 182 legionnaires who became ill, died. An additional 39 individuals who were in or passed by the hotel developed pneumonia. In all, 34 died from the disease or associated complications during this outbreak (49). The illness, eventually named Legionnaires’ disease, took its name from the Philadelphia outbreak. Initially, a viral etiology was suggested due to the influenza-like symptoms manifested in
these people. Indeed, influenza and other bacterial pneumonias are still common diagnostic considerations by clinicians presented with symptoms of \textit{L. pneumophila} infection (99).

Failure to immediately isolate the causative agent following the Philadelphia epidemic caused the news media to heighten concerns about the presence of a new microbial killer. These embellished reports undoubtedly expedited the epidemiological and microbiological investigations following the outbreak and contributed to subsequent recognition of this important human pathogen. Although investigators at the Centers for Disease Control (CDC) in Atlanta announced the cause of the disease six months after the outbreak, they could not identify the mode of transmission but only pointed out that a common-source epidemic had occurred.

The inability to stain or grow the organism using conventional microbiological procedures proved the main problems in identifying the etiological agent of the disease. Dr. Joseph McDade at CDC finally isolated the organism using techniques established for the recovery and identification of rickettsiae. The yolk sacs of embryonated eggs were inoculated with lung samples from fatal cases and the bacteria were visualized by Gimenez stain which contains carbol fucshin rather than safranin. The latter fails to stain \textit{Legionella} species and, as it is the usual component of the gram stain, offers explanation of the failure to visualize the agent initially (85). It was subsequently shown that the organism and the disease it caused were not new, and previously unresolved epidemics were retrospectively attributed to this
organism. Among these was an outbreak of a febrile illness with a high attack rate that occurred in Pontiac, Mi. in 1968 and, due to its non-pneumonic form, was later called "Pontiac Fever" (59). The explanation for the variability of symptoms of Legionnaires' disease and Pontiac Fever is unknown. The spectrum of disease caused by this and related microorganisms is termed legionellosis.

After attempted cultivation on 17 commonly used bacteriological media, Feeley et al. (43) reported that Mueller-Hinton agar supplemented with 1% hemoglobin and 1% IsovitalexR supported sparse L. pneumophila growth. Feeley-Gorman agar, which contained added L-cysteine hydrochloride and soluble ferric pyrophosphate, was an improvement upon this medium (42). Finally, Edelstein (29) introduced a useful but expensive solid medium which contained α-ketoglutarate (BCYEα) that supported substantial bacterial growth after a 48-72 h incubation period at 37°C. This remains the medium of choice for the cultivation of legionellae in clinical and research laboratories.

Subsequent investigations have demonstrated that L. pneumophila is an environmental organism readily found in streams, lakes and potable water and when delivered in appropriate aerosolized form enters the respiratory tract of humans and replicates within alveolar macrophages of the lung (13, 111). Air conditioners, humidifiers, evaporative condensers, cooling towers and other water systems capable of generating aerosols are potential sources of disease. Human-to-human spread has never been recorded.

Experimental study of the pathogenic aspects of the organism has
utilized a number of different laboratory animals, but to date none have fully simulated all aspects of legionellosis. Guinea pigs, rats, mice, rabbits and monkeys have been experimentally infected with *L. pneumophila* by a number of different inoculation routes and these manifest some, but not all the symptoms of clinical disease. Guinea pigs are used most extensively in such studies, but restricted availability, high costs and legislative pressures have forced researchers to seek alternative systems to study pathogenesis of the organism.

1.3 The Organism:

1.3.1 Taxonomy and nomenclature. The classification and nomenclature of this organism is difficult. Brenner et al. (15, 16) used DNA homology studies as the primary tool to establish that the Legionnaires' disease bacterium of the Philadelphia outbreak was a member of a new family (*Legionellaceae*) of the genus *Legionella* and species *pneumophila*. Serological diversity within this species is well established and numerous additional species, sub-species and serogroups have been described since that time (Table 1.1).

The diversity of the genus has been noted (15) and attributed to the failure of traditional biochemical, immunological and genotypic techniques to yield corroborative results. Analysis of fatty acid and isoprenoid quinone content have been useful, but do not accurately differentiate all species and are not appropriate for use outside of a large research laboratory (15). The determination of DNA relatedness has been and remains the main criterion for
Table 1.1  Taxonomy and classification of the genus *Legionella*

<table>
<thead>
<tr>
<th>Species</th>
<th>Serogroup</th>
<th>Isolated From</th>
<th>Humans</th>
<th>Environment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. pneumophila</em>¹</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. micdadei</em>²</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. bozemanii</em>³</td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. dumoffii</em>³</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. gormanii</em>³</td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. longbeachae</em></td>
<td>1</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. jordanis</em></td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. oakridgensis</em></td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. wadsworthii</em></td>
<td></td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. feelii</em></td>
<td>1</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. sainthelensi</em></td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. anisa</em></td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. maceachernii</em></td>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. jamestowniensis</em></td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. rubrilucens</em></td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><em>L. erythra</em></td>
<td></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
L. hackeliae | 1 | Yes | No
| 2 | Yes | No

L. spiritensis | No | Yes
L. parisiensis | No | Yes
L. cherrii | No | Yes
L. steigerwaltii | No | Yes
L. santicruces | No | Yes
L. israelensis | No | Yes
L. birminghamensis | Yes | No
L. cincinnatensis | Yes | No
L. moravica | No | Yes
L. brunensis | No | Yes
L. quinlivanii | No | Yes
L. tucsonensis | Yes | No


1 *L. pneumophila* contains three subspecies: *L. pneumophila* subspecies *pneumophila*, *L. pneumophila* subspecies *pascuali* and *L. pneumophila* subspecies *fraseri*.

2 Alternate genus name: *Tatlockia*.

3 Alternate genus name: * Fluoribacter*. 
classification. However, practical identification of *Legionella* species has depended on phenotypic characteristics which include gram reaction as well as growth on BCYEα with added L-cysteine and the absence of growth on blood agar or BCYEα without added L-cysteine.

The classification of *Legionella* species has been somewhat controversial. Disagreement continues as to the degree of genetic divergence that constitutes the establishment of a separate species as well as the disparity with which researchers have applied criteria to make these distinctions. Brown *et al.* (54) proposed alternate criteria for classification purposes in which two new genera, *Tatlockia* and *Fluoribacter*, were created. These would contain organisms otherwise called *L. micdadei* and the autofluorescing *Legionella* species *L. bozemanii*, *L. dumoffii* and *L. gormanii*, respectively (Table 1.1). However, this scheme has not found universal acceptance and most workers accept the single genus terminology of *Legionella* within which all species are located. It is clear that further work is necessary to establish uniform guidelines for the classification of the legionellae and related species.

### 1.3.2 Morphological characteristics

Morphological properties of *L. pneumophila* are shown in Table 1.2. Transmission electron microscopy of thin-sectioned *L. pneumophila* cells has demonstrated the typical morphological characteristics associated with gram-negative bacteria (105). *L. pneumophila* organisms were shown to be pleomorphic gram-negative rods that measure 2 to 20 μm in length and 0.3 to 0.9 μm in width. Artificial media giving poor or
Table 1.2 Morphological properties of *L. pneumophila*¹

<table>
<thead>
<tr>
<th>Property</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short rods</td>
<td>(from autopsied lung 0.6 x 1-2 μm, from agar 0.4 x 2-3 μm)</td>
</tr>
<tr>
<td>Tapered ends</td>
<td></td>
</tr>
<tr>
<td>Vacuolated surfaces, convoluted or smooth surfaces</td>
<td></td>
</tr>
<tr>
<td>Long forms</td>
<td><em>(in vivo</em> up to 20 μm in length, <em>in vitro</em> &gt;50 μm)</td>
</tr>
<tr>
<td>Internal PBH granules</td>
<td>deformed in freeze-dried fracture</td>
</tr>
<tr>
<td>Division</td>
<td>by nonseptate binary fission</td>
</tr>
<tr>
<td>Flagella and fimbriae (pili)</td>
<td>present</td>
</tr>
<tr>
<td>Blebs and granules on surface subunits</td>
<td>no evidence of acid polysaccharide capsule</td>
</tr>
<tr>
<td>F-1 antigen (serogroup specific)</td>
<td>located at bacterial surfaces</td>
</tr>
<tr>
<td>Limiting cell envelope</td>
<td>&quot;unit membrane&quot;, 10 nm thick</td>
</tr>
<tr>
<td>Peptidoglycan-like mucoprotein layer present</td>
<td>after partial plasmolysis</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>&quot;unit membrane&quot;, 10 nm thick</td>
</tr>
<tr>
<td>Rich in ribosomes</td>
<td>25 nm diameter</td>
</tr>
</tbody>
</table>

¹Adapted from reference 107, Rodgers, F.G., Legionellosis.
slow bacterial growth resulted in organisms with an elongated or filamentous form with tapered ends. Those rods visualized from autopsied lung were consistently shorter (21, 105).

The cytoplasm of each organism is enclosed within a double cell envelope of approximately 25 nm thickness with an electron-lucent zone or periplasm between the inner and outer membranes indicating, like the aquatic pseudomonads, an apparent absence of peptidoglycan (105). However, peptidoglycan and diaminopimelic acid have been demonstrated following partial plasmolysis and enzymatic digestion of the organism by electron microscopy and by other biochemical techniques (46, 105, 107, 108). The outer membranes of organisms contain substantial amounts of the bacterial lipopolysaccharide component, 2-keto-3-deoxyoctonate (46).

Cytoplasmic contents consist of evenly dispersed, electron-dense ribosomes, each of 25 nm diameter, arranged in small groups or polysomes and a fine skein of nuclear material (105, 110, 111). Electron-lucent vacuoles have been demonstrated as a regular feature of the cytoplasm of L. pneumophila whether present in human lung cells, guinea pig spleen, peritoneal macrophages or from bacteriological media (107). The reported size of these vacuoles varied from 30 to 200 nm (13) but larger forms up to 0.5 μm have been found (107). These structures have been confirmed as poly-β-hydroxybutyrate (PBH) granules by their appearance in freeze-fractured preparations (107, 108).
Negative-stain and scanning electron microscopy of *L. pneumophila* revealed bacterial cells with nonparallel sides, tapered ends and smooth undulating surfaces. Flagella and fimbriae (pili) have been demonstrated in vitro as surface appendages of *L. pneumophila* (110). The presence of small blebs and evaginations of the outer membrane have been noted on the surfaces of organisms by thin-section, scanning and freeze-fracture electron microscopy, but an extracellular mucopolysaccharide layer has not been detected. In addition, freeze-fracture electron microscopy of *L. pneumophila* demonstrated four short ridges enclosing the bacterial cytoplasm which corresponded to the double-unit membrane seen in thin-sectioned material (107, 108). Fractured aspects of the peptidoglycan were not evident in the intramembranous region of bacilli even after partial plasmolysis.

Poly-β-hydroxybutyrate granules were surrounded by a non-fracturable membrane evident as a single ridge in freeze-fracture preparations and confirmed reports of electron-lucent vacuoles in thin-sections. In keeping with the nature of PBH granules, contents of the granules in *L. pneumophila* underwent plastic deformation as an artifact of the fracture process and were evident as long, drawn out structures (107, 108).

1.3.3 Biochemical properties. *L. pneumophila* is a non-spore forming, aerobic, non-acid fast organism. All species of the genus demonstrate weak catalase and peroxidase activity. *L. pneumophila* also expresses gelatinase, phospholipase and β-lactamase as well as the ability to hydrolyze starch. Many
reports describe a number of exoproteases produced by the organism and these degrade a wide variety of proteins (10, 79). Keen and Hoffman (79) have isolated and partially characterized an exoprotease of \textit{L. pneumophila} that exhibited cytotoxic and hemolytic activity.

\textit{L. pneumophila} contains an unusually large proportion of branched-chain fatty acids located in the cell wall (15) but reports demonstrating the presence of an extracellular acid polysaccharide layer by ruthenium red staining have been contradictory (66, 105). The organism has been shown to possess a high molecular weight antigen or lipopolysaccharide (LPS), termed the F-1 antigen (34, 76) which had endotoxic activity, inhibited serological reactions and was localized on the surface of the bacterium. The LPS of \textit{L. pneumophila} serogroup 1 was found to be tightly bound to the major outer membrane protein (MOMP), reacted specifically with the sera of patients with \textit{L. pneumophila} serogroup 1 infection and was distinctly different from that of most enteric bacteria (136).

The MOMP of \textit{L. pneumophila} has a molecular weight of 24 to 29 kDa (18, 53, 67, 76, 89) and is expressed on the surface of the bacterium (34). Reports have suggested that the MOMP may be associated with LPS (67) and peptidoglycan (52). Gabay and Horwitz (52) showed that this molecule formed ion-permeable channels similar in function to those found in \textit{Escherichia coli} upon exposure to lipid membranes. Butler \textit{et al.} (18) suggested that the MOMP is part of a larger, four sub-unit 95 kDa complex.

Investigations have shown that amino acids are the major source of
energy for growth (55, 80), although the specific amino acid requirements are as yet undetermined. However, it is generally agreed that arginine, threonine, methionine, serine, isoleucine, valine, and cysteine are necessary. In addition, several groups have demonstrated that L. pneumophila can be grown in chemically defined media composed of amino acids (55, 94). While purines, pyrimidines, vitamins and other growth factors are not required, it is known that this organism has an unusually high requirement for L-cysteine.

The Krebs cycle is the primary route for carbon assimilation and energy production for L. pneumophila (55, 68, 122). These studies have shown that all the enzymes of this cycle were present but those of the glyoxylate shunt were marginal or absent in cell-free extracts of this bacterium.

Legionellae do not appreciably catabolize carbohydrates. Early reports showed negative sugar fermentation results but subsequent studies indicated that the Entner-Douderoff and pentose phosphate pathways but not the Embden-Meyerof pathway functioned to a limited degree in glucose catabolism (134). However, the gluconeogenic anabolic enzymes of the Embden-Meyerof pathway were responsible for sugar synthesis (55).

L. pneumophila growth is enhanced by trace metals, most notably ferric iron, but this may not be an absolute requirement (102, 121). Other important metals include Cu, Mg, Mn, Co, Ca, Mo, Ni, V, and Zn. In addition, Reeves et al. (103) have reported the absence of phenolate or hydroxamate ferric-binding compounds in Legionella species. The function of charcoal and α-ketoglutarate in the growth media of this organism has been questioned. Some investigators
believe that these compounds neutralize oleic acid produced in the media upon autoclave sterilization while others have suggested that they may serve to scavenge toxic oxygen radicals released from the yeast extract present in the medium upon exposure to fluorescent light (69). However, the precise nature of this requirement has not been elucidated.

The electron transport chain of *L. pneumophila* is composed of cytochromes c, b, a and d types but the organization of the respiratory chain remains undetermined (68). The concentrations of these heme-containing proteins were much lower in *Legionella* compared with those of other aerobes such as *Pseudomonas* or *Campylobacter*. Researchers have concluded that the respiratory chain is quite complex in *L. pneumophila* but is punctuated by at least three terminal oxidases (68). The biochemical properties of *L. pneumophila* are summarized in Table 1.3.

1.3.4 Physico-chemical properties. The legionellae have an optimum pH range for growth of 6.7 to 6.9 in BCYEα but may grow in other media with pH values as low as 6.0 (47, 55). Clinical isolates have an optimal temperature range of 35 to 37° C but environmental isolates prefer an optimum of 30° C. Isolation of the legionellae from water at 50 to 65° C has been reported (47).

1.3.5 Genetics. Genetic analysis of *L. pneumophila* has proved difficult. The fastidious nature of the organism and lack of conventional genetic transfer systems for the legionellae have limited our knowledge in this area. Plasmids have been found in *L. pneumophila* but have not been shown to contribute to or detract from the virulent properties of the organism. Recent efforts to clone the
Table 1.3 Biochemical properties of *L. pneumophila*

- non-spore forming
- aerobic
- non-acid fast
- motile (most strains)
- high proportion of branched chain fatty acids in cell wall
- amino acids as carbon and energy sources

- oxidase (+/-)
- catalase (+)
- peroxidase (+)
- nitrate reductase (-)
- urease (-)
- hippurate hydrolysis (-, most strains)
- gelatinase (-)
- B-lactamase (+)
- exoproteases (+)
genes for Legionella antigens in E. coli have been successful (27, 37, 38). In addition, Cianciotto et al. (23) were able to render a previously virulent strain of L. pneumophila non-infectious for cell cultures through the genetic deletion of a 24 kDa surface protein. Studies such as these may help further our knowledge concerning the pathogenic mechanisms of this organism.

1.3.6 Environmental aspects. The Legionellaceae are ubiquitous in natural aquatic habitats and man-made water systems. L. pneumophila has been isolated from lakes, streams, rivers, cooling water, hot water and potable water systems (48, 86). There is conclusive epidemiological evidence that the generation of aerosols from domestic and industrial air-conditioners, evaporative condensers, cooling, hot water and other aquatic systems which release micron-sized water droplets into the air serve as potential sources for legionellosis (86). An appropriate infectious dose of L. pneumophila may be delivered by aerosolized droplets 5 to 15 μm in diameter which can readily enter the nasopharynx and penetrate alveolar airspaces of the lung.

L. pneumophila has been primarily isolated from warm or thermally polluted freshwater systems. Although the organism has been found in a wide variety of aquatic niches, it is estimated to comprise a very small portion of the total bacterial population in the aquatic environment.

The association of Legionella with blue-green algae and freshwater protozoans including free-living amoebae Acanthamoeba, Tetrahymena and Cyclidium species has been demonstrated (3, 113, 124). Such interactions have been hypothesized to provide legionellae with an abundance of nutritional
support and a host in which microbial numbers can be amplified. Evidence presented by Berendt (11) suggested that the survival of *L. pneumophila* in an aerosolized form was enhanced by its association with various species of the blue-green algae, *Fischerella*, and that dissemination away from the source of a generated aerosol may be aided by particular algal components. Results from other studies (127) indicated that legionellae may be carried in aerosolized form in cooling tower plumes 20 to 50 kilometers downwind and illustrated the potential danger posed by this pathogen.

The eradication of legionellae from water systems is rarely possible and studies should center on determining acceptable microbial densities for the organism. Effective biocidal treatments are being investigated for use as control and preventative measures. The most effective potable water treatments have been shown to be hyperchlorination, especially of hot water supplies and heating to above 50° C. Acceptable treatments for non-potable water included chlorination, use of quaternary ammonium compounds and halamines (118).

### 1.4 The Disease

#### 1.4.1 Epidemiology.

Legionellosis can exist in two forms: the severe pneumatic type with associated high fever known as Legionnaires' disease and the non-pneumonic form known as Pontiac Fever. Table 1.4 lists the symptoms for these conditions. The disease can occur as epidemics, sporadic cases, or nosocomial infections. Epidemic legionellosis has been documented in many countries including the United States, Great Britain, Spain, The Netherlands, Germany, Sweden, France, Canada and Italy and shows an...
Table 1.4 Comparison of symptoms of patients with Legionnaires' disease or Pontiac Fever.

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Legionnaires' Disease</th>
<th>Pontiac Fever</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Sputum production</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Hemoptysis</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Myalgias</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Upper respiratory symptoms</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Headache</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Confusion</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Nausea or vomiting</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Fever</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
increase in incidence during the summer months of July, August and September (Figure 1.1). *L. pneumophila* serogroup 1 is responsible for 85% of reported cases of legionellosis while *L. micdadei* causes approximately 6% (136). A number of species of *Legionella* have been isolated from human and environmental sources. The most recognized form of legionellosis, Legionnaires' disease, has an incubation period of 2 to 10 days with an attack rate of 1 to 5% and a case-fatality ratio of 0 to 40% (84, 139). It has been estimated to be responsible for 1 to 29% of community acquired pneumonia. Assessments of this nature have been difficult due to the environmental nature of *Legionella* and the difficulty in establishing an etiology for many undiagnosed pneumonias. Table 1.5 illustrates the clinical presentation of legionellosis.

The disease exhibits a predilection for immunocompromised individuals. The risk of development of Legionnaires' disease increases in the elderly and in those who excessively smoke and/or drink alcoholic beverages. In addition, underlying illnesses such as diabetes mellitus, congestive heart failure, malignancy, obstructive pulmonary disease or use of immunosuppressive medication may increase susceptibility to this opportunistic pathogen.

It has been demonstrated that airborne transmission of aerosolized organisms is the mechanism by which the organism and hence the disease disseminates, but identification of pin-point sources of the infection in epidemic legionellosis are often difficult. Investigation of the Philadelphia outbreak did not conclusively demonstrate the source of the epidemic, but studies showed that attendance in a 14th floor hospitality room of the Bellevue-Stratford Hotel
Figure 1.1 Average incidence of Legionnaires' disease cases reported to the CDC from 1978 to 1981. Note higher incidence during the summer months of each year. Reprinted with permission from Legionellosis, S. M. Katz (ed.), copyright CRC Press, Inc. Boca Raton, Fl.
Table 1.5  Clinical presentation of legionellosis

Two forms: Legionnaires’ disease (LD) and Pontiac Fever (PF)
Genotypically and phenotypically identical

<table>
<thead>
<tr>
<th></th>
<th>LD</th>
<th>PF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Named for:</td>
<td>Philadelphia outbreak</td>
<td>Pontiac out break</td>
</tr>
<tr>
<td>Attack rate:</td>
<td>1-5%</td>
<td>95%</td>
</tr>
<tr>
<td>Incubation period</td>
<td>2-10 days</td>
<td>1-2 days</td>
</tr>
<tr>
<td>Symptoms:</td>
<td>Pneumonia</td>
<td>Non-pneumonic</td>
</tr>
<tr>
<td>Other organs affected:</td>
<td>Kidneys, G.I. tract, CNS</td>
<td>None</td>
</tr>
<tr>
<td>Case:Fatality rate</td>
<td>Variable 0-40%</td>
<td>0%</td>
</tr>
</tbody>
</table>
may have been a significant risk factor. However, the development of illness in people who never entered the hotel but were in close proximity was suggestive of airborne transmission. That evaporative condensers and air handling equipment have been identified as the source of other outbreaks further substantiated this hypothesis (84, 86).

The first reported case of Pontiac Fever was recognized retrospectively following the events of the Philadelphia epidemic. The Pontiac Fever outbreak occurred in July 1968 in a health department in Pontiac, Mi. and this episode provided significant evidence of airborne transmission of organisms from a evaporative condenser (59). Between the time that the first illness was reported until two days later, 89 of the facility's 100 employees and 15 visitors became sick. All cases had no common exposures outside the building and people working in all areas of the building were exposed. However, attack rates were higher in persons exposed during the morning than in people newly exposed in the afternoon suggesting increased risk after the air-conditioning system was first turned on each day. Subsequent investigation revealed that the central air-conditioning unit for the building was contaminated by an adjacent evaporative condenser.

Similar instances have been documented where burst exposure of individuals to aerosols or air handling equipment contaminated with \textit{L. pneumophila} have led to illness. Most notably, a study of recurrent Legionnaires' disease in a hotel in Spain revealed that members of married couples who were the first to shower in the morning had contracted
legionellosis in five of six cases (5). These data may indicate that inoculum size of \textit{L. pneumophila} is a contributing factor to the initiation of disease.

Sporadic cases of legionellosis outnumber epidemic cases by a two to one ratio. This ratio may even be higher given the probability that only 0.8 to 2% of these cases are reported each year. According to the surveillance system of the CDC, from the period of 1978 to 1981, sporadic cases had been reported in every one of the states of the USA, occurred primarily in the summer months and had an incidence ranging from less than 0.25 to more than 1.0 cases per 100,000 people (Figure 1.2). Sixty-six percent of cases were in persons greater than 50 years old (84).

Tobin \textit{et al.} (125) were the first to suggest that hospital water systems other than cooling units could act as reservoirs for \textit{L. pneumophila}. Subsequent investigations have substantiated that potable water is a potential source of nosocomial or hospital-acquired legionellosis (1). Environmental sampling in several hospitals has led to the isolation of \textit{L. pneumophila} and other species from a variety of sources including shower heads, tap water, medical drills and respiratory therapy equipment (1, 4, 17). Reports vary as to the incidence of hospital-acquired \textit{Legionella} infections but these range from 0 to as high as 30%. In general, investigators actively searching for \textit{Legionella} as a cause of pneumonia tend to find it to a greater frequency.

The incidence of nosocomial Legionnaires' disease can be attributed to the ubiquity of the organism in hospitals and the presence of a susceptible population. Haley \textit{et al.} (63) have found that length of stay and compromised
Figure 1.2 Reported sporadic cases of legionellosis to the CDC from 1978 to 1981 indicating average incidence per 100,000 individuals per state. Reprinted with permission from Legionellosis, S. M. Katz (ed.), copyright CRC Press, Inc. Boca Raton, Fl.
immunity were the greatest risk factors for acquisition of disease by patients. Interestingly, the widespread presence of legionellae in water sources of hospitals does not correlate well with the incidence of disease. Explanations for this phenomenon include colonization of water lines with non-virulent legionellae, presence of sub-infective doses of organisms and variations in the susceptibility of patients. Further studies are needed to determine the pre-existing conditions within a hospital which are most likely responsible for the incidence of nosocomial Legionnaires’ disease.

The most perplexing issues concerning the epidemiology of legionellosis is the manifestation of two separate illnesses, Legionnaires’ disease and Pontiac Fever, from infection with the same organism. No differences can be attributed to the bacterium or to conditions surrounding outbreaks to explain discrepancies in symptoms, attack rate or incubation period. It is possible that either the infectious dose for each disease varies or alternatively that patient-related differences may be responsible. To date no evidence has accumulated to support either of these hypotheses. Further work is needed to determine whether this phenomenon is related to other pathogenic features exhibited by _L. pneumophila_.

1.4.2 Clinical features. Legionellosis is two to three times more common in men than in women and infections in children are rare. The disease presents as an atypical lobar pneumonia and has an incubation period of 2 to 10 days. The infection begins with acute fever, rigors, headache and myalgia. Upper respiratory tract symptoms are often absent and a non-productive dry cough
may appear three to four days later. On occasions when sputum is produced it is often non-purulent. Dyspnea and respiratory difficulties are common. Extrapulmonary symptoms such as gastroenteritis, fatigue and confusion are often reported and commonly lead to misdiagnoses by clinicians (see Tables 1.4 and 1.5). Legionellae are difficult to isolate from respiratory secretions but may be readily demonstrated in direct lung biopsy by immunofluorescence.

1.4.3 Pathology. X-ray radiographs of patients with Legionnaires' disease have been variable and do not uniquely identify *Legionella* pneumonia. An average of three days occurs between the onset of symptoms and the appearance of radiographic pathology (40, 81). Deterioration of lung has been seen in two-thirds of cases and the most common features noted are the appearance of infiltrates and pleural effusions. Usually one lobe is affected but bilateral involvement has occurred in approximately one-quarter of reported cases (81). The resolution of radiographic problems has often taken longer than that of clinical recovery and may require an extended period of convalescence. Post-mortem lung samples have demonstrated the development of fibrino-purulent disease in one or both lungs with focal or lobar consolidation, edema, congestion and grey hepatization. In most cases, a fibrinous exudate is present in the pleural cavity. Macroscopic lesions are not always present but can include hemorrhagic areas and focal abscess formation (14).

Microscopic examination has revealed a severe inflammatory response within the distal airspaces of the lung. Terminal bronchioles and alveoli are
filled with proteinaceous material, fibrinous exudate and contain numerous
macrophages and polymorphonuclear leukocytes (PMNLs) presenting in
varying proportions. Proximal bronchi and bronchioles usually remain
uninvolved throughout the disease process. Alveolar damage with hyaline
membranes and desquamation of lining cells has been noted in lung biopsies
(90, 136).

Shortly after the initial outbreak in 1976, Rodgers et al. (111) first
demonstrated the presence of \textit{L. pneumophila} within lung cells by electron
microscopy and described ultrastructural degenerative changes associated with
further dissemination of the organism into alveolar extracellular spaces. These
findings were subsequently confirmed by other ultrastructural reports which
described the intracellular nature of the organism (105, 106, 107).

Several investigators have demonstrated that patients with severe illness
have developed extrathoracic pathology that involved the kidneys, heart, liver,
spleen and brain. Instances of pyelonephritis, hepatic abscesses, cerebral
myoglial reactions, pericarditis and endocarditis have been reported (4, 136).
Degenerative changes have been found in these organs, but macroscopic
lesions are uncommon.

Unusual complications such as dual infection with multiple species (26)
or subtypes (78) of \textit{Legionella} or other opportunistic pathogens have been
reported. In addition, serologic confirmation of sub-clinical infection and diverse
clinical presentation of disease following repeated exposure to the same source
of \textit{L. pneumophila} has been described (58, 136).
1.4.4 Pathogenesis. *L. pneumophila* is a facultative intracellular pathogen which replicates within alveolar macrophages of the lung. During the course of infection, the bacteria are actively phagocytosed by host cells and enter phagosomes associated with ribosomes and mitochondria. Phagocytosis of *L. pneumophila* by macrophages is independent of, but enhanced by, the presence of opsonizing antibody and complement. However, phagocytosis by PMNLs requires the presence of antibody, is not bactericidal and does not support the growth of *L. pneumophila*. Direct penetration of bacteria into eukaryotic cells has been suggested but uptake of *L. pneumophila* by guinea pig macrophages has been shown to occur by a conventional phagocytic process (36).

Studies have shown that macrophages exhibited an oxidative burst upon internalization of virulent *L. pneumophila* but subsequent events such as phagolysosomal fusion and acidification of the phagosome were inhibited (71, 73). Rapid intracellular multiplication of *L. pneumophila* was followed by cellular lysis with release of organisms and continued spread to adjacent host cells. Further spread of the organism via the bloodstream has been described and may explain the extrapulmonary pathology observed during advanced stages of the disease process (31, 105, 136).

Although no single microbial feature has been shown to confer virulence on *L. pneumophila*, a number of bacterial products have been implicated as potential factors which contribute to its pathogenicity. Release of toxins which neutralize the bactericidal pathways of phagocytes (50) and induce necrotizing
inflammatory and cytolytic responses in human tissues (6, 10) may be the most important pathogenic aspects of the legionellae.

1.4.5 Diagnosis. Difficulties involved in the diagnosis of \textit{L. pneumophila} infections are due primarily to the similarity between symptoms of \textit{Legionella} infections with those of other bacterial and viral pneumonias and the non-pathognomic radiographic appearance of the disease. In addition, failure to stain the organism in clinical samples further complicates positive identification of legionellae as the causative agent. These problems make it necessary to rely on culture, serology, and detection of bacterial nucleic acid or antigen as the main methods for the diagnosis of Legionnaires' disease. Table 1.6 illustrates clinical and laboratory diagnostic indicators of legionellosis.

The mainstay of laboratory diagnosis remains culture on selective bacteriological media (31, 137). Growth on BCYE\textsubscript{a} with supplemented L-cysteine in the absence of growth on a blood agar media is indicative of \textit{L. pneumophila}. Recovery of clinical specimens by culture will identify 50 to 80\% of positive samples and this has been reported to have a specificity of 100\% (137). Colonial morphology is distinct and requires two to five days for positive identification. Once isolated, confirmation of these tests should be made by a gram stain in which carbol fuchsin is substituted for safranin as the counter-stain along with immunofluorescence. Figure 1.3 illustrates the rationale for determining the laboratory detection of \textit{L. pneumophila}.

The development of a variety of serological tests including indirect immunofluorescence assays (IFA) has been useful in the identification of
Table 1.6  Diagnosis of Legionnaires' Disease

Clinical diagnosis
Onset
fever
rigors
headaches
weakness
gastrointestinal abnormalities

Acute phase
unremitting fever
radiographic abnormalities
non-productive cough
nervous system manifestations
dyspnea

Laboratory diagnosis
Organism detection methods (culture, immunofluorescence, nucleic acid probes, etc.)
Slightly elevated white blood cell count
hyponatremia
proteinuria
hematuria
Identification Scheme for *L. pneumophila*

Growth on BCYEα of gram-negative bacillus

<table>
<thead>
<tr>
<th>No</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not <em>L. pneumophila</em></td>
<td>Potential <em>L. pneumophila</em></td>
</tr>
</tbody>
</table>

Growth on BCYEα without L-cysteine at 37 °C in 3 days

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probably not <em>L. pneumophila</em></td>
<td>Possible <em>L. pneumophila</em></td>
</tr>
</tbody>
</table>

Growth on blood containing agar

| Not *L. pneumophila* |

Immunofluorescence or Agglutination

<table>
<thead>
<tr>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presumptive <em>L. pneumophila</em></td>
<td>Probable <em>L. pneumophila</em></td>
</tr>
</tbody>
</table>

Biochemical Analysis for confirmation

---

Figure 1.3. Flow scheme for the identification of *L. pneumophila*. 
Legionella antibody for diagnostic purposes. Although, IFA has a sensitivity of approximately 75% and a specificity of almost 100%, problems have arisen in its use for diagnosis of sporadic cases of Legionnaires' disease. This is due to the incidence of cross-reactivity reported with the use of *L. pneumophila* antibody and the inherent difficulty involved in the subjective interpretation of the assay. Detection of seroconversion to a four-fold titer by serological methods is useful, but yields retrospective results and should be interpreted with caution (4, 64, 137).

Direct observation of *L. pneumophila* antigen in clinical specimens by IFA has provided relatively rapid diagnoses but with low sensitivity. Problems due to reagent cross-reactivity with other bacteria and the environmental presence of the legionellae have lead to false-positive results (4, 64).

Commercial nucleic acid probes are now available and exhibit better sensitivity than IFA, and although they are gaining widespread acceptance, further examination is needed before they replace IFA completely. Radioimmunoassay, latex agglutination, microagglutination, counter immunoelectrophoresis, hemagglutination and enzyme immunoassay techniques have been used or are currently being investigated for application as rapid, sensitive and specific diagnostic tools (64, 114).

1.4.6 Immunity. The immune response to Legionnaires' disease is not well understood. However, epidemiological evidence has suggested that susceptibility and resistance to *Legionella* infection are directly related to risk factors which usually immunocompromise the host. Advanced age, underlying
disease, excessive cigarette smoking and immunosuppressive therapy have been shown to influence attack and fatality rates of Legionnaires' disease. Experimental studies have demonstrated that the cellular arm of the immune system may have a significant role in resistance to infection with \textit{L. pneumophila}. Freidman et al. (51) demonstrated that lymphokine activated macrophages did not support the growth of ingested legionellae while Horwitz et al. (72) showed that non-specific activation of these cells also inhibited intracellular replication of \textit{Legionella}. That untreated alveolar macrophages of mice did not support intracellular growth of this organism (140) further suggested that aspects of the bacteria-macrophage interaction have a central function in the immune response to legionellosis.

The role of humoral immunity has not been well-defined. Theoretically, the introduction of anti-\textit{L. pneumophila} antibodies would facilitate opsonization of bacteria with detrimental results to the host. However, animal studies have demonstrated that passive transfer of antibody against \textit{L. pneumophila} is effective against subsequent challenge with whole organisms (104, 136).

It has been difficult to establish whether individuals who have contracted legionellosis are susceptible to re-infection. Researchers have shown that immunization of guinea pigs with killed antigen is protective in some cases, but not in others (7, 33). Winn and Davis (136) were able to protect guinea pigs for one month after infection by aerosol or intratracheal route with a sub-lethal inoculum of \textit{L. pneumophila}.

Human defenses against legionellosis most likely involve synergistic action of the cellular and humoral immune system. Studies that have elucidated a
defense system in animals have demonstrated limited bacterial killing or bacteriostasis of *L. pneumophila*. Further work is needed to fully delineate the mechanisms of human resistance to Legionnaires' disease.

1.4.7 Antimicrobial Therapy. Erythromycin is the current clinical drug of choice for legionellosis. Studies have shown that the clinical reaction to this antibiotic may range from an immediate response to no therapeutic results at all. Relapse of infection and development of severe phlebitis with intravenous use of erythromycin have been reported (62). Rifampicin is generally considered the most effective drug *in vitro* and has been used effectively in combined clinical therapy with erythromycin, but can not be used alone due to the high frequency with which bacterial resistance is induced. Consequently, alternative antimicrobial therapeutic regimens are being sought for the treatment of clinical legionellosis.

*L. pneumophila* is sensitive to a wide variety of antibiotics *in vitro* (30, 60, 123). However, these sensitivity levels are not always reflected in clinical trials. Antimicrobial agents with an effective minimum inhibitory concentration (MIC) such as the aminoglycosides, cephalosporins and penicillins are reported as ineffective in the treatment of human Legionnaires' disease (82) while others such as erythromycin, rifampicin and the tetracyclines have an effective role in therapy (2). Discrepancy between experimental and clinical trials may be explained by the inability of various antibiotics to penetrate host cell membranes and reach intracellular bacteria. Indeed, it has been reported that ciprofloxacin, erythromycin and rifampicin (65, 130) inhibit intracellular growth of *L. pneumophila* in cell cultures, while the β-lactam antibiotics such as ampicillin, cefoxitin and cefotaxime do not (142).
Of the new antibiotics examined, the quinolones have shown great promise in vitro and in animal studies (44, 45, 60, 128, 129). Ciprofloxacin, the best studied compound of this group, is a broad spectrum antibiotic which offers exceptional oral bioavailability and achieves high tissue concentrations. It is well-tolerated by patients and has been used successfully in the clinical treatment of various urinary and respiratory tract infections. Results from animal studies indicated that the efficacy of ciprofloxacin is equal to or greater than that of erythromycin and rifampicin in treatment of experimental Legionella infections (44, 45, 128, 129). Further studies are in progress to develop practical in vivo screening methods for the evaluation of new antimicrobial agents for treatment of clinical Legionnaires' disease.

Despite the massive accumulation of data concerning the nature of L. pneumophila since its initial isolation and identification following the epidemic in Philadelphia, Legionnaires’ disease remains a major cause of pneumonia in humans. Enhanced awareness of this syndrome and aggressive therapeutic regimens have helped in the maintenance and treatment of legionellosis, but further studies are needed to establish prophylactic or preventative measures especially for “at risk groups”. Elucidation of the pathogenic mechanisms of the organism and the role chemotherapeutic agents play in the treatment of disease as well as the investigation of the mechanisms of attachment and penetration used by L. pneumophila to initiate the disease process may lead to new developments in the control of this important human disease.
Section II

2. Hypothesis and Specific aims.

*L. pneumophila* is the causative agent of a fatal form of pneumonia known as Legionnaires' disease. The organism is a facultative intracellular pathogen which attaches to, penetrates and replicates within alveolar macrophages of the lung. Continued growth and spread of *L. pneumophila* leads to the presentation of well-defined clinical features as well as to extensive pulmonary and extrapulmonary pathology. The mechanisms by which *L. pneumophila* recognizes and attaches to host cells are crucial to the initiation of disease in a susceptible host. The process of binding was investigated using human cells in culture in order to elucidate these earliest stages of microbial infection. It is postulated that determination of these adherence events will lead to better understanding of the nature of this infectious disease and to the development of more effective control measures. Of the present therapeutic regimens, erythromycin is the preferred drug for treatment of Legionnaires' disease. However, patients in the advanced stages of severe illness often respond poorly to such antimicrobial treatment. The *in vitro* activity of established antibiotics along with novel, potentially useful drugs were evaluated against the organism *per se* to define the kinetics and mode of action of these antimicrobial agents. Furthermore, the *in vivo* pathogenesis and pathology induced in experimental *L. pneumophila* infection in the chick embryo were
examined and the ability of clinically proven or potentially useful antibiotics to control or ameliorate the disease process was investigated in this animal system. From the results of these combined studies it is anticipated that novel preventative or control measures may be forthcoming for the treatment of Legionnaires' disease.

Specific aims of this project were:
1) to establish the experimental parameters leading to the characterization of Legionella pneumophila adherence factors by using the human cell lines U937 (macrophage-like) and MRC-5 (lung fibroblast) to partially define these adhesins;
2) to elucidate the in vitro activity of selected antibiotics on the growth and morphology of Legionella pneumophila;
3) to delineate the cellular and sub-cellular pathology induced in the chick embryo following experimental infection with Legionella pneumophila and to define the pathogenesis of infection in this system;
4) to examine the role of antimicrobial agents in the disease and recovery processes.
3.1 Abstract

The mechanisms by which the facultative intracellular pathogen, *Legionella pneumophila*, attached to macrophage-like (U937) and human embryo lung (MRC-5) cells *in vitro* was examined. Bacteria and host cell surfaces were modified or treated with degradative enzymes, detergents, fixatives, lectins, oxidizing chemicals, and sugars prior to adherence assays in order to elucidate putative bacterial adhesins and eukaryotic receptors responsible for mediating specific bacterial binding to these cell lines. Bacterial attachment to host cells was determined by viable count and indirect immunofluorescence assay methods. Results from these studies suggested that one or more lectin-like structures on the surface of *L. pneumophila* organisms may potentiate the specific recognition of eukaryotic host cells.
3.2 Introduction

The specific recognition of host cells by pathogenic bacteria forms a necessary prelude to the initial stages of the disease state (8). Duguid (28) first coined the term "adhesin" in 1959 to describe the adhesive structures on bacteria which facilitate their ability to attach to susceptible eukaryotic cells. This work remained largely unnoticed until Gibbons (56) reported on the adherence of bacteria to specific areas of the oral cavity. Since that time, numerous and extensive investigations (9, 25, 39, 41, 92, 93, 95, 96, 97, 98, 100, 117, 119, 120, 126, 131, 132) concerning the interaction of pathogenic organisms such as Escherichia coli, Neisseria gonorrhoeae, Vibrio cholerae and Streptococcus pyogenes with host cells have increased our understanding of how bacteria initiate disease. These reports described experiments in which the in situ blocking, inhibition or enzyme degradation of putative bacterial adhesins and eukaryotic receptors were designed to identify these structures and characterize the nature of microbial adherence to host cell surfaces. Further studies demonstrating the inhibition of binding by these isolated bacterial components/adhesins or by adhesin/receptor specific antibody provided additional evidence that these molecules were responsible for bacterial attachment. Although a number of these structures have been implicated by these methods, pili are the best studied (98, 119) and have been shown for a number of bacterial species to act sequentially or concomitantly with other attachment factors (39, 131) during the binding process.

The elucidation of adhesins and their receptors has led to the
development of novel strategies for the prevention and control of infectious
diseases. Prophylactic or post-infection treatment with anti-adherence vaccines
and purified adhesin/receptor or adhesin/receptor analogues used in a
competitive fashion have yielded encouraging clinical results (8). In addition,
studies have indicated that sublethal doses of antibiotics may alter the ability of
some bacteria to adhere to host cell surfaces (22).

*L. pneumophila* is a facultative intracellular pathogen which replicates
within alveolar macrophages in clinical disease but has been shown to multiply
within a wide variety of eukaryotic cells *in vitro* (36, 74, 75, 91, 138). The
preliminary steps involved in the attachment of this organism to potential host
cells prior to invasion have not been elucidated. However, *L. pneumophila* has
been shown to possess pili, fimbriae and flagella (108, 110) on their surface
and these, as pointed out by Rodgers (106) and Figure 3.1, may potentiate
binding by bridging the gap between the negatively charged surfaces of the
invading pathogen and host target cells.

Oldham and Rodgers (91) studied the interaction of *L. pneumophila* with
MRC-5 cells, a human embryonic lung cell line, both quantitatively (viable
counts following eukaryotic cell lysis) as well as qualitatively (by electron
microscopy). Attachment of the legionellae to cells was followed by a
thickening of eukaryotic cell membranes and the proliferation of microvilli which
led to bacterial endocytosis by a process which they termed "bacteriopexis".
More recently, Cianciotto *et al.* (23, 37) used U937 cells, a macrophage-like,
human histiocytic lymphoma cell line, as a cellular model of infection and
suggested that a 24-kDa surface protein of \textit{L. pneumophila} potentiated infectivity and subsequent intracellular replication. In addition, because similar results with explanted human alveolar macrophages were obtained, these investigators have demonstrated the relevance of this transformed cell line in the study of \textit{L. pneumophila} pathogenesis \textit{in vitro}.

Patients with systemic Legionnaires' disease often respond poorly to antibiotic therapy. Vaccines prepared to stimulate antibody production against whole \textit{L. pneumophila} organisms lead to avid opsonophagocytosis by the "preferred" host cells (macrophages) and may prove counterproductive to therapy. The objective of this study was to conduct preliminary studies on the attachment of \textit{L. pneumophila} to U937 and MRC-5 cells as a prerequisite to infection at the cellular level. Specific goals included the development and establishment of the experimental parameters for adherence assays as well as the partial characterization of bacterial "adhesins" and eukaryotic receptors involved in the "recognition" process. Fixatives, lectins, detergents, oxidizing chemicals, degradative enzymes and sugars were used to modify either bacterial or eukaryotic cell surfaces. Following these treatments, \textit{L. pneumophila} was added to the test cells and adherence assay experiments performed. Viable counts of organisms (following eukaryotic cell lysis) and immunofluorescence assays (IFA) (for direct counts of labelled bacteria) were done and the number of bound organisms following adherence was determined. Percent inhibition of binding was ascertained by direct comparison of results from treated and untreated control trials.
Due to the complexity of the bacterial and host-cell membranes, data derived from studies of this nature are often hard to interpret and the experimental procedures often lead to contradictory results making definitive adherence criteria difficult to elucidate (8, 70, 120). For these reasons, researchers have based their conclusions upon well-controlled, stringently analyzed experiments. It has been generally accepted that only those experimental treatments that inhibited bacterial adherence at a level equal to or greater than 50% of untreated controls were significant. In the present study, similar inhibition levels were used to measure the experimental significance of treatments for the binding of \textit{L. pneumophila} to U937 or MRC-5 cells. In addition, because quantitative measurements of bacteria binding are often difficult to assess accurately, two different types of assay methods were used, and the results cross-referenced, to evaluate bacterial binding to host cells.

The identification of the factors that lead to bacterial adherence will help characterize the initial stages of bacterial invasion of host cells and increase our understanding of the pathogenic properties of \textit{L. pneumophila}. The results from these studies may suggest potential clinical regimens for blocking infection at the cellular level or the development of split vaccines leading to novel approaches in the prevention and control of Legionnaires' disease.

3.3 Materials and Methods

Reagent formulations and preparations along with detailed procedures are given in Appendices 1 and 4.

**Bacterial strain and cultivation.** \textit{L. pneumophila} serogroup 1, strain
Nottingham N7, was isolated from the sputum sample of a fatal case of Legionnaires' disease, subsequently passaged on bacteriological media, and maintained fully virulent in a frozen state in serum sorbitol at -70°C. Cultures were grown on buffered charcoal yeast extract agar supplemented with \( \alpha \)-ketoglutarate (BCYE\( \alpha \)) (29) as previously described (128, 129) at 37°C for 48 h. To ensure the use of a consistent population of organisms in all experiments, *L. pneumophila* were harvested from plates, inoculated into buffered yeast extract broth supplemented with \( \alpha \)-ketoglutarate (BYE\( \alpha \) broth) (112) to give an initial concentration of approximately \( 10^5 \) organisms/ml and incubated in a shaking water bath for 24 h at 37°C until the culture had reached approximately \( 2-3 \times 10^8 \) colony forming units (cfu)/ml as determined by subsequent viable counts. Bacteria were harvested by centrifugation at 3000 \( \times \) g in a Beckman Microfuge 12 (Beckman Instruments, Palo Alto, Ca.). This procedure did not alter either the ability of organisms to adhere to eukaryotic cells or influence the LD\( _{50} \) for the fertile hen's egg (see Section 5). Organisms were washed in Hank's Balanced Salt Solution (HBSS) (Irvine Scientific, Santa Ana, Ca.) and diluted 1:10 in HBSS prior to adherence assays. The ratio of bacteria to cells was approximately 100:1 for both cell lines used in these trials. The rationale for the selection of this multiplicity of infection (MOI) as the inoculum for both cell lines was based on data derived from a) bacterial adherence experiments (see Adherence to U937 and MRC-5 cells, p. 44) and b) adherence studies on other microorganisms in which an inocula of 100 MOI was used.
Eukaryotic cells and growth conditions. U937 cells. U937 cells, derived from a histiocytic lymphoma cell-line, were obtained from the ATCC (Rockville, Md.) and maintained as replicative, non-adherent monocyte-like cells in Minimal Essential Medium with Earle's salts (MEM) (Irvine Scientific, Santa Ana, Ca.) supplemented with 10% bovine calf serum (Hyclone Laboratories, Inc., Logan, Utah) and 3mM L-glutamine (Sigma Chemicals, St. Louis, Mo.). The cells were grown in T-75 cm² flasks (Costar, Cambridge, Ma.) and harvested for use in adherence trials at points when the culture reached late exponential phase of growth (1-2 x 10⁶ cells/ml). At this stage, the cells were differentiated into a non-replicative, adherent macrophage-like cell line with 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma Chemicals, St. Louis, Mo.), which was added at a final concentration of 10⁻⁸ M for 48 h. Cells were removed from flasks with 0.2% EDTA, washed with media, and distributed into 24-well plastic plates (Costar, Cambridge, Ma.) at a cell density of 4-8 x 10⁵ cells/well for use in viable count adherence assays or into similar 6-well plates containing glass coverslips at a cell density of 5-8 x 10⁵ cells/well for use in IFA counts.

MRC-5 cells. MRC-5 cells, a semi-continuous, adherent line of human embryonic lung fibroblasts, were obtained from Flow Laboraties (McLean, Va.) and maintained in MEM supplemented with 10% bovine calf serum and 3 mM L-glutamine. Cells were grown to a confluent state in 24-well or 6-well plates (on glass coverslips) and used directly for viable count or IFA L. pneumophila adherence assays. Confluent cell monolayers reached a density of
approximately $1-2 \times 10^5$ cells/well in 24 well plates and approximately $2-3 \times 10^5$ cells/cover slip in 6 well plates.

**Adherence to U937 and MRC-5 cells.** To determine the maximum bacterial binding capacity to both cell types in these assays, *L. pneumophila* was grown in BYEα broth for 24 h as described. Cultures were pooled, centrifuged, serially diluted ten-fold and each dilution added to 6 and 24-well plates containing either U937 or MRC-5 cells for 1 h. The number of organisms bound to each cell type was assayed by viable count and IFA methods to determine the specificity of bacterial attachment to these cells.

**Inhibition of adherence.** **Bacterial treatments.** The role of *L. pneumophila* surface proteins and sugar moieties in the adherence process was examined. *L. pneumophila* was treated with degradative enzymes, detergents, fixative and oxidizing chemicals and adherence determined by the viable count and IFA methods described below (p. 45-47). The type, treatment time, concentrations and mode of action of the agents used to modify the surface of the bacteria are shown in Table 3.1. All agents, with the exception of glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, Pa.), were obtained from Sigma Chemicals (St. Louis, Mo.) and prepared at the concentration specified in HBSS prior to testing. Bacteria were treated with the test agent for the time indicated, washed three times in HBSS and used in the adherence assays (p. 45)

**Eukaryotic treatments.** The role of eukaryotic surface protein, glycoprotein, lipid, glycolipid or sugar was examined as possible adhesins in
the binding process. The type, treatment time, concentrations, and mode of action of the degradative enzymes, detergents, fixatives, oxidizing chemicals and lectins used to modify the surfaces of U-937 and MRC-5 cells are shown in Table 3.2. All agents were obtained from Sigma Chemicals (St. Louis, Mo.) with the exception of the glutaraldehyde and formaldehyde (Electron Microscopy Sciences, Ft. Washington, Pa.) and prepared at the specified concentration in HBSS prior to testing. Cells were treated with agents for the time indicated and washed three times with HBSS prior to adherence assays.

**Sugars.** A number of sugars were examined as possible inhibitors of the attachment process by conducting adherence assays in the presence of these carbohydrates. Monosaccharides (Sigma Chemicals, St. Louis, Mo.) used in these studies are shown in Table 3.3. Sugars were prepared at the specified concentrations in HBSS, adjusted to pH 7.2 and added to cells 10 minutes prior to the addition of *L. pneumophila* for adherence experiments.

**Adherence assays.** U937 and MRC-5 cells were washed three times with HBSS prior to adherence experiments. Following either treatment of bacteria and/or eukaryotic cells with the agents to be tested or after competitive binding, cells were washed with HBSS and *L. pneumophila* added to wells of 6 and 24-well plates and allowed to adhere to eukaryotic cells for 1 h. Appropriate control organisms and cells were treated with HBSS. After the binding period, unattached organisms were removed by three-fold washings with HBSS and host-cell bound organisms assayed by viable count and IFA methods as follows:
Viable counts. After removal of non-adherent *L. pneumophila*, eukaryotic cells were lysed by the addition of 1 ml of sterile distilled water. This procedure did not affect the viability of the bacteria as ascertained by viable count studies of similarly treated organisms. Bacterial counts were made on lysates from each of 10 separate wells by inoculating 10μl in duplicate of appropriate dilutions onto BCYEα plates. Plates containing 30 to 300 colonies after an incubation period of 72 h were used to calculate average viable counts of lysates for each well as previously described (91). Ratios of cfu/eukaryotic cell were determined by dividing average lysate values for each well by the number of eukaryotic cells seeded per well. These values were compared to untreated control values within each experiment for determination of the percent reduction of adherence following each treatment used.

Non-specific binding of organisms to the plastic culture plates was determined for each experimental trial. For every well, viable count estimations of unbound organisms contained in washes made following the 1 h incubation period for adherence assays were determined. This value was added to the corresponding lysate value for the appropriate well and subtracted from the inoculum. The resultant figure was used to estimate the presence of bacteria non-specifically stuck to exposed plastic not covered by the cell monolayers. In multiple experiments, this value was found to be a consistently small percentage of the inoculum. The level of nonspecific binding was found to be unaffected by bacterial or eukaryotic cell treatments and was not altered by the
addition of de-ionized water.

**Immunofluorescence.** Following bacterial or eukaryotic cell treatments and binding experiments, U937 and MRC-5 cells were fixed in 10% buffered formalin for 12 h, washed in HBSS, and treated in situ on the glass coverslips with a 1:100 dilution of rabbit anti-*L. pneumophila* antibody (see Appendix 4) for 1 h at 37°C. Unbound globulin was removed with three-fold washings of HBSS. Cells were then treated with a 1:200 dilution of goat anti-rabbit FITC conjugated antibody (Cooper Biomedical, Malvern, Pa.) for 1 h at 37°C. Unbound globulin was removed by repeated washings and cells counterstained with 1% (w/v) propidium iodide for 5 min. Glass coverslips were mounted in 1% (w/v) 1,4-diazabicyclo (2.2.2.) octane (DABCO) (Sigma Chemicals, St. Louis, Mo.) in glycerin onto microscope slides and viewed with an Olympus BH-2 epifluorescence microscope using an excitation filter emitting a wavelength of 490 nm and a barrier filter blocking wavelengths above 515 nm. The number of fluorescing organisms bound to 200 eukaryotic cells was determined and expressed as adherent organisms/cell. These were compared to control values within each experiment to determine the percent reduction of adherence. Non-specific binding of labelled organisms to areas of glass not covered by eukaryotic cells was ascertained by IFA. Although this was noted to be insignificant in all experiments, these organisms were not counted in the IFA assay for adherence to host cells.

3.4 Results

**Adherence to U937 and MRC-5.** Bacterial binding to U937 and
MRC-5 cells is illustrated by IFA in plate 3.1 and binding curves for both cell lines are summarized in Figures 3.2-3.5. For each cell type, the use of approximately 5 x 10^8 cfu/ml as the inoculum gave adherence kinetics approaching saturation of the eukaryotic receptors. This was consistent for either U937 (Figures 3.2 and 3.3) or MRC-5 (Figures 3.4 and 3.5) cells as determined by both assay techniques. The saturation points corresponded to an average cfu/cell ratio of 2.5 and 11 for U-937 (Figure 3.2) and MRC-5 (Figure 3.4) cells, respectively, as assayed by viable counts. IFA equivalent ratios were 3.1 and 6.9 organisms/cell for U937 (Figure 3.3) and MRC-5 (Figure 3.5) cells, respectively. At this high inoculum level, marked non-specific binding to the plastic of culture dishes and glass coverslips were noted by both viable counts and IFA. However, the use of an inoculum with 10-fold fewer organisms (approximately 5 x 10^7 cfu/ml) gave non-specific binding levels that were dramatically reduced. This inoculum was equivalent to about 100 bacteria/eukaryotic cell (or 100 MOI) and approached the saturation point of eukaryotic receptors of both cell lines.

**Inhibition of adherence studies.** Bacterial treatments. Figures 3.6-3.9 illustrate the percent reduction of adherence following modification of \( \text{L. pneumophila} \) with the agents listed in Table 3.1. Prior treatment of \( \text{L. pneumophila} \) with glutaraldehyde, \( \beta \)-galactosidase, chymotrypsin, lipase, protease, trypsin and sodium metaperiodate all effected a greater than 50% reduction in adherence to U937 cells as compared with controls in both viable count and immunofluorescence assays. For MRC-5 cells only bacterial
treatments with glutaraldehyde, chymotrypsin, protease, trypsin and sodium metaperiodate effected a similar response; i.e., unlike for U937 cells, lipase and β-galactosidase treatment did not significantly influence bacterial binding to MRC-5 cells. Bacterial adherence or inhibition of binding to both U937 and MRC-5 cells was consistently similar whether assayed by viable count or IFA methods.

**Eukaryotic treatment.** Figures 3.10-3.13 illustrate the percent reduction in adherence following modification of U937 and MRC-5 cell surfaces with the agents listed in Table 3.2. Concanavalin A, wheat germ agglutinin, sodium metaperiodate, nonidet P40, glutaraldehyde and formaldehyde reduced binding of *L. pneumophila* to treated U937 cells by greater than 50% (Figures 3.10 and 3.11). Similar results were obtained by viable count and IFA generated data. It was noted that none of the enzymatic treatments reduced bacterial binding to U937 cells. In addition, modification of U937 cell surfaces with cytochalasin B, a eukaryotic microtubule formation inhibitor, did not result in differences between treated and untreated cells as measured by either assay technique. These results were difficult to interpret but suggested that bacterial uptake by the phagocytic U937 cells may be independent of attachment since the difference between the two assays was not great.

The percent reduction of adherence to MRC-5 cells following treatment with the modifiers listed in Table 3.2 are illustrated in Figure 3.12 and 3.13. Concanavalin A, wheat germ agglutinin, sodium metaperiodate, nonidet P40, glutaraldehyde and formaldehyde effected significant reductions in bacterial
binding as measured by viable counts and IFA. However, viable counts but not IFA indicated that lipase and chymotrypsin treatment of cells reduced adherence by greater than 50%.

In all eukaryotic treatments with either cell line assayed by both techniques, concanavalin A, wheat germ agglutinin, glutaraldehyde and formaldehyde were the strongest inhibitors of bacterial binding.

**Sugars.** Co-incubation of L. pneumophila with either cell line in the presence of those sugars listed in Table 3.3 are illustrated in Figures 3.14-3.17. Of those tested, no monosaccharide was able to significantly reduce bacterial binding to U937 or MRC-5 host cells. Viable count and IFA techniques yielded similar results for all sugars tested.

3.5 Discussion.

The adherence of microorganisms to host cells as a prelude to the disease process has been well documented (8). A number of bacterial components, including pili and fimbriae, have been shown to mediate the specific attachment of microbes to eukaryotic cells (8, 70, 116), but evidence has suggested that these and other structures may act concomitantly or sequentially (39, 131) in this process. In addition, the role of van der Waals, thermodynamic forces and hydrophobic interactions in the initial attachment and eventual binding of bacteria to cell surfaces is still unclear. The parameters for both an effective bacterial binding assay and a rationale for determining adherence were established. These criteria were used to partially characterize the nature of "adhesins" involved in L. pneumophila adherence.
The adherence curves indicated that a specific interaction between \textit{L. pneumophila} and both cell types occurred. Bacterial binding to eukaryotic receptors approached apparent saturation levels when an inoculum of approximately $10^8$ cfu/ml was used in adherence assays. However, this data required caution in interpretation as increasing quantities of non-specific binding to plastic wells were noted to exceed acceptable levels at this point.

Determination of adherence as measured by viable count and IFA techniques yielded consistent and corroborative results and provided evidence that a lectin-like adhesin(s) associated with the surface of \textit{L. pneumophila} was in part, responsible for mediating attachment to U937 and MRC-5 cells. Degradation and immobilization of bacterial surface proteins with enzyme and fixative treatments as well as periodate oxidation of microbial sugars significantly reduced \textit{L. pneumophila} adherence to both types of host cells. That lipase and β-galactosidase treatment of bacteria prior to adherence experiments significantly reduced adherence to U937 cells, but not to MRC-5 implied that lipid and lactose moieties may also be important components in the bacterial binding process. Several gram-negative bacteria possess lectin-like molecules associated with pili, fimbriae, or flagella which enable them to adhere to host cell surfaces \cite{8, 70, 120}. \textit{L. pneumophila} possesses analogous bacterial appendages \cite{108 and Figure 3.1} which may facilitate bacterial binding by similar mechanisms. Isolation of these structures in pure form for use as potential blocking agents in similar adherence experiments will further elucidate their role as adhesins.
Treatment of eukaryotic cell surfaces with the lectins concanavalin A and wheat germ agglutinin substantially reduced bacterial binding and corroborated our results from the bacterial treatment trials. That oxidation of eukaryotic cell surface sugars with sodium metaperiodate inhibited bacterial binding further supported these findings. However, co-incubation of bacteria and cells in the presence of mannose or N-acetyl glucosamine (sugars specific for concanavalin A and wheat germ agglutinin) failed to appreciably inhibit attachment. This may be due to the presence of dissacharides or complex oligosaccharides on the surfaces of the U937 and MRC-5 cells that contain the appropriate sub-unit monosaccharides specific for concanavalin A and wheat germ agglutinin. Therefore, the addition of simple monosaccharides as potential blocking agents in these experiments may not be recognized by \textit{L. pneumophila} lectin-like surface molecules and consequently failed to reduce adherence. Use of dissacharides or oligosaccharides in similar competitive blocking studies may elucidate putative receptors for \textit{L. pneumophila}. However, determination of the nature of these "docking" sites by these methods are easily complicated by steric considerations such as the presence of disaccharide sugar receptors conjugated with proteins, lipids or other cell membrane moieties. In this study, efforts were focused on the bacterial component of \textit{L. pneumophila} adherence to host cells.

That fixation of cell surfaces drastically reduced \textit{L. pneumophila} binding to U937 and MRC-5 cells while treatment with proteolytic enzymes did not, may signify a requirement for mobile, complex proteins or glycoproteins to facilitate
the adherence of bacteria to these cells. In addition, oxidation of eukaryotic cell surface sugars with sodium metaperiodate actively inhibited bacterial binding. A possible role for these molecules could be selective steric masking or exposure of receptors specific for adhesins on the surface of \textit{L. pneumophila}. In addition, treatment with nonidet P-40 blocked adherence of \textit{L. pneumophila} to both cell lines whereas similar treatment with lipase was generally not effective (reducing adherence to MRC-5 cells by 53\% as assayed by viable counts only). These results suggested membranous lipid moieties as possible components of eukaryotic receptors as well. It was clear from the compiled data that a simple and uniform receptor system for \textit{L. pneumophila} may not exist on the surface of U937 and MRC-5 cells. Indeed, synergistic, sequential, steric and hydrophobic interactions between different membrane components of host cells may have a role in the recognition process and further complicate the elucidation of host cell receptors specific for \textit{L. pneumophila}.

The cytochalasins inhibit the internalization processes of eukaryotic cells and have been used to determine whether pathogens enter host cells by means of active invasion or phagocytosis. Elliott and Winn (36) have shown that \textit{L. pneumophila} fails to actively penetrate cytochalasin-treated guinea pig alveolar macrophages \textit{in vitro}. However, the attachment of bacteria to phagocyte membranes was not inhibited in these trials. In the present studies, adherence experiments following treatment of the macrophage-like U937 cells with cytochalasin B resulted in similar binding capacities as assayed by viable counts and IFA when compared to untreated controls. Given that viable counts
measure attached and internalized organisms while IFA staining facilitate the
detection of only bound bacteria these experiments indicate that significant
numbers of legionellae were not phagocytosed during the one hour binding
assay and that a process of "uptake-independent-adherence" (UIA) occurred for
*L. pneumophila*.

Caution should be exercised when extrapolating *in vitro* results derived
from laboratory experimental conditions to the context of the clinical situation.
Neither eukaryotic cell type used in this study were the natural host cell of *L.
pneumophila* in human clinical disease. Although both are human cell lines,
U937 cells are macrophage-like cells derived from a histiocytic lymphoma and
may possess characteristics not resembling those of primary alveolar
macrophages. MRC-5 cells are a semi-continuous fibroblast cell from the lung
and exhibit few of the properties of alveolar macrophages. Other potential
factors include environmental differences that exist between cells propagated *in
vitro* as compared with those grown *in vivo*. Exposure to disparate nutritional
sources, regulatory signals and other microorganisms could effect the
expression of host target cell receptors. Similarly, varying nutritional conditions
between the *in vitro* and clinical environment will most likely affect the growth of
microorganisms and may alter expression of adhesins responsible for
attachment to eukaryotic cells.

The data from these studies offer evidence that a lectin-like adhesin(s)
mediates attachment to U937 and MRC-5 cells. The nature of this receptor(s)
remains to be elucidated. Host cell receptors most likely consist of
oligosaccharide and lipid structures that interact to form complex but as yet unresolved "docking site" for the organism. The finding that lectin-like adhesins occur on other gram-negative bacteria possessing appendages such as pili, fimbriae, or flagella in association with these adhesive molecules suggest that \textit{L. pneumophila} may utilize similar attachment strategies. Studies using these and other fractionated bacterial structures as blocking agents along with wider classes of saccharides in adherence assays could further characterize or identify specific components responsible for mediating attachment. The elucidation of factors which contribute to the binding of \textit{L. pneumophila} to host cells will increase our understanding of the pathogenic mechanisms used by this intracellular pathogen to initiate disease and may eventually influence the prevention and control of clinical legionellosis.
Legionella organism showing A surface features and B cell wall structure. C "unit membrane" structure of a eucaryotic cell.

Figure 3.1. *Legionella pneumophila*. Schematic representation of potential "adhesins" and eukaryotic cell receptors.
(Reproduced with kind permission from reference 38, Oldham and Rodgers, Zbl. Bakt. I Abt. Orig. A.)
Table 3.1. Bacterial treatments prior to adherence assays

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration</th>
<th>Treatment Time (min)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-galactosidase</td>
<td>100 units/ml</td>
<td>60</td>
<td>Degradation of protein/sugar-containing adhesins on surface of L. pneumophila</td>
</tr>
<tr>
<td>chymotrypsin</td>
<td>250</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>lipase</td>
<td>100</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>neuraminidase</td>
<td>20</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>protease</td>
<td>5</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>trypsin</td>
<td>250</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Fixative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>glutaraldehyde</td>
<td>0.1%</td>
<td>10</td>
<td>Immobilization of protein moieties</td>
</tr>
<tr>
<td>Oxidizing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agent</td>
<td>Concentration</td>
<td>Treatment Time (min)</td>
<td>Function</td>
</tr>
<tr>
<td>sodium</td>
<td>10mM</td>
<td>30</td>
<td>Oxidation of carbohydrate moieties</td>
</tr>
<tr>
<td>metaperiodate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agent</td>
<td>Concentration</td>
<td>Treatment</td>
<td>Function</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------</td>
<td>-----------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td></td>
<td>Time (min)</td>
<td>Time (min)</td>
<td></td>
</tr>
<tr>
<td>Enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>0.1</td>
<td>30</td>
<td>Degradation of enzyme function</td>
</tr>
<tr>
<td>Lipase</td>
<td>100</td>
<td>30</td>
<td>sensitive receptors</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>1.0</td>
<td>30</td>
<td>on host cells</td>
</tr>
<tr>
<td>Pepsin</td>
<td>100</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Protease</td>
<td>0.005</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>50</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agent</td>
<td>Concentration</td>
<td>Treatment</td>
<td>Function</td>
</tr>
<tr>
<td></td>
<td>Time (min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>0.1%</td>
<td>10</td>
<td>Immobilization of protein-con</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1.0%</td>
<td>10</td>
<td>containing moieties</td>
</tr>
<tr>
<td>Nonidet P40</td>
<td>0.005%</td>
<td>60</td>
<td>Action on membrane lipids</td>
</tr>
<tr>
<td>Sodium metaperiodate</td>
<td>5mM</td>
<td>10</td>
<td>Oxidation of carbohydrate moieties</td>
</tr>
<tr>
<td>cytochalasin B</td>
<td>3ug/ml</td>
<td>60</td>
<td>Inhibition of microfilament formation/phagocytosis</td>
</tr>
<tr>
<td>Lectins</td>
<td>Concentration</td>
<td>Treatment</td>
<td>Function</td>
</tr>
<tr>
<td></td>
<td>Time (min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>100ug/ml</td>
<td>60</td>
<td>Binds to mannose</td>
</tr>
<tr>
<td>Wheat Germ Agglutinin</td>
<td>100ug/ml</td>
<td>60</td>
<td>Binds to N-acetyl glucosamine</td>
</tr>
<tr>
<td>Sugars</td>
<td>Concentration</td>
<td>Function</td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>---------------</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>1) N-acetyl-D-galactosamine</td>
<td>100mM</td>
<td>Competitive binding</td>
<td></td>
</tr>
<tr>
<td>2) N-acetylneuraminic acid</td>
<td>100mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3) N-acetylglucosamine</td>
<td>100mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4) α-D(-)-Fucose</td>
<td>100mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5) β-D(+) -glucose</td>
<td>100mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6) D(+) -mannose</td>
<td>100mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7) D(+) -galactose</td>
<td>100mM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.2 Bacterial binding of *L. pneumophila* to U937 cells as measured by viable counts of organisms released from lysed cells. Ratios of cfu per cell were calculated by dividing the average lysate value obtained from each inoculum by the average eukaryotic cell seed per well. For this experiment the average cell seed equaled $5.7 \times 10^5$ cells/well. Bacterial attachment to eukaryotic cell receptors approached saturation when an inoculum of approximately $5 \times 10^8$ cfu/ml was added to cell cultures (arrow). This corresponded to a ratio of 2.5 cfu/eukaryotic cell. Each point represents an average of three separate trials.
Figure 3.3 Bacterial binding of *L. pneumophila* to U937 cells as measured by IFA. The number of fluorescing organisms bound to approximately 200 eukaryotic cells is expressed as a ratio of organisms/eukaryotic cell for each inoculum used. An inoculum of about $5 \times 10^8$ approached saturation of eukaryotic receptors (arrow). This corresponded to a ratio of 3.1 organisms/eukaryotic cell.
Figure 3.4 Bacterial binding of *L. pneumophila* to MRC-5 cells as measured by viable counts of organisms released from lysed cells. Ratios of cfu per cell were calculated by dividing the average lysate value obtained from each inoculum by the average eukaryotic cell seed per well. For this experiment the average cell seed equaled $1.6 \times 10^5$ cells/well. Bacterial attachment to eukaryotic cell receptors approached saturation when an inoculum of approximately $5 \times 10^8$ cfu/ml was added to cell cultures (arrow). This corresponded to a ratio of 11 cfu/eukaryotic cell. Each point represents an average of three separate trials.
Figure 3.5 Bacterial binding of *L. pneumophila* to MRC-5 cells as measured by IFA. The number of fluorescing organisms bound to approximately 200 eukaryotic cells is expressed as a ratio of organisms/eukaryotic cell for each inoculum used. An inoculum of about $5 \times 10^8$ approached saturation of eukaryotic receptors (arrow). This corresponded to a ratio of 6.9 organisms/cell.
Figure 3.6  Bacterial treatments, U937 cells. Percent reduction of *L. pneumophila* binding to U937 cells as measured by viable count estimation of eukaryotic lysates following treatment of bacteria with the agents listed in Table 3.1. Each value represents the average of 10 separate trials. Error bars indicate standard error of the mean for each treatment.
Figure 3.7 Bacterial treatments, U937 cells. Percent reduction of *L. pneumophila* binding to U937 cells as measured by counts of fluorescing organisms bound to approximately 200 eukaryotic cells following treatment of bacteria with the agents listed in Table 3.1.
Figure 3.8 Bacterial treatments, MRC-5 cells. Percent reduction of *L. pneumophila* binding to MRC-5 cells as measured by viable count estimations of eukaryotic lysates following treatment of bacteria with the agents listed in Table 3.1. Each value represents the average of 10 separate trials as compared with untreated controls. The standard error of the mean is indicated for each treatment.
Figure 3.9 Bacterial treatments, MRC-5 cells. Percent reduction of *L. pneumophila* binding to MRC-5 cells as measured by counts of fluorescing organisms bound to approximately 200 eukaryotic cells following treatment of bacteria with the agents listed in Table 3.1.
Figure 3.10 Eukaryotic cell treatments, U937 cells. Percent reduction of *L. pneumophila* binding to U937 cells as measured by viable count estimations of eukaryotic lysates following treatment of host cells with the agents listed in Table 3.2. Each value represents the average of 10 separate trials. Error bars indicate standard error of the mean for each treatment.
Figure 3.11 Eukaryotic cell treatments, U937 cells. Percent reduction of *L. pneumophila* binding to U937 cells as measured by counts of fluorescing organisms bound to approximately 200 eukaryotic cells following treatment of host cells with the agents listed in Table 3.2.
Figure 3.12 Eukaryotic cell treatments, MRC-5 cells. Percent reduction of *L. pneumophila* binding to MRC-5 cells as measured by viable count estimation of eukaryotic lysates following treatment of host cells with the agents listed in Table 3.2. Each value represents the average of 10 separate trials. Error bars indicate the standard error of the mean for each treatment.
Figure 3.13 Eukaryotic cell treatments, MRC-5 cells. Percent reduction of L. pneumophila binding to MRC-5 cells as measured by counts of fluorescing organisms bound to approximately 200 eukaryotic cells following treatment of host cells with the agents listed in Table 3.2.
Figure 3.14 Competitive binding studies with sugars, U937 cells. Percent reduction of bacterial adherence following co-incubation of \textit{L. pneumophila} and U937 cells in the presence of monosaccharide sugars listed in Table 3.3 as measured by viable counts of eukaryotic lysates. Concentration of added sugars was 100 mM. Each value represents the average of 10 separate trials. Standard error of the mean is indicated for each treatment. None of the sugars used in these experiments effected a significant reduction of adherence.
Figure 3.15 Competitive binding studies with sugars, U937 cells. Percent reduction of bacterial adherence following co-incubation of *L. pneumophila* and U937 cells in the presence of monosaccharide sugars listed in Table 3.3 as measured by counts of fluorescing organisms bound to approximately 200 eukaryotic cells. None of the sugars used in these experiments effected a significant reduction of adherence. Concentration of added sugars was 100 mM.
Figure 3.16 Competitive binding studies with sugars, MRC-5 cells. Percent reduction of bacterial adherence following co-incubation of *L. pneumophila* and MRC-5 cells in the presence of monosaccharide sugars listed in Table 3.3 as measured by viable counts of eukaryotic lysates. Concentration of added sugars was 100 mM. Each value represents the average of 10 separate trials. Standard error of the mean is indicated for each treatment. None of the sugars used in these experiments effected a significant reduction of adherence.
Figure 3.17 Competitive binding studies with sugars, MRC-5 cells. Percent reduction of bacterial adherence following co-incubation of *L. pneumophila* and MRC-5 cells in the presence of monosaccharide sugars listed in Table 3.3 as measured by counts of fluorescing organisms bound to approximately 200 eukaryotic cells. Concentration of added sugars was 100 mM.
Plate 3.1 *L. pneumophila* binding to U937 and MRC-5 cells in an indirect immunofluorescence assay. Organisms are labelled with FITC (green) while eukaryotic cells are counterstained with propidium iodide (red).

a) *L. pneumophila* attachment to U937 cells. Note incidence of bacterial binding to host cell surfaces (X 660).

b) Higher magnification of *L. pneumophila* binding to MRC-5 cells. Note single organism attached along its length to the host cell surface (X 1,650).
Section IV

The Effect of Antibiotics That Inhibit Cell Wall, Protein and DNA Synthesis on the Growth and Morphology of *Legionella pneumophila*

4.1 Abstract

The response of *L. pneumophila* to cell wall, protein synthesis and DNA synthesis inhibitory antibiotics was examined by electron microscopy, MIC estimations and viable count assays. Cefotaxime, methicillin, rifampicin and ciprofloxacin, each used separately at 20 times their respective MIC values, showed activity against *L. pneumophila* in these studies. The cell wall inhibitors, cefotaxime and methicillin, effected the greatest bactericidal activity and induced the most extensive morphological changes. Organisms treated with these antibiotics lost cytoplasmic contents through membranous lesions induced in their cell walls. In terms of ultrastructural damage and loss of viability, the protein and DNA synthesis inhibitors were less efficacious than antibiotics that acted on the microbial cell wall. Rifampicin treated cells possessed irregular membranes and were partially or fully lysed while ciprofloxacin induced abnormally elongated organisms with intermittently lysed and detached inner membranes. These results illustrated the ability of clinically putative antibiotics with diverse modes of action to affect microbial cytology at the ultrastructural level as well as the viability of *L. pneumophila* in vitro.
4.2 Introduction

*Legionella pneumophila*, the causal agent of a fatal form of lobar pneumonia known as Legionnaires' disease, has been studied by negative stain, thin-section, freeze-etching, and scanning electron microscopy (105, 108). The response of this human pathogen to treatment with antimicrobial chemotherapeutic agents has been investigated using various *in vitro* and *in vivo* techniques (19, 30, 35, 44, 45, 57, 60, 65, 83, 107, 109, 112, 128, 129, 142). Although several antibiotics have excellent activity against *L. pneumophila* *in vitro* as assessed in minimum inhibitory concentration (MIC) studies, few show similar results in clinical trials.

Erythromycin is the current drug of choice for the treatment of legionellosis; however, inherent problems arise in its use. These include reports of relapse of infection, onset of phlebitis with intravenous antibiotic administration and potential complications associated with the development of bacterial resistance to therapy. In addition, studies in which Elliott and Rodgers (35) examined the morphological and growth response of *L. pneumophila* to erythromycin and ampicillin illustrated that erythromycin had limited bactericidal activity *in vitro*. Rifampicin has proven effective in clinical trials but is not used as the sole therapeutic drug because of the high frequency with which resistance is induced. For these reasons, a number of other antimicrobial agents have been investigated as potential alternatives for clinical use in the treatment of legionellosis. Of those examined, the quinolone antibiotics have been the most promising. Ciprofloxacin, the best studied of this group, is a lipid
soluble antimicrobial which inhibits bacterial DNA gyrase and has yielded encouraging results against experimental \textit{L. pneumophila} infection \textit{in vivo} \cite{44, 45, 128, 129}.

In the present study, the morphological characteristics and growth response of \textit{L. pneumophila} was examined following exposure to antimicrobials derived from three groups of antibiotics (bacterial cell wall, protein synthesis and DNA inhibitors). The evaluation of the efficacy of these drugs was determined using MIC estimations, viable count data and ultrastructural investigations.

4.3 Materials and Methods.

Reagent formulations and preparation along with detailed procedures are given in the appendices 1 and 3.

**Bacterial strain and growth conditions.** \textit{L. pneumophila} serogroup 1, strain Nottingham N7 was isolated from a sputum sample of a fatal case of Legionnaires' disease, was subsequently passaged twice on bacteriological media and maintained fully virulent in a frozen state in serum sorbitol at -70° C. Cultures were grown on buffered charcoal yeast extract agar supplemented with \( \alpha \)-ketoglutarate (BCYE\( \alpha \)) \cite{29} at 37° C for 48 h. Organisms were harvested from plates and inoculated into \( \alpha \)-ketoglutarate enriched buffered yeast extract (BYE\( \alpha \)) broth containing 10 g/l yeast extract (Difco), 10g/l ACES buffer (Sigma), 1.0g/l \( \alpha \)-ketoglutarate (Sigma), 0.4 g/l L-cysteine (Sigma) and 0.25 g/l ferric pyrophosphate (Sigma). The broth was adjusted to pH 6.9.
with KOH and filter sterilized through a 0.2μm filter to give a final concentration of approximately 10^5 colony forming units (cfu)/ml. Organisms were incubated in static culture at 37°C and 5 ml aliquots removed at 4 to 6 h intervals to develop growth curves for this organism in the broth to be used for subsequent antibiotic activity studies.

**Antibiotics.** Dilutions of cefotaxime (sodium salt) (Hoechst-Roussel Pharmaceuticals), methicillin (sodium salt) (Sigma Chemical Company), rifampicin (Sigma Chemical Company), and ciprofloxacin (Miles Pharmaceuticals) were prepared in BYEα broth. Each antibiotic was added in the mid-exponential phase of organism growth to give a final concentration of 20 times the MIC of each antimicrobial agent. Aliquots of 5 ml were removed after incubation for 6 and 24 h in the presence of antibiotics. Organisms were harvested by centrifugation at 600 g for 10 min, washed twice in broth and either serially diluted for viable counts or prepared for electronmicroscopy. Control samples of untreated organisms were harvested and prepared in the same manner.

**Viable count technique.** Bacterial counts were determined in triplicate by inoculating 10 μl aliquots of serially diluted antibiotic-treated cultures onto BCYEα agar. Resultant cfu/ml were counted from those plates containing 30 to 100 colonies as previously described (91) to define the influence of these antibiotics on bacterial viability.

**Electron microscopy.** Bacteria were fixed in 3% (v/v) glutaraldehyde
in 0.05M sodium cacodylate containing 10mM MgSO₄ (35). For negative stain electron microscopy, 25 µl of each sample was mixed with an equal volume of 1% (w/v) phosphotungstic acid pH 6.7, and applied to 400 mesh formvar-carbon coated copper electron microscope grids. For scanning electron microscopy, samples were applied to specimen stubs, dehydrated in a graded ethanol series, and treated with hexamethyldisilazane (HMDS) (Polysciences, Inc.) (Warrington, Pa.) (87). Prior to examination, samples were coated with 20 nm of either gold/palladium with a target to specimen distance of 5 cm, at a current of 15 mA for 4 min in a Hummer V sputter coater. For thin-section electron microscopy, all samples were post-fixed in 1% (w/v) osmium tetroxide, dehydrated in an ethanol series and embedded in epon-araldite mixture. Sections of approximately 60 nm thickness were cut on an LKB Ultratome III with a diamond knife and stained with uranyl acetate 5% (w/v) for 1 min and lead citrate 0.4% (w/v) for 20 sec (105, 108).

**MIC determination.** MICs were determined by the broth dilution method (133). Briefly, serial two-fold dilutions of each antibiotic were prepared in BYEα broth and inoculated with cultures in the mid-exponential phase of growth to give a final concentration of 10⁵ cfu/ml. The broths were incubated for 16 h and the lowest concentration of antibiotic which inhibited growth was taken as the MIC (Table 4.1)

**4.4 Results.**

**Viability studies.** MIC values for all antibiotics are shown in Table 4.1.
The growth characteristics and effect on viability of *L. pneumophila* by the antibiotics investigated are illustrated in Figures 4.1-4.6 (data on ampicillin and erythromycin are from reference 35 with permission and are included for a complete comparative profile). *L. pneumophila* grown in BYEα broth, had a mean generation time of 1.5 h. The bacterial cell wall inhibitors, cefotaxime and methicillin, effected the most rapid decline in viable counts during the first 6 h of treatment, while the remaining antibiotics also showed excellent activity against *L. pneumophila* throughout the course of these experiments. Of those tested, cefotaxime most effectively reduced organism viability and induced the most dramatic morphological changes at the time intervals investigated.

**Morphological studies. Untreated.** Negative stain, scanning and thin-section electron microscopy studies of normal control organisms revealed the typical appearance of legionellae and confirmed the findings of others. Most cells were 2-10 μm long and 0.25-0.5 μm wide. The bacterial surface of normal cells was rugose and ruffled in appearance. Organisms showed a cell wall structure consistent with that of gram-negative bacteria. A lipid bilayer, or cytoplasmic membrane, enclosed the cytoplasm which consisted of ribosomes, a small number of intracellular vacuoles and a fine skein of nuclear elements. The latter were distributed evenly throughout the bacterial cytoplasm. The outer membrane enveloped the periplasm which showed little structural evidence of a peptidoglycan layer (plate 4.1).

**Cell wall inhibitors. Cefotaxime treatment.** Cefotaxime at 20 times
the MIC caused extreme damage to microorganisms. Incubation with this antibiotic for 6 h resulted in the formation of lesions and lytic points in the cell walls of a greater number of bacteria than any of the antibiotics studied at this time period. Extrusion of the bacterial cytoplasmic contents through such lytic points was the most common feature noted. Bulbous vesicles were observed on the surface of cells and many organisms possessed diffuse electron-lucent cytoplasmic contents. Extension of treatment with cefotaxime to 24 h resulted in an increase in damage to these cells in terms of the degree of membranous lesions and vesicles induced in the cell surfaces as well as the number of organisms affected. In addition, many bacteria lacked either cytoplasmic contents or prokaryotic structure. The formation of spheroplasts was not observed (plates 4.2 and 4.3).

**Methicillin treatment.** Organisms grown in the presence of 20 times the MIC of methicillin showed extensive morphological changes. Affected cells exhibited cell wall and membrane abnormalities and developed several lytic points through which the cytoplasmic contents extruded into the surrounding menstruum. Although many organisms appeared normal, spheroplast formation was evident at this stage. Incubation with methicillin for 24 h induced a greater degree of cell wall and membrane damage than the lesser incubation period. The outer membrane of the majority of organisms was found to have separated from the remaining cell wall and the development of small vesicles on the cell surfaces was evident. As a consequence of lysis of the inner and outer membranes, a loss of cytoplasmic material occurred (plates 4.4-4.8).
Protein synthesis inhibitor. **Rifampicin treatment.** Exposure of *L. pneumophila* to 20 times the MIC of rifampicin induced marked damage to bacteria. Incubation for 6 h in the presence of this antimicrobial induced membrane damage to the organism but this appeared less pronounced than for the other antibiotics examined. Separation of outer membranes from the remaining cell wall and breakage points in the inner membranes were evident in affected microorganisms. In addition, the ribosomes of these cells were enlarged, much increased in electron density and associated with areas of cytoplasmic clearing. Extended treatment with rifampicin elicited a more pronounced effect in which lytic points in the membrane of cells and loss of intracellular contents led to eventual collapse (plates 4.9-4.12).

DNA inhibitor. **Ciprofloxacin treatment.** Organisms treated for 6 h with 20 times the MIC of ciprofloxacin exhibited marked morphological changes. The majority of cells were abnormally elongated forming filaments each of which possessed centrally located "pinched zones" suggestive of arrested division. The inner membranes of many legionellae were intermittently lysed and separated from the remaining cell wall material. In addition, the intracellular contents of cells were more densely packed due to an apparent overall increase in the size of individual ribosomes. Further exposure to this antibiotic induced damage to 98% of the *Legionella* organisms present. Twisted, convoluted bacteria were found with depressed, partially collapsed areas on their cell surface. In addition, some organisms at this time had lost their intracellular contents through breakage points in the cell wall while others
maintained the densely packed cytoplasmic appearance found in 6 h antibiotic treated organisms (plates 4.13-4.19).

Figure 4.7 shows the relative proportion of lysed and partially damaged cells compared with those of normal morphology following antibiotic treatment for both 6 and 24 h and Table 4.2 summarizes the morphological findings.

4.5 Discussion

Erythromycin is the preferred drug for the treatment of Legionnaires' disease. However, because patients with severe illness often respond poorly to antimicrobial therapy, new antibiotics are being studied for use as alternate therapeutic regimens. *In vitro* screening of these potential agents to determine the activity and mode of action of these antibiotics must be performed in order to evaluate subsequent *in vivo* and clinical trials. In the present study, the *in vitro* effect of clinically relevant antibiotics, including the new quinolone, ciprofloxacin, on the growth and morphology of *L. pneumophila* was examined.

Electron microscopy studies have been important for the delineation and understanding of antimicrobial activity against many pathogenic microorganisms (61, 77, 141). Elliott and Rodgers (35, 107, 109) evaluated the morphological and growth response of *L. pneumophila* following exposure to ampicillin and erythromycin for 6 and 24 hours using electron microscopy, MIC determinations and microbial viable count data. In that investigation, ampicillin exhibited greater bactericidal activity and induced more ultrastructural changes than erythromycin *in vitro*. Ampicillin treatment caused the appearance of vacuole-like lesions in the cell walls of organisms which led to extensive
bacterial lysis and collapse of legionellae while exposure of *L. pneumophila* to erythromycin effected occasional breakage points in the cell wall along with cytoplasmic clearing, formation of membranous vesicles and some cell lysis.

In this study, all antibiotics tested were effective against *L. pneumophila* as assayed by MIC data, the reduction in bacterial viable counts and morphological response induced. Methicillin showed extreme bactericidal activity against multiplying organisms in broth culture. This was confirmed by the morphological studies in which many lysed and empty cells were evident after 6 and 24 h exposure periods. Formation of spheroplasts following treatment with 1280 µg/ml of this antibiotic was demonstrated and confirmed previous observations by Elliott and Rodgers of this phenomenon with another cell wall inhibitory antibiotic, ampicillin (35). These data suggested that the formation of minicell-like organisms and spheroplasts upon exposure to cell wall inhibitory antibiotics such as methicillin and ampicillin may constitute a mechanism by which osmotically protected legionellae survive within host cells and continue infection following reversion to vegetative forms upon discontinuation of therapy. Such findings may offer an explanation for the reported apparent "reactivation" infections due to *L. pneumophila* and may reflect ineffective killing of the organism *in vivo* in human lung.

Chan et al. (19) reported similar spheroplast formation for methicillin used at 100 µg/ml, but could not demonstrate this phenomenon with increased doses of up to 1000 µg/ml of methicillin. It is possible that the addition of
magnesium salts to the fixatives and buffers used in the present study could have stabilized fragile bacterial membranes and may account for the differences in spheroplast detection.

Cefotaxime showed the greatest bactericidal activity and this resulted in the most rapid decline in numbers of viable organisms. Indeed, this third generation cephalosporin induced the greatest morphological changes in microbial cytology at either of the exposure times. These results conflicted with those of Chan et al. (19) who reported no morphological changes in response to treatment of L. pneumophila with different doses of this antibiotic. It was interesting to note that this drug failed to induce spheroplast formation by L. pneumophila although a similar finding was reported following treatment of other gram negative bacteria with increased levels of cephalothin (88).

Although most Legionella species produce β-lactamases, the β-lactam antibiotics are most effective against actively dividing L. pneumophila cells in vitro. In this study, antibiotics were added in the mid-exponential phase of growth, which for L. pneumophila using the broth selected and an inoculum of 10^5 cfu/ml was achieved 24 h into the growth cycle, to allow these agents to function at their full capacity. Failure of Chan et al. (19) to detect similar morphological changes due to this antibiotic may have reflected the non-replicative status of the organisms subjected to antimicrobial treatment.

Ciprofloxacin and rifampicin showed effective bactericidal activity in viable count estimations at all the times studied. Despite showing the greatest activity in MIC assays, these antibiotics induced only moderate structural
damage after short term exposure. However, such changes increased with treatment time. That bacterial viability was lost prior to the induction of extensive ultrastructural damage probably reflected the mode of action of these antimicrobial agents against \textit{L. pneumophila} cells in the exponential phase of growth.

Results from this and other studies (35, 107, 109) demonstrated that the cell wall inhibitory antibiotics were the most effective in terms of their ability to reduce \textit{L. pneumophila} viability and induce morphological damage \textit{in vitro}. Although all the antibiotics investigated in this study showed \textit{in vitro} antimicrobial activity against \textit{L. pneumophila}, it has been reported that the aminoglycosides, cephalosporins, and penicillins including ampicillin and methicillin were clinically ineffective in the treatment of legionellosis (82). Because of its intracellular nature, \textit{L. pneumophila} can avoid the effects of antimicrobial agents that lack the ability to penetrate host cell membranes. Indeed, it has been reported that ciprofloxacin, erythromycin and rifampicin (65, 130) inhibit intracellular \textit{L. pneumophila} growth while the \(\beta\)-lactam antibiotics such as ampicillin, cefoxitin and cefotaxime do not (142). This most likely explains the efficacy of erythromycin and rifampicin in the clinical treatment of human legionellosis and emphasizes the need for further \textit{in vivo} testing of ciprofloxacin as a potential alternative to current clinical therapeutic regimens.

That bacteria with apparently normal morphology were found following extended incubation with all antibiotics examined in this study and in similar experiments performed by Elliott and Rodgers (35) with ampicillin and
erythromycin may offer further explanation for failure of current antimicrobial therapeutic protocols for the treatment of clinical Legionnaires' disease. In addition, these results considered in combination with reports of increasing incidence of bacterial resistance to standard antibiotic regimens indicated a continued need for thorough in vitro investigation of the response of pathogenic agents to antimicrobial chemotherapy.

These data illustrate the in vitro activity and mode of action of clinically relevant antibiotics for the treatment of Legionnaires' disease. This information is necessary to the understanding and evaluation of subsequent in vivo trials investigating the activity of these agents for the treatment of experimentally infected animal systems and for potential therapy of clinical disease.
Table 4.1 Susceptibility of *L. pneumophila* to selected antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/ml)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell wall inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>cefotaxime</td>
<td>8.0</td>
</tr>
<tr>
<td>methicillin</td>
<td>64</td>
</tr>
<tr>
<td>(ampicillin)</td>
<td>0.4²</td>
</tr>
<tr>
<td><strong>Protein synthesis inhibitor</strong></td>
<td></td>
</tr>
<tr>
<td>rifampicin</td>
<td>0.03</td>
</tr>
<tr>
<td>(erythromycin)</td>
<td>0.5²</td>
</tr>
<tr>
<td><strong>DNA inhibitor</strong></td>
<td></td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td>0.08</td>
</tr>
</tbody>
</table>

¹For viability and ultrastructural studies antibiotics were added to broth cultures at a final concentration of 20 times the MIC, respectively.

²Data for ampicillin and erythromycin reproduced with permission (Elliott and Rodgers, *J. Med. Microbiol.*, reference 35) and are included here to complete the comparative profile. Data were derived in a similar manner as for the other antibiotics.
Table 4.2  Morphological response of *L. pneumophila* following exposure to antibiotics for 6 and 24 hours.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Cef 6h</th>
<th>Cef 24h</th>
<th>Meth 6h</th>
<th>Meth 24h</th>
<th>Amp 1 6h</th>
<th>Amp 1 24h</th>
<th>Ery 1 6h</th>
<th>Ery 1 24h</th>
<th>Rif 6h</th>
<th>Rif 24h</th>
<th>Cipro 6h</th>
<th>Cipro 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>formation of lesions in cell wall</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td></td>
<td>+/</td>
</tr>
<tr>
<td>cytoplasmic clearing</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-/</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-/</td>
</tr>
<tr>
<td>membrane vesicle formation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>spheroplast formation</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>membrane abnormalities</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>enlargement of ribosomes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>plasmolysed cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>convoluted organisms</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-/+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 4.1 Growth of *L. pneumophila* in BYEα broth (■ ■ ■). The effect on bacterial viability of cefotaxime (♦ ♦ ♦ ) added during the mid-exponential phase of growth (arrow) is shown. This antibiotic was very effective at reducing bacterial viability.
Figure 4.2 Growth of *L. pneumophila* in BYEα broth ( ■ ■ ). The effect on bacterial viability of methicillin ( ♦ ♦ ) added during the mid-exponential phase of growth (arrow) is shown. This antibiotic was very effective at reducing bacterial viability.
Figure 4.3 Growth of *L. pneumophila* in enriched blood broth. The effect on bacterial viability of ampicillin added during mid-exponential phase of growth (arrow) is shown. This antibiotic effected a rapid decline in viable counts but was not bactericidal for the entire bacterial population. Data reproduced with kind permission (Elliott and Rodgers, *J. Med. Microbiol.*, reference 35) and included here to complete comparative analysis. Growth of *L. pneumophila* in this broth had a mean generation time of 2.0 h.
Figure 4.4 Growth of *L. pneumophila* in enriched blood broth. The effect on bacterial viability of erythromycin added during mid-exponential phase of growth (arrow) is shown. This antibiotic was the least effective in terms of bactericidal activity. Data reproduced with kind permission (Elliott and Rodgers, *J. Med. Microbiol.*, reference 35) and included here to complete comparative analysis. Growth of *L. pneumophila* in enriched blood broth had a mean generation time of 2.0 h.
Figure 4.5 Growth of *L. pneumophila* in BYE$\alpha$ broth (■■). The effect on bacterial viability of rifampicin (◇◇) added during the mid-exponential phase of growth (arrow) is shown. This antibiotic showed marked antibacterial activity.
Figure 4.6 Growth of *L. pneumophila* in BYEα broth (■■■■). The effect on bacterial viability of ciprofloxacin (♦♦♦♦) added during the mid-exponential phase of growth (arrow) is shown. This antibiotic showed marked antibacterial activity.
Figure 4.7 Proportion of bacterial cells showing normal morphology (■) and those damaged (□) after exposure for 6 or 24 h to the various antibiotics at 20 times their respective MIC values. Percentages were calculated from counts of 100 or more cells for each agent and for each treatment time. Data in boxed area is reproduced with permission (Elliott and Rodgers, J. Med. Microbiol., reference 35) and is included for the purposes of comparison.
Plate 4.1 Untreated, control *Legionella* organisms showing normal morphology. The bacterial surface is ruffled and the cell wall structure is composed of a double track membrane with little visual evidence of a peptidoglycan.

a) negative stain (X 18,000), b) scanning (X 30,000), c) thin-section (X 40,000). Bars=0.5 μm.
Plate 4.2 *L. pneumophila* organisms exposed to cefotaxime for 6 h.
a) note bulbous vesicles on the surface of the organism, scanning (X 20,000),
b) organism showing loss of intact membrane structure surrounding diffuse,
electron-lucent cytoplasmic contents, thin-section (X 40,000),
c) bacterium with advanced membrane damage and associated loss of cytoplasm, thin-section
(X 60,000). Bars=0.25 μm.
Plate 4.3. Legionellae exposed to cefotaxime for 24 h.
a) formation of lytic points (arrow) in the bacterial cell wall, negative stain (X16,000), b) collapsed organism with abnormal surface structure, scanning (X 30,000), c) plasmolyzed organism with loss of prokaryotic structure, (thin-section (X 80,000). a, b Bars=0.5 μm, c Bar=0.25 μm.
Plate 4.4 *L. pneumophila* exposed to methicillin for 6 h.
a) *L. pneumophila* organism with breakage point in cell wall (arrow), negative-stain (X 16,000), b) advanced stage of lysis-cytoplasmic contents extruding through lytic point in the cell wall of an organism, negative-stain (X 18,000).
Bars=0.5 μm.
Plate 4.5 Legionellae exposed to methicillin for 6 h.
a) damaged bacterial cells surrounding an apparently normal cell, thin-section (X 30,000), b) spheroplast formation is evident along with deformed and normal cells, thin-section (X 40,000). Bars=0.25 μm.
Plate 4.6 *L. pneumophila* exposed to methicillin for 6 h.

a) severely damaged cells with abnormal membrane distortion (arrow), thin-section (X 40,000), b) plasmolyzed organism with pinching and ballooning of membranes, thin-section (X 80,000). Bars=0.25 μm.
Plate 4.7 *Legionella* organisms exposed to methicillin for 24 h. 
a) note loss of cytoplasmic contents through advanced lytic point in bacterial cell wall (arrow), negative-stain (X 12,000), b) induction of numerous blebs on the microbial surface, scanning (X 20,000). Bars=0.5 μm.
Plate 4.8 *L. pneumophila* exposed to methicillin for 24 h.
a) complete loss of bacterial cytoplasmic contents with extreme damage to the microbial cell walls, thin-section (X 40,000), b) organism devoid of cytoplasm with associated inner and outer membrane separation, thin-section (X 40,000). Bars=0.5 μm.
Plate 4.9. Organisms exposed to rifampicin for 6 h.
a) note numerous breakage points in the cell wall and loss of cytoplasm from cell (arrows), negative-stain (X 16,000), b) extrusion of cytoplasm was evident (short arrow) as well as regions of bacterial collapse (long arrow), scanning (X 25,000). Bars=0.5 μm.
Plate 4.10 *L. pneumophila* exposed to rifampicin for 24 h.

a) darkly staining surface lesions and numerous small vesicles present, negative-stain (X 16,000), b) distorted, partially collapsed organisms, scanning (X 15,000), c) disruption of outer membrane and increased size and density of ribosomal content of cells, thin-section (X 70,000).

a, b Bars=0.5 μm, c Bar=0.25 μm.
Plate 4.11 Organisms exposed to rifampicin for 24 h. Note localized zones of increased density and size of ribosomes within cytoplasm as well as distorted membrane structure of cells (arrow), thin-section (X 34,000). Bar=0.25 μm.
Plate 4.12 Organisms exposed to rifampicin for 24 h. Increased density of cytoplasmic contents is evident in addition to separation of cell walls from remaining cell contents, thin-section (X 34,000).
Plate 4.13 *L. pneumophila* exposed to ciprofloxacin for 6 h. 

a) darkly staining lesions in cell wall of a partially collapsed organism, negative-stain, (X 24,000), b) convoluted organism with centrally located pinched zone, scanning (X 20,000). Bars=0.5 μm.
Plate 4.14 *L. pneumophila* exposed to ciprofloxacin for 6 h. Note severely deformed cell (arrow) with areas of cytoplasmic clearing and abnormal membrane structure, thin-section (X 34,000). Bar=0.25 μm.
Plate 4.15 *L. pneumophila* exposed to ciprofloxacin for 6 h. Abnormal distribution of ribosomes along with areas of localized clearing within the bacterial cytoplasm of cells, thin-section (X 40,000). Bar=0.25 μm.
Plate 4.16 Legionellae exposed to ciprofloxacin for 6 h. Note vesiculation of bacterial cytoplasm (arrow) and increased size and density of ribosomes, thin-section (X 60,000). Bar=0.25 μm.
Plate 4.17 *L. pneumophila* exposed to ciprofloxacin for 6 h. Single cell showing very dense ribosomal contents within bacterial cytoplasm, thin-section (X 60,000). Bar=0.25 μm.
Plate 4.18 *L. pneumophila* exposed to ciprofloxacin for 24 h.
a) twisted, convoluted organism with multiple pinched zones, negative-stain (X 12,000), b) filamentous, twisted organisms are evident, scanning (X 10,000), c) partially collapsed organism with centrally located pinched zone, scanning (X 34,000).  a, b Bars=0.5 µm, c Bars=0.25 µm.
Plate 4.19  *L. pneumophila* exposed to ciprofloxacin for 24 h. Organisms showing various types of morphological response to treatment. Some cells have lost their cytoplasmic contents (short arrow) while others exhibit increased ribosomal density (long arrow). Note twisted, abnormally shaped cell morphologies, thin-section (X 24,000). Bar=0.5 μm.
Section V

Pathogenesis, Pathology and Chemotherapy of Experimental 
*Legionella pneumophila* Infection in the Chick Embryo

5.1 Abstract

The pathogenicity of *Legionella pneumophila*, serogroup 1, strain Nottingham N7, was assessed in terms of LD<sub>50</sub> data and the ability of the organism to induce histopathological and ultrastructural lesions in the fertile hen's egg. Histological examination of embryo organs after inoculation with 1, 10, 100 and 1000 times the yolk sac LD<sub>50</sub> revealed a disseminated infection. Systemic spread of the organism resulted in the appearance of pathological lesions and the generation of edema fluid. These were particularly severe in the liver, heart, spleen and kidney. Electron microscopy of the pathology induced in organs of chick embryos previously infected with 100 times the YSLD<sub>50</sub> of *L. pneumophila* confirmed these observations. Subtle pathological changes were noted three days post-inoculation while more extensive cellular and sub-cellular necrotic changes occurred at four days post-inoculation. The infection elicited an inflammatory cell response that consisted of polymorphonuclear leucocytes and lymphocytes. Selected antimicrobial agents were investigated in therapeutic studies for their capacity to ameliorate, or control disease processes in this test system. Of those examined, ciprofloxacin was most effective at reducing or reversing the incidence of
lesions in these tissues and for prolonging embryo viability. Rifampicin, and to a lesser degree, erythromycin and doxycycline, also showed antimicrobial activity in these in vivo trials. These results illustrated the efficacy of the fertile hen's egg as a useful alternative in vivo assay system for the evaluation of clinically putative antimicrobial agents in the treatment of Legionnaires' disease. In addition, this study demonstrated the superior antimicrobial activity of ciprofloxacin in the treatment of experimentally induced Legionella infections.
5.2 Introduction

*L. pneumophila* is the etiological agent of legionellosis which in the severe pneumonic form presents as Legionnaires' disease (85). Despite the accumulation of considerable data describing the invasive nature of the microorganism, the pathogenic mechanisms by which this gram-negative rod causes infection are not well understood. In human disease and experimental animal infections, the legionellae are facultative intracellular pathogens which survive and replicate within alveolar macrophages.

Although primarily recognized as a pneumonic illness, Legionnaires' disease has been shown to produce a widespread bacteremia along with the appearance of soluble *L. pneumophila* antigen in clinical samples of serum and urine (12) in association with extrapulmonary pathology (13, 115). Multi-organ inflammatory lesions due to *Legionella* bacteremia are not common but have been described (24). Because Legionnaires' disease first presents as a classic pneumonic infection, the extra-thoracic aspects of the disease have been overlooked, but it is evident that the serious pathological sequelae that result from *L. pneumophila* infection may play a larger role in the overall disease process than was previously suspected.

Numerous antibiotics with different modes of action have shown activity against *L. pneumophila* in vitro (30, 35, 60, 109, 112, 123). However, many of these antibiotics with effective minimum inhibitory concentrations (MIC) are reported as ineffective in the treatment of Legionnaires' disease (82) while others such as erythromycin, rifampicin, and to a lesser extent, the tetracyclines...
have been efficacious in therapy (2). Erythromycin is considered the drug of choice for Legionnaires' disease but variable clinical responses in patients to this antibiotic and problems associated with low dose or intravenous erythromycin therapy have been reported (62). Consequently, other therapeutic options are currently sought.

The quinolones are antimicrobial agents that effectively inhibit bacterial DNA gyrase activity. One member of this group, ciprofloxacin, is a promising new lipid soluble antibiotic with broad spectrum activity and the ability to penetrate eukaryotic cells. This antibiotic has few side-effects, offers oral bioavailability, achieves high tissue concentrations and has been used successfully in the treatment of various urinary and respiratory tract infections. Ciprofloxacin has been shown to be very active against \textit{L. pneumophila} \textit{in vitro} (60, 65) and in experimental \textit{Legionella} infections of guinea pigs (44, 45, 135).

Because treatment of human legionellosis often commences well after onset of disease, controlled clinical studies exploring the use of alternative chemotherapeutic agents are difficult to perform and force researchers to depend on animal studies for data accumulation. Although \textit{in vivo} testing of antibiotic efficacy in guinea pigs has thus far correlated well with clinical data, investigators using this animal system for these studies require specialized equipment, animal laboratory facilities, specialized expertise, and, according to current regulations, qualified staff, animal care and use permission, and in some countries animal licenses. In addition, the cost and availability of these animals limits the use of significant numbers in experiments and makes
interpretation of data difficult. It is clear that the ability to test alternative therapeutic regimens for the treatment of Legionnaires' disease has become increasingly important and necessitates the development of a simplified and reproducible in vivo antimicrobial assay system.

Lewis et al. (83) introduced the use of embryonate eggs to examine in vivo prophylactic and post-infection antibiotic therapy of experimental legionellosis following the initial outbreak of Legionnaires' disease in Philadelphia in 1976. This study evaluated antimicrobial therapy based on the ability of antibiotics to reduce mortality rates of infected embryos and yielded results in keeping with clinical findings. In the present study, the chick embryo was used to delineate the baseline histological and ultrastructural pathology associated with the progression of experimental infection in this animal with *L. pneumophila* in order to determine the therapeutic value of antimicrobial agents introduced into this animal following experimental infection. Results from these investigations were used to evaluate the chick embryo as an alternative in vivo assay system for the testing of new and/or putative antimicrobial agents in the treatment of human legionellosis. In addition, the quinolone antibiotic ciprofloxacin was examined for its chemotherapeutic activity and compared with clinically relevant antibiotics for its effectiveness at regulating infection in the chick embryo animal system. Elucidation of the mechanisms of pathogenesis and growth of *L. pneumophila* in this in vivo host system and the role of antimicrobial therapy in the disease process may have an impact on current procedures for the maintenance and care of patients with legionellosis.
5.3 Materials and Methods.

Reagent formulations and preparations along with detailed procedures are given in Appendices 1, 2, 3, and 4.

Preparation of bacterial inocula. *L. pneumophila*, serogroup 1, strain Nottingham N7, was isolated from the sputum of a fatal case of Legionnaires' disease, subsequently passaged twice on bacteriological media and maintained frozen at -70°C in 10% serum with added 1% sorbitol. Thawed aliquots were grown at 37°C on buffered charcoal yeast extract agar enriched with L-cysteine, ferric pyrophosphate and α-ketoglutarate (BCYEα) (29) and incubated aerobically for 48 hours. Organisms were harvested in 5 ml phosphate buffered saline pH 6.9 (PBS) to give a density equivalent to 10^8 colony forming units (cfu)/ml as determined by a Klett-Summerson photoelectric colorimeter. Organism mass, expressed in terms of viability, was determined in duplicate as cfu on BCYEα to confirm opacity data.

Egg type, incubation, inoculation route and determination of LD50s. Antibiotic free, fertile White Leghorn hens' eggs (UNH Poultry Farm) were incubated at 36°C in a humid atmosphere. Organisms were introduced into eggs by four inoculation routes: allantoic and amniotic sacs (at 10 days of embryo incubation), chorioallantoic membrane (CAM) and yolk sac (at 7 days of embryo incubation) (Figures 5.1 and 5.2). Sixty embryonate eggs were used for each inoculation route. Serial 10-fold dilutions of *L. pneumophila* in PBS were made and those ranging from 10^1 to 10^6 cfu/ml were inoculated at a rate of 10
eggs per dilution. Inoculated eggs were incubated and candled twice daily to check for embryo viability. Mortality and protection experiments were performed three times and the LD$_{50}$ for each inoculation route was determined from the averaged mortality data by the method of Reed and Muench (101).

**Recovery of organisms and histopathology studies.** Organism mass was calculated as described and the inocula adjusted to give inoculation doses equivalent to 1, 10, 100 and 1000 times the yolk sac LD$_{50}$ (YSLD$_{50}$).

Each multiple dose LD$_{50}$ of *L. pneumophila* was inoculated into the yolk sacs of fertile eggs and these were harvested at 24 hour intervals from one to eight days post-inoculation. Organ samples including liver, heart, spleen and kidney were removed aseptically from embryos, rinsed thoroughly in PBS and weighed. Organs were homogenized in a Sorval Omni Mixer, serially diluted and aliquots inoculated onto BCYE$_a$ for viable count assay. Colony counts were expressed as cfu/gram of tissue and were calculated for each organ at each time interval in triplicate. Similarly inoculated and control eggs were harvested and whole embryos as well as samples of liver, heart, spleen and kidney were fixed in 10% buffered formalin and embedded in paraffin wax in a Lab Tek Tissue Processor. Four micron thick sections were cut, deparaffinized and routinely stained with hematoxylin and eosin for histopathological examination.

**Immunofluorescence.** Deparaffinized tissue sections and tissue homogenates were incubated with a 1 in 50 dilution of rabbit anti-*L.*
pneumophila serum, followed by incubation with a 1 in 200 dilution of FITC-
conjugated goat anti-rabbit serum (Cappel Laboratories, Malvern, Pa.). All
labelling steps were followed by 3-fold washings in PBS to remove unbound
globulins.

Electron microscopy studies. Eggs were inoculated with 100 times
the YSLD₅₀ by the yolk sac route, and the embryo organs, liver, heart, spleen
and kidney, harvested at 24 h intervals and prepared for electron microscopic
examination. Tissue samples were rinsed in 0.1 M cacodylate buffer, pH 7.2
(CB) fixed in 3% (v/v) glutaraldehyde in CB for 24 hours, post-fixed in 1% (w/v)
osmium tetroxide (OsO₄) in CB for two hours and dehydrated in a graded
ethanol series. Samples were embedded in an epon-araldite resin mixture and
polymerized for 24 hours at 60°C (105, 108). Thin sections, cut on an LKB
Ultratome III with a diamond knife, were stained with 5% (w/v) uranyl acetate
and 0.4% (w/v) lead citrate and examined by transmission electron microscopy
in a Hitachi H600 electron microscope used at 80kV.

Antibiotic therapy studies. MIC values were calculated for
ciprofloxacin (Miles Pharmaceuticals, West Haven, Ct.), erythromycin,
doxycycline and rifampicin (Sigma Chemicals, St. Louis, Mo.) by a standard
agar dilution technique (133) using BCYEα. For these experiments, eggs were
infected by the yolk sac route with 100 times the YSLD₅₀ of L. pneumophila.

For histopathological studies, one or ten times the MIC of each of the four
antimicrobial agents were administered intra-allantoically at one day post-
inoculation. Mortality data were calculated with time for each antibiotic and bacterial viable counts determined from embryo organ homogenates. Similar samples were harvested and prepared for histological examination to assess the ability of each antibiotic to inhibit either an inflammatory cell response or the development of pathological lesions in embryo organs.

Antibiotic therapy of chick embryos was commenced at three days post-infection for electron microscopic investigations. This schedule was selected because electron microscopic studies of untreated control samples showed that this time interval allowed for the earliest expression of ultrastructural pathological lesions to develop after the introduction of *L. pneumophila* into the chick embryo. In such treatments, 10 times the MIC levels of each of the four antibiotics were administered intra-allantoically and organ samples of liver and kidney from viable embryos were harvested for electron microscopy at four and eight days post-inoculation (one and five days post-therapy, respectively).

### 5.4 Results

**Pathogenesis.** The LD$_{50}$ values for *L. pneumophila* introduced into the embryonate egg were determined and are illustrated in Table 5.1. The inocula required to induce embryo death ranged from $1.4 \times 10^2$ cfu/ml for the amniotic sac to $5.5 \times 10^4$ cfu/ml for the allantoic sac. The LD$_{50}$ studies demonstrated that those inoculation routes which accessed the immediate environment or the nutritional source of the embryo required lower organism numbers to induce fatal infections compared with more peripheral routes.

Inoculation of multiple doses of the YSLD$_{50}$ into fertile hens' eggs
resulted in dissemination and growth of *Legionella* organisms in the liver, heart, spleen and kidney. A tissue response and dose effect was observed in that viable counts on harvested organs of embryos infected with 1 or 10 times the YSLD<sub>50</sub> revealed that exponential bacterial growth began at four days post-inoculation in the liver and heart but after five days in the spleen and kidney (Figures 5.3 and 5.4). In addition, bacterial assays of organs from embryos inoculated with 100 and 1000 times the YSLD<sub>50</sub> showed that organism growth began at four days post-inoculation in all examined organs (Figures 5.5 and 5.6). Microbial assay of tissues at eight days post-inoculation with 1000 YSLD<sub>50</sub> yielded counts ranging from 3.3 X 10<sup>4</sup> to 9.9 X 10<sup>5</sup> cfu/gram in the kidney and liver, respectively (Figure 5.6). Organism concentration in organs was highest in the liver and lowest in the kidney irrespective of the multiple dose used as inoculum.

**Histopathology.** Gross examination of the organs of embryos infected by various routes of inoculation with multiple doses of the YSLD<sub>50</sub> showed no ostensible lesions. Inoculated CAMs failed to demonstrate visible pocks or pathological changes. Following yolk sac inoculation, histopathological examination on organs of embryos infected with 1, 10, 100 and 1000 times the YSLD<sub>50</sub> revealed pathological changes in the liver, heart, spleen and kidney and were most evident in regions rich in blood vessels (plates 5.1-5.5). With inocula of 1, 10, or 100 times the YSLD<sub>50</sub> an inflammatory cell response appeared simultaneously at five days post-inoculation in all organs examined.
but at four days following administration of 1000 times the YSLD$_{50}$. At this stage, congestion of the blood vessels within tissues were most evident (plates 5.1-5.2). Heart tissue showed marked vascular congestion with evidence of an edematous response (plate 5.2). In the kidney, pathological changes in the glomeruli, with an associated cell exudate, were evident as compared with the relatively undamaged convoluted tubules (plate 5.4).

Histopathological examination of sections at higher magnification showed degenerative in each organ typified by breakdown of cell and nuclear membrane structure, loss of cell components, and margination of nuclear chromatin. Darkly staining PMNLs and leucocytes were also common (plate 5.5). These results are summarized in Table 5.2.

**Immunofluorescence.** The presence of *L. pneumophila* organisms in situ in tissue sections and homogenates was confirmed by specific immunofluorescence. Labelled organisms were visualized along with tissue damage in liver, heart, spleen and kidney sections of embryos previously inoculated with multiple doses of the YSLD$_{50}$.

**Ultrastructural pathology.** Electron microscopic examination of the liver, heart, spleen, and kidney of infected chick embryos revealed extensive pathological damage at the cellular and sub-cellular levels. These observations are summarized in Table 5.3. The ultrastructural tissue damage induced by the organism was similar in all organs examined and the earliest, most tenuous pathological changes were observed at three days post-inoculation. Mitochondrial damage was evident at this time (plate 5.6 and 5.7).
Major ultrastructural changes occurred at four days post-inoculation and consisted of extensive degenerative changes which included fatty degeneration and cytoplasmic clearing. These changes were characterized by ballooning and breakdown of the cellular and nuclear membranes, nuclear clearing, margination of chromatin material, and cytoplasmic vesiculation (plate 5.8). A hemolytic process (plate 5.9) was evident in these tissues. During latter stages of the infection, many lysed, swollen or apparently empty mitochondria were distributed throughout the cytoplasm of affected cells. At this stage, the nuclei of many cells exhibited pyknosis or karyolysis (plate 5.10-5.12). In addition, lysosomal bodies were present within cells presumably involved with autolysis of these tissues. Occasionally, small numbers of legionellae were observed within cells of embryo organs (plate 5.13).

**Antibiotic therapy studies.** The MICs of ciprofloxacin, doxycycline, erythromycin and rifampicin for *L. pneumophila* are shown in Table 5.4. Of the four antibiotics, ciprofloxacin at 10 times the MIC proved most effective in delaying tissue damage and preventing an inflammatory cell response prior to eight days post-inoculation as assessed by histopathological examination. Erythromycin, doxycycline or rifampicin effected a delay in the development of these lesions, but their effect was much less pronounced. These results are shown in Table 5.4. Only embryos protected with ciprofloxacin survived to term. In addition, the efficacy of these antibiotics was evaluated in bacterial viable count assays performed on organs of embryos previously infected eight days previously with 100 times the YSLD$_{50}$ of *L. pneumophila*. Results are shown in
Figure 5.7. At 10 times their MIC, ciprofloxacin was more efficient at reducing organism numbers in these tissues than either erythromycin or rifampicin.

Figure 5.8 shows the results of embryo viability after inoculation with 100 times the YSLD\textsubscript{50} of \textit{L. pneumophila} with administration 24 hours later of 10 times the MIC levels of ciprofloxacin, erythromycin or rifampicin. Ciprofloxacin gave the highest percentage of embryo survival throughout the trials. Eight days post-inoculation the survival rate for embryos protected with ciprofloxacin was 83\% compared with rifampicin at 33\% or erythromycin at 10\%. By 14 days post-inoculation all rifampicin or erythromycin treated embryos had succumbed to infection.

Ultrastructural observations of infected embryo organs treated three days subsequently with 10 times the MIC of these antibiotics showed similar changes and are summarized in Table 5.5. Only viable embryos were processed for electron microscopic examination. Ultrastructural tissue damage in erythromycin (plate 5.14) and doxycycline (plate 5.15) treated embryos was less severe than unprotected tissues at four days post-inoculation. Indeed, the cellular changes at this stage in the presence of erythromycin were similar in appearance to those at day three of infection in the absence of antibiotic. Tissues from rifampicin treated embryos also showed less damage than controls and were comparable at four and eight days post-inoculation (plates 5.16 and 5.17). However, tissues from ciprofloxacin treated embryos were found to be consistently less damaged at eight days than at four days post-infection (plates 5.18 and 5.19) indicating that the degenerative process
associated with infection at the ultrastructural level had been reversed.

5.5 Discussion

In keeping with current pressures and legislation to reduce vertebrate animal use in research and diagnosis, the self-contained embryonate hen's egg offers an alternative in vivo antibiotic assay system for \textit{L. pneumophila}. Eggs are relatively easy to handle, inexpensive, and can be used in sufficiently large numbers to test each antibiotic so as to give significant assessment levels normally unattainable with other laboratory animals. In addition, no specialized animal facilities, equipment, personnel or licenses are required.

The LD$_{50}$ studies revealed the high degree of pathogenicity of \textit{L. pneumophila} for this animal. These findings reflected the relative few numbers of microorganisms required to achieve these mortality data and illustrated the extreme infectious nature of the bacterium for the chick embryo and its tissues. The rapid growth of \textit{L. pneumophila} in the liver, heart, spleen and kidney following inoculation with multiple doses of the YSLD$_{50}$ indicated that growth of the organism in the chick embryo was characterized by a bacteremia with satellite infections in these organs. Microbial assays also revealed that irrespective of the inoculum dose, extensive organism growth occurred within organs all organs examined, most notably in the liver but to a lesser degree in the kidney. These data supported similar observations by other workers that organisms were found within the extrapulmonary organs of humans and animals previously infected with \textit{L. pneumophila}.

Correlation exists between human legionellosis and experimental

131
infections in various animal models. Pyrexia, antibody production, cell-mediated immunity and bacteremia have been demonstrated in various laboratory animals. In addition, the production of inflammatory cells in response to experimental and natural infections with the organism is often similar and consists of a mixed infiltrate of PMNLs and macrophages. Histopathological examination of the liver, heart, spleen and kidney of the chick embryo following inoculation with multiple doses of *L. pneumophila* revealed the simultaneous appearance of vascular cells in all organs and suggested a large scale inflammatory cell response consisting primarily of PMNLs and lymphocytes.

Experimental infection of fertile hens' eggs with *L. pneumophila* elicited severe cellular and sub-cellular pathological changes in all embryo organs examined by electron microscopy. That ultrastructural tissue damage was found to be similar and occurred synchronously three days post-inoculation in these organs supported histopathological observations of a systemic-type infection in this animal (128). The ultrastructural appearance of tissues of infected embryo organs was comparable to similar features exhibited in human legionellosis (20, 21, 30, 105, 111).

Although *L. pneumophila* is sensitive to a variety of antimicrobial agents in vitro, the ability of this pathogen to replicate intracellularly limits the use of many of these agents in vivo. Antibiotics which have been used successfully in treatment include erythromycin, rifampicin and the tetracyclines. Animal experiments with guinea pigs previously exposed to aerosol suspensions of *L. pneumophila* and treated with clinically proven antibiotics readily demonstrated
the efficacy of rifampicin and to a lesser extent erythromycin. Effective antimicrobial agents were able to decrease mortality and clear viable *L. pneumophila* from the lungs but only rifampicin was able to reverse histopathological lesions (57). Similar results were obtained using *L. pneumophila* infected fertile hens' eggs in which mortality data was reduced by treatment with rifampicin and erythromycin administered either prophylactically or post-infection (83). To date, additional antimicrobial investigations have not been performed to corroborate and extend these results.

Ciprofloxacin has shown excellent *in vitro* activity against *L. pneumophila* in preliminary studies with agar and broth dilution tests as well as cell cultures (65). Effective ciprofloxacin treatment of experimental *Legionella* infection in guinea pigs has also been demonstrated. In these studies, ciprofloxacin was as effective as rifampicin and better than erythromycin in preventing death of infected guinea pigs (44).

In the present study, the fertile hen's egg was used as an *in vivo* assay system for the critical evaluation of clinically useful antibiotics. Antimicrobial agents were administered one day post-inoculation and tested for their ability to delay the development of histopathological lesions and the appearance of infiltrative cells, reduce organism numbers in tissues and decrease embryo mortality. Ciprofloxacin, erythromycin, doxycycline and rifampicin were all effective against *L. pneumophila* infection of the embryonated egg in all aspects of testing as compared with PBS controls. However, results from these investigations demonstrated the superior activity of ciprofloxacin in these *in vivo*
The treatment of human legionellosis often commences well after the disease process is initiated. In this study, the progression of disease was monitored at the ultrastructural level and antimicrobial therapy administered to eggs to correspond with the appearance of the earliest cellular and sub-cellular pathological changes in tissues. It was evident that ciprofloxacin was the most effective antibiotic investigated based on its consistent ability to reverse ultrastructural lesions induced in response to infection with *L. pneumophila*.

Although no animal system completely simulates the conditions of human therapy, these results established the efficacy of the chick embryo to evaluate clinically putative antibiotics for the treatment of human legionellosis and to investigate the pathology associated with otherwise fatal experimental *L. pneumophila* infections. The usefulness of ciprofloxacin in the treatment of such infections in this animal was also demonstrated in all aspects of these trials. Rifampicin, erythromycin and doxycycline also showed antimicrobial activity in this assay system, but to a lesser degree. Evidence from this and other reports suggested that the use of ciprofloxacin for the treatment of *L. pneumophila* experimental infections in animals requires further investigation.

In addition, studies are necessary to determine whether the use of this drug may be efficacious in the treatment of clinical legionellosis.
Table 5.1  LD$_{50}$ values for *L. pneumophila* in the embryonated hen's egg.

<table>
<thead>
<tr>
<th>Route of Inoculation</th>
<th>cfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>allantoic sac</td>
<td>5.5 x 10$^4$</td>
</tr>
<tr>
<td>amniotic sac</td>
<td>1.4 x 10$^2$</td>
</tr>
<tr>
<td>chorioallantoic membrane (CAM)</td>
<td>3.3 x 10$^4$</td>
</tr>
<tr>
<td>yolk sac</td>
<td>6.6 x 10$^2$</td>
</tr>
</tbody>
</table>
Table 5.2 Histopathological observations on organs of embryos inoculated with 1, 10, 100 or 1000 times the YSLD<sub>50</sub> of <i>L. pneumophila</i>.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Days post-inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1, 2, 3, 4</td>
</tr>
<tr>
<td>systemic degenerative changes in organs</td>
<td>-</td>
</tr>
<tr>
<td>edema</td>
<td>-</td>
</tr>
<tr>
<td>congestion of blood vessels</td>
<td>-</td>
</tr>
<tr>
<td>cellular damage</td>
<td>-</td>
</tr>
<tr>
<td>inflammatory cell response</td>
<td>-</td>
</tr>
</tbody>
</table>

+++ = extensive damage, - = not found

¹All features described reflect findings with inoculas of 1, 10 and 100 times the YSLD<sub>50</sub> with the exception of 1000 YSLD<sub>50</sub>, which were found at four days post-inoculation.
Table 5.3 Ultrastructural observations on cells of embryo organs inoculated with 100 times the YSLD$_{50}$ of *L. pneumophila*.

<table>
<thead>
<tr>
<th>Cell Damage</th>
<th>Days post-inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1,2</td>
</tr>
<tr>
<td>Breakdown of cell and nuclear membrane</td>
<td>-</td>
</tr>
<tr>
<td>Cytoplasmic clearing</td>
<td>-</td>
</tr>
<tr>
<td>Fatty degeneration</td>
<td>-</td>
</tr>
<tr>
<td>Ballooning of nuclear membrane</td>
<td>-</td>
</tr>
<tr>
<td>Pyknosis</td>
<td>-</td>
</tr>
<tr>
<td>Karyolysis</td>
<td>-</td>
</tr>
<tr>
<td>Mitochondrial damage</td>
<td>-</td>
</tr>
<tr>
<td>Margination of chromatin and nuclear</td>
<td>-</td>
</tr>
<tr>
<td>clearing</td>
<td></td>
</tr>
<tr>
<td>Intercellular spaces and cellular vacuoles</td>
<td>-</td>
</tr>
<tr>
<td>Hemolysis</td>
<td>-</td>
</tr>
</tbody>
</table>

++++ = extensive in all cells examined; +++ = common in many cells; + = rarely found; - = never found
Table 5.4 Appearance of inflammatory cells in chick embryo organs infected with 100 times the YSLD$_{50}$ of *L. pneumophila* and protected with 10 times the MIC of each antibiotic.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC ($\mu g/ml$)</th>
<th>Days post-inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (100 YSLD$_{50}$)</td>
<td>-----</td>
<td>5</td>
</tr>
<tr>
<td>ciprofloxacin</td>
<td>0.08</td>
<td>8</td>
</tr>
<tr>
<td>doxycycline</td>
<td>0.80</td>
<td>6</td>
</tr>
<tr>
<td>erythromycin</td>
<td>0.50</td>
<td>6</td>
</tr>
<tr>
<td>rifampicin</td>
<td>0.03</td>
<td>6</td>
</tr>
</tbody>
</table>
Table 5.5 Ultrastructural cell damage in embryo organs previously inoculated with 100 times the YSLD$_{50}$ of *L. pneumophila* and protected with antibiotic

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/ml)</th>
<th>Dose administered per egg$^1$ (µg/ml)</th>
<th>Days post-inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 (1 day post-therapy)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.08</td>
<td>28</td>
<td>+++2</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.03</td>
<td>10.5</td>
<td>+++</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.50</td>
<td>175</td>
<td>+</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.80</td>
<td>280</td>
<td>+</td>
</tr>
</tbody>
</table>

$^1$Dose per egg was based upon 10 times the MIC for each drug and an average egg volume of 35 mls.

$^2$Degree of ultrastructural damage observed, +++= marked, + = slight
Figure 5.1 Schematic illustrating various routes of inoculation and compartments of the chick embryo.
Figure 5.2 Schematic illustrating the chorioallantoic route of inoculation of the fertile hen's egg.
Figure 5.3 Bacterial viable counts assays on embryo organs from eggs inoculated with the YSLD\textsubscript{50} of \textit{L. pneumophila}. Note rapid bacterial growth in all organs examined. Organisms were found most concentrated in the liver.
Figure 5.4 Bacterial viable count assays on embryo organs from eggs inoculated with 10 times the YSLD$_{50}$ of *L. pneumophila*. Note increased bacterial growth rate in all organs examined. The liver contained the densest microbial population.
Figure 5.5 Bacterial viable count assays on embryo organs from eggs inoculated with 100 times the YSLD$_{50}$ of L. pneumophila. Bacterial growth in organs was similar in all organs examined but was less rapid than growth in organs of embryos inoculated with 10 times the YSLD$_{50}$. 
Figure 5.6 Bacterial viable count assays on embryo organs from eggs inoculated with 1000 times YSLD$_{50}$ of *L. pneumophila*. Note increased bacterial growth in liver and heart tissue after 6 days post-inoculation.
Figure 5.7 Bacterial growth of *L. pneumophila* in embryo organs previously inoculated with 100 times the YSLD$_{50}$ and treated one day post-inoculation with 1 or 10 times the MIC of antibiotic. Treatment with ten times the MIC of ciprofloxacin was most effective at reducing bacterial growth in the majority of organs examined.
Figure 5.8 Viability rate of embryos previously infected with 100 times the VSLD$_{50}$ of $L.\,$pneumophila and treated with 10 times the MIC of each antibiotic. PBS control (■), ciprofloxacin (■), erythromycin (▲) and rifampicin (□). Ciprofloxacin was the most efficacious antibiotic used in these trials.
Plate 5.1 Histological sections of liver tissue from the chick embryo. Hematoxylin and eosin stain.
a) normal tissue with dispersed red blood cells (RBCs) (X 165), b) liver tissue five days after inoculation with the YSLD$_{50}$ of *L. pneumophila*. Note edematous tissue and densely staining inflammatory response within congested central vein (X660).
Plate 5.2 Histological sections of heart tissue from the chick embryo. 
Hematoxylin and eosin stain.

a) normal tissue (X 165), b) heart tissue five days after inoculation with the 
YSLD50 of *L. pneumophila* with marked vascular congestion (arrows) (X 165).
Plate 5.3 Histological sections of spleen from the chick embryo. Hematoxylin and eosin stain.
a) normal tissue (X 165), b) spleen 5 days after inoculation with the YSLD$_{50}$ of 
{	extit{L. pneumophila}}. Inflammatory cells are evident within central artery and 
surrounding tissue (X185).
Plate 5.4 Histological sections of kidney from the chick embryo. Hematoxylin and eosin stain.
a) normal tissue showing glomeruli, convoluted tubules and circulating RBCs of the kidney (X 165), b) kidney five days after inoculation with the YSLD<sub>50</sub> of <i>L. pneumophila</i>. Note damaged sustained by glomeruli (short arrow) as compared to the convoluted tubules (long arrow). Inflammatory cells are distributed thoughout tissue the tissue (X 660).
Plate 5.5 Histological section of liver at five days post-inoculation at high magnification showing influx of inflammatory cells. Note incidence of PMNLs (short arrow) and lymphocytes (long arrow) as well as damaged hepatocytes (X 1650). Hematoxylin and eosin stain.
Plate 5.6 Electron micrograph of kidney three days after inoculation with 100 times the YSLD₅₀ of *L. pneumophila*. Subtle pathological changes within the mitochondria of cells were evident at this time (arrows) along with fatty degeneration and vacuole formation (X 8,000). Bar=1 μm.
Plate 5.7 Electron micrograph of heart three days after inoculation with 100 times the YSLD$_{50}$ of *L. pneumophila*. Note extreme damage to mitochondria (arrows) and initial stages of cytoplasmic clearing (X 10,000).
Bar=1 μm.
Plate 5.8 Electron micrograph of spleen four days after inoculation with 100 times the YSLD$_{50}$ of _L. pneumophila_ illustrating ballooning of nuclear membranes (short arrow), margination of chromatin material (long arrow), vesicle formation within cytoplasmic space (open arrow) and the presence of "ghost mitochondria" (curved arrow) (X 6,000). Bar=5 μm.
Plate 5.9 Electron micrograph of kidney four days after inoculation with 100 times the YSLD$_{50}$ of \textit{L. pneumophila}. Note the various stages of the hemolytic process (X 8,000). Bar=1 μm.
Plate 5.10 Electron micrograph of liver five days after inoculation with 100 times the YSLD$_{50}$ of *L. pneumophila* illustrating advanced stages of the infection. Necrotic hepatocytes are bordered by hemolysed RBCS. Cell membranes of liver cells are intermittently lysed (X 4,000). Bar=5 μm.
Plate 5.11 Electron micrograph of heart five days after inoculation with 100 times the YSLD$_{50}$ of *L. pneumophila* showing pathological changes at this stage of the infection. Fatty degeneration, intercellular and intracellular clearing, margination of chromatin material and membrane lysis are apparent (X 4,000). Bar=5 μm.
Plate 5.12 Electron micrograph of spleen five days after inoculation with 100 times the YSLD$_{50}$ of *L. pneumophila*. Pyknosis (short arrow) and karyolysis (long arrow) of nuclei are evident along with the formation of many cytoplasmic vesicles (X 6,000). Bar=5 μm.
Plate 5.13 Electron micrograph of spleen tissue four days after inoculation with 100 times the YSLD$_{50}$ of *L. pneumophila*. Cell with intracellular organism associated with cellular and sub-cellular pathology (X 8,000). Inset: higher magnification of *L. pneumophila* organism (X 20,000). Bars=1 μm.
Plate 5.14 Electron micrograph of kidney from embryos treated with 10 times the MIC of erythromycin three days after bacterial infection. Thin-sectioned tissue at four days post-infection (one day post-therapy) showing improved tissue as compared to untreated controls at the same stage of infection. Erythromycin did not sustain this level of activity with time (X 10,000).

Bar=1 μm.
Plate 5.15 Electron micrograph of kidney from embryos treated with 10 times the MIC of doxycycline three days after bacterial infection. Thin-sectioned material at four days post-infection (one day post-therapy) illustrating improved presentation of tissue as compared with untreated controls at the same stage of infection. Doxycycline was not able to maintain this level of activity with time (X 8,000). Bar=1 μm.
Plate 5.16 Electron micrograph of kidney from embryos treated with 10 times the MIC of rifampicin three days after bacterial infection. Thin-sectioned material at four days post-infection (one day post-therapy). Rifampicin treated tissue showed some improvement as compared to untreated tissue at the same stage of infection (X 4,000). Bar=5 μm.
Plate 5.17 Electron micrograph of kidney from embryos treated with 10 times the MIC of rifampicin three days after bacterial infection. Thin-sectioned material at eight days post-infection (five days post-therapy). No improvement of the condition of the tissue was noted with time (X 4,000). Bar=5 $\mu$m.
Plate 5.18 Electron micrograph of liver from embryos treated with 10 times the MIC of ciprofloxacin three days after bacterial infection. Thin-sectioned material at four days post-infection (one day post-therapy) exhibiting no significant improvement of tissue condition as compared to untreated controls at the same stage of the infection (X 4,000). Bar=5 μm.
Plate 5.19 Electron micrograph of liver from embryos treated with 10 times the MIC of ciprofloxacin three days after bacterial infection. Thin-sectioned material at eight days post-infection (five days post-therapy). Note much improved condition of tissue as compared with treated embryos at 4 days post-infection (X 4,000). Bar=5 μm.
6. General Discussion

Data from these studies indicated that a molecule or molecules with lectin-like activity on the surface of this organism may be responsible, at least in part, for \textit{L. pneumophila} binding to host target cells. Further identification and isolation of \textit{L. pneumophila} surface structures which facilitate attachment to target cells may lead to the development and production of potentially useful anti-adherence vaccines. The process by which \textit{L. pneumophila} recognized and adhered to host cell surfaces represents the earliest, most critical stage of bacterial infection. The ability to block these initial events with such prophylactic treatments would illustrate a novel strategy for the prevention of clinical legionellosis.

The chick embryo was established as a powerful tool for the evaluation of \textit{L. pneumophila} virulence and proved a viable alternative to other animal systems for the study of the pathology and chemotherapy of the extrapulmonary manifestations of experimental \textit{L. pneumophila} infections. Because of the intracellular nature of this organism, \textit{in vitro} MIC data do not reflect \textit{in vivo} or clinical results. These studies demonstrated the fertile hen's egg as a readily accessible \textit{in vivo} system for the evaluation of putative antimicrobial therapy for legionellosis and may prove of significant value for the evaluation of antibiotic regimens for other facultative intracellular pathogens such as \textit{Brucella}, \textit{Yersinia}, \textit{Listeria}, \textit{Chlamydia} and many others. Although, erythromycin (the drug of choice for Legionnaires' disease), rifampicin and doxycycline showed antimicrobial activity in this animal system,
ciprofloxacin, a newer broad spectrum quinolone antibiotic was the most efficacious in these trials.

Studies which characterized the morphological response of L. pneumophila to clinically relevant antibiotics provided indicators of the bacteriostatic versus bactericidal nature of these agents and yielded some information concerning the structural nature of the organism. In addition, examination of the microbial damage induced by selected antimicrobials was necessary for the interpretation of results derived from further in vivo evaluations of these antibiotics.

L. pneumophila is a human pathogen that causes fatal disease. Immunocompromised individuals are particularly susceptible to infection and respond variably to the current antibiotic regimens. The present investigation partially characterized the adherence of L. pneumophila to host cells and explored the possibilities of novel preventative measures in the form of anti-adherence vaccines for human disease. Furthermore, these data delineated the pathogenesis and pathology of L. pneumophila in experimental animal infections and illustrated the role of clinically relevant antibiotics in the outcome of disease. These results may lead to the development of aggressive combination therapeutic regimens in which protective vaccines and new broad-spectrum antimicrobial agents can be used for the prophylaxis and control of clinical legionellosis. Finally, this work clearly demonstrated the superior antimicrobial activity of the quinolones and their potential for treatment of human Legionnaires' disease.
Section VII

7. Possible future studies:

This work has taken a number of interesting turns and has revealed a variety of problems which could usefully be addressed concerning the further characterization of the pathogenesis of *L. pneumophila*:

1) role of humoral and cell-mediated immunity of the chick embryo to *L. pneumophila* infection and the outcome of disease.

2) examination of the potential intracellular colonization of the inflammatory cells reported here by *L. pneumophila*.

3) characterization of the role of *L. pneumophila* toxins in the disease process in the chick embryo.

4) role of bacterial toxins and antibiotics in intracellular survival of *L. pneumophila*.

5) comprehensive characterization of specific *L. pneumophila* adhesins (outer membrane proteins, fimbriae, pili, lectins) involved in adherence to host cells.

6) identification of host cell receptors for *L. pneumophila*.

7) electron microscopic examination of *L. pneumophila* adherence to U937 and subsequent internalization processes.

8) effect of selected antibiotics (especially at sub-MIC levels) on *L. pneumophila* adherence to host cells.
9) evaluation of immunosuppressive regimens on the outcome of experimental legionellosis in the chick embryo system.

10) determination of the synergistic action of selected antimicrobial agents in vitro and in vivo.

11) characterization of the mechanisms of L. pneumophila uptake by host cells.
8. List of References


172


APPENDICES

Appendix 1

Media used in biological studies.

1. Cell cultures

1.1 De-ionized water

All water used to make up media was obtained from a Millipore Milli-Q filtration system. Ten Megohm water was collected, filter sterilized through a 0.2 µm filter, and one liter aliquots stored at 4° C.

1.2 Phosphate buffered saline (PBS)

Solutions of PBS were made in de-ionized water as follows and sterilized by autoclaving and stored in 100 ml aliquots in glass bottles at 4° C.

<table>
<thead>
<tr>
<th>component</th>
<th>grams/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.15</td>
</tr>
</tbody>
</table>

1.3 Hanks’ Balanced Salt Solution (HBSS)

A concentrated 10 X sterile solution of HBSS with 0.35 g/l sodium bicarbonate and phenol red was obtained from Irvine Scientific. For use, the stock solution was diluted ten-fold under aseptic conditions with sterile de-ionized water. The stock and working strength solutions were stored in sterile glass bottles at 4° C.
1.4 Sodium bicarbonate solution (NaHCO₃)

A stock 7.5% solution of sodium bicarbonate was made by dissolving 75 g NaHCO₃ in 1 liter of de-ionized water and sterilized by filtration. Aliquots of 100 ml were placed in sterile bottles and stored at 4°C.

1.5 Bovine calf serum

Sterile bovine calf serum supplemented with iron was obtained from Hyclone Laboratories, distributed in 50 ml volumes in sterile plastic tubes and stored at -20°C.

1.6 Glutamine

This was obtained in powdered form from Sigma, dissolved in sterile de-ionized water to make a 100 X stock (300mM) solution and filter sterilized. Aliquots were stored at -20°C.

1.7 Minimal Essential Media with Earles salts and non-essential amino acids (MEM)

This medium was obtained from Irvine Scientific as a sterile 10 X concentrated solution with added phenol red. For use, stock solutions were diluted ten-fold with sterile de-ionized water in an aseptic manner. Stock and working strength solutions were stored in sterile glass bottles at 4°C.

1.8 Trypsin-EDTA

Trypsin and sodium versenate (EDTA) were obtained in powdered form from Sigma and dissolved in 100 ml of warm PBS to make a working solution of 0.05% trypsin and 0.02% EDTA. Aliquots of 10 ml were stored at -20°C.
Growth medium

MEM with Earles salts was prepared with the following sterile constituents:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM with Earles salts</td>
<td>450 ml</td>
</tr>
<tr>
<td>Bovine calf serum</td>
<td>50 ml</td>
</tr>
<tr>
<td>Sodium Bicarbonate (7.5%)</td>
<td>10 ml</td>
</tr>
<tr>
<td>Glutamine</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

2. Organism growth

2.1 Solid medium

Buffered yeast extract agar supplemented with $\alpha$-ketoglutarate (BCYE$\alpha$) was obtained from Gibco Laboratories (Madison, Wisconsin) and prepared according to the manufacturer's instructions. Components of this medium were as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>grams/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>yeast extract</td>
<td>10.0</td>
</tr>
<tr>
<td>ACES buffer</td>
<td>10.0</td>
</tr>
<tr>
<td>ferric pyrophosphate</td>
<td>0.25</td>
</tr>
<tr>
<td>$\alpha$-ketoglutarate</td>
<td>1.0</td>
</tr>
<tr>
<td>agar</td>
<td>15.0</td>
</tr>
<tr>
<td>charcoal, activated</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Four ml of a filter sterilized solution of 10% L-cysteine HCl was then aseptically added to 1 liter of medium according to manufacturer's instructions.
2.2 **Broth medium**

Buffered yeast extract broth supplemented with α–ketoglutarate (BYEα) was prepared as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>grams/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>yeast extract</td>
<td>10.0</td>
</tr>
<tr>
<td>ACES buffer</td>
<td>10.0</td>
</tr>
<tr>
<td>ferric pyrophosphate</td>
<td>0.25</td>
</tr>
<tr>
<td>α–ketoglutarate</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Constituents were added to one liter of distilled water, allowed to dissolve, filter sterilized through a 0.2μm filter and collected aseptically. Four ml of a filter sterilized 10% solution of L-cysteine HCL was aseptically added to the medium prior to use.
Appendix 2

1. Reagents and materials used in histological studies.

1.1 Harris’ Alum Hematoxylin

This solution was comprised of the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>hematoxylin crystals</td>
<td>5.0 g</td>
</tr>
<tr>
<td>absolute alcohol</td>
<td>50 ml</td>
</tr>
<tr>
<td>aluminum ammonium sulfate</td>
<td>100 g</td>
</tr>
<tr>
<td>de-ionized water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>mercuric oxide</td>
<td>2.5 g</td>
</tr>
</tbody>
</table>

Hematoxylin crystals were dissolved in alcohol and the aluminum ammonium sulfate dissolved in water. The two solutions were then mixed and rapidly brought to a boil. Mercuric oxide was added slowly and the mixture reheated for 15-20 min. This was kept covered in a glass coplin jar ready for use.

1.2 Stock 1% aqueous eosin solution

The following components comprised this solution:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>water soluble eosin</td>
<td>10 g</td>
</tr>
<tr>
<td>de-ionized water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>glacial acetic acid</td>
<td>2.0 ml</td>
</tr>
</tbody>
</table>

This were mixed and the solute allowed to dissolve. The stock solution was kept in a glass stoppered bottle and stored at 20°C. A working solution was made by mixing 50 ml of the stock solution with 120 ml absolute alcohol, 30 ml de-ionized water and 1.0 ml glacial acetic acid.

1.3 Acid alcohol solution

This was made by adding 70% (710 ml of 95% alcohol with 290 ml...
deionized water) to 10 ml concentrated hydrochloric acid, and stored in a stoppered bottle at 20° C.

1.4 Saturated lithium carbonate

This was made by dissolving 4.5 g of lithium carbonate in 450 ml deionized water and stored in a stoppered bottle at 20° C.

1.5 Buffered formalin solution

A 10% solution of formaldehyde (VWR) was prepared in PBS and stored in a stoppered glass bottle at room temperature.

1.6 Preparation of histological sections

Tissue samples were harvest from embryos and fixed in 10% buffered formalin. Samples were then embedded in paraffin with a Lab Tek Tissue Processor and trimmed with a razor blade. Four micron sections were cut with a American Optical rotary microtome, attached to glass slides and prepared for staining.

1.6.1 Hematoxylin and eosin staining procedure

The following staining procedure was used:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histoclear (two changes)</td>
<td>2 min</td>
</tr>
<tr>
<td>100% alcohol (two changes)</td>
<td>1 min</td>
</tr>
<tr>
<td>95% alcohol (two changes)</td>
<td>1 min</td>
</tr>
<tr>
<td>de-ionized water</td>
<td>3 dips</td>
</tr>
<tr>
<td>Harris' Hematoxylin</td>
<td>6 min</td>
</tr>
<tr>
<td>Running tap water</td>
<td>2 min</td>
</tr>
<tr>
<td>Acid alcohol</td>
<td>6-8 dips</td>
</tr>
<tr>
<td>de-ionized water</td>
<td>3 dips</td>
</tr>
<tr>
<td>lithium carbonate</td>
<td>7 dips</td>
</tr>
<tr>
<td>running tap water</td>
<td>15 min</td>
</tr>
<tr>
<td>eosin</td>
<td>1-4 min</td>
</tr>
<tr>
<td>95% alcohol (two changes)</td>
<td>15 sec</td>
</tr>
<tr>
<td>100% alcohol (two changes)</td>
<td>15 sec</td>
</tr>
</tbody>
</table>
Appendix 3

Reagents and materials used in electron microscopy studies.

1. Negative staining

1.1 Grids

Copper 400 mesh grids were supplied by Electron Microscopy Sciences (EMS) and were used in all studies.

1.2 Formvar

This was obtained from EMS as a dry powder. A stock 2% solution was made by dissolving one gram of formvar in 50 ml electron microscopy grade ethylene dichloride (EMS). Remaining solid after vigorous shaking dissolved upon standing overnight. Stock solution was stored in glass stoppered bottles in a desiccator until use.

Working solution was made up freshly as a 0.2% solution by mixing 18 ml ethylene dichloride with 2 ml stock formvar solution.

1.3 Formvar coating of grids

A clean dry glass slide was dipped into 20 ml 0.2% working strength formvar solution in a Coplin jar, removed and drained. When dry, the film was cut in situ into suitable sized squares using a sharp scalpel, and these squares were floated off onto the surface of de-ionized water by carefully submerging at a shallow angle the formvar coated slide. In reflected light, the formvar films could be seen as pale opalescent squares floating on the surface of the de-ionized water. Copper grids were placed (matt side down) on top of the films and collected from the water by carefully placing pieces of Whatman No. 1 filter paper over the films. Filter papers with adherent formvar films and grids were
collected and dried.

1.4 Phosphotungstic acid (PTA) negative stain

A 1% (w/v) solution was made by dissolving potassium phosphotungstate in 100 ml de-ionized water and the pH was adjusted to 6.7 using fresh aqueous potassium hydroxide. Any turbidity remaining after standing at room temperature for 24 hours was removed by centrifugation at 10,000 RPM in a Beckman Microfuge 12. The stain was stored at room temperature and used as required.

1.5 Negative staining procedure

Equal volumes of a bacterial suspension of approximately 5 x 10^6 cfu/ml were mixed with 1% PTA on a glass slide and a drop of the resultant mixture was placed onto a formvar coated grid. The mixture was allowed to remain on the grid for about 10-15 seconds before being drained off by touching the edge of the grid with a piece of Whatman No. 1 filter paper. The rate at which the bacteria-stain mixture drained from the grid into the filter paper was indicative of the suitability of the preparation. Drops that drained quickly usually contained insufficient material.

2. Embedding and thin-sectioning

2.1 Grids

EMS 400 mesh copper grids were used in all studies.
2.2 **Cacodylate buffer**

This was made by adding the appropriate amount of sodium cacodylate (EMS) to one liter of de-ionized water to give the following concentrations:

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>bacteria</td>
<td>0.05 M (supplemented with 10mM MgSO₄)</td>
</tr>
<tr>
<td>tissue</td>
<td>0.10 M</td>
</tr>
</tbody>
</table>

These solutions were adjusted to a pH of 7.2 using hydrochloric acid and stored at 4°C until required.

2.3 **Glutaraldehyde**

Glutaraldehyde was obtained from EMS as an electron microscopy grade 25% (v/v) aqueous solution. The stock solution was stored in at 4°C and working 3% (v/v) solutions were made freshly in cacodylate buffer as required.

2.4 **Osmium tetroxide**

A stock 2% (w/v) solution of OsO₄ (Stevens Metallurgic Corporation) was made by dissolving the contents of a 1g ampule in 50 ml cacodylate buffer. OsO₄ emits a dangerous vapor and was always handled in a fume hood. The solute was completely dissolved after standing overnight at 4°C. The stock solution was stored at 4°C in a tightly capped bottle covered with aluminum foil. The working 1% solution was made by mixing equal volumes of the stock 2% solution and cacodylate buffer.

2.5 **Ethanol series**

A graded ethanol series was made by mixing appropriate amounts of dry absolute ethanol and cacodylate buffer. The 50% and 70% solutions were stored at 4°C and the 90%, 95% and absolute ethanol solutions were stored at 194°C.
room temperature. (Percentages refer to the concentration of ethanol).

2.6 Propylene oxide

This was obtained from EMS and was supplied ready for use. The container was kept tightly stoppered at all times.

2.7 Resin mixture

The resin used in all studies is described in 2.7.1 below. All volumes were measured in disposable 50 ml tri pour beakers from VWR except for dibutyl phthalate and DMP-30 which were measured using one ml disposable syringes without needles.

2.7.1 Epon-araldite resin mixture

The resin mixture was comprised of the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Epon 812 (resin)</td>
<td>5 parts</td>
</tr>
<tr>
<td>2) Dodecenyl succinic anhydride (DDSA)</td>
<td>11 parts</td>
</tr>
<tr>
<td>3) Araldite (resin)</td>
<td>3 parts</td>
</tr>
<tr>
<td>4) Dibutyl phthalate (plasticizer)</td>
<td>0.8 parts</td>
</tr>
<tr>
<td>5) Methyl nadic anhydride (DMP 30) (accelerator)</td>
<td>0.4 parts</td>
</tr>
</tbody>
</table>

Components were added to a glass bottle in this order and vigorously shaken for 10 min with the exception of DMP 30 which was added just before resin was required for use. Complete resin (with DMP-30) was then vigorously shaken for an additional three to five min. This resin mixture was polymerized at 60° C for 24 h.
2.8 Embedding and thin-sectioning procedure

For bacterial samples, organisms were harvested, fixed in 3\% buffered glutaraldehyde, washed three times in 0.05 M cacodylate buffer containing 10mM MgSO₄ and pre-embedded in 3\% (w/v) Noble agar. Cubes of 1 mm³ thickness were cut and post-fixed in 1\% OsO₄ in cacodylate buffer for 1 h.

Tissue samples were harvested, rinsed in 0.1 M cacodylate buffer and fixed in 3\% buffered glutaraldehyde. One mm³ cubes were cut and post-fixed in 1\% OsO₄ in cacodylate buffer for 1 h. All samples were rinsed in cacodylate buffer three times followed by a dehydration procedure of immersion for five min in each of 50\%, 70\%, 90\% and 95\% ethanol in cacodylate buffer. Dehydration of samples was completed by two rinses of ten min each in absolute alcohol. Ethanol was removed by pipette and the specimens rinsed twice in propylene oxide. Specimens were not allowed to dry during any step of this procedure.

Resin infiltration was done by placing the samples in a 2:1 mixture of propylene oxide and complete resin for one hour, and this mixture was then replaced by a 1:2 mixture of propylene oxide and complete resin for an additional hour. Specimens were placed in complete resin for one hour after which the resin was changed for fresh complete resin which had been degassed for 20 min in a vacuum desiccator. These remained in the degassed resin for one hour, removed and located in embedding molds. The molds were filled with degassed complete resin. Specimens were manipulated using wooden swab sticks sharpened at one end to form a flexible spatula which was pre-treated by dipping in degassed complete resin. These treated spatulas
were placed at 60° C for one hour prior to use to allow the resin end to dry and so as to prevent the introduction of air bubbles around the specimen. Blocks were labelled with hardened paper and polymerized in a 60° C oven for 24 h. After removal from the oven, blocks were left at room temperature for two or three days to allow complete hardening of the resin prior to their removal from the molds. Embedding of samples was completed in one day and was followed by a 24 h polymerization, the whole procedure taking approximately 30 h to complete. The embedding schedule and times required are summarized in Table 1.

Polymerized blocks were placed in the round chuck of a LKB Ultratome III ultramicrotome and trimmed mechanically with a glass knife made with an LKB knife maker. When blocks had been appropriately trimmed, ultra thin sections were cut using the thermal advance on the ultramicrotome and using a Diatom diamond knife filled with clean de-ionized water. The diamond knife edge was cleaned with a soft clean pith stick. Sections giving grey or silver interference colors (60-90 nm thickness) were chosen. Individual sections and small ribbons were picked up on copper grids. Grids were submerged in the boat and sections located above them with a mounted eyelash. Sections were removed by lifting the grid out of the water and the grids blotted dry by touching the edge to a piece of filter paper.

Ultrathin sections were stained with 5% aqueous uranyl acetate for 60 seconds by floating grids, sections downward, on a drop of stain placed on parafilm and covered by a glass petri dish lid. Grids were removed and
sections washed thoroughly in a running stream of de-ionized water using approximately 15 ml per grid, after which they were blotted and allowed to dry. The grids were then held in fine forceps and a drop of lead citrate placed on them for 20 seconds, after which they were washed as before using approximately 25 to 30 ml de-ionized water per grid. Grids were blotted and allowed to dry as before, Stain was removed from the stock bottles using a fine pasteur pipette with the tip located about 5mm below the meniscus. The first drop in the pipette was always discarded, thus ensuring minimal stain deposit.

Thin-sectioning was performed under strictly dust free conditions and all materials coming into contact with sections, knife or boat water were stringently cleaned and rinsed under running de-ionized water. Sections were examined and photographed in a Hitachi H600 electron microscope at 75 kV, using various instrumental manipulations.

2.9 Stains for ultra thin sections

2.9.1 Uranyl acetate

A 5% (w/v) solution of uranyl acetate was made by dissolving 1 g in 20 ml de-ionized water. The solid was found to dissolve completely upon standing overnight at room temperature. The solution was filtered through a Whatman No. 2 filter paper and stored in a dark glass-stoppered bottle at room temperature.

2.9.2 Lead citrate

One pellet sodium hydroxide was dissolved in 15 ml boiled cooled de-ionized water. This solution was added to 0.1 g lead citrate in a glass screw-capped bottle and dissolved by shaking. A further 10 ml boiled cooled de-
ionized water was added, and the stain was stored tightly capped at room temperature.

3. Scanning electron microscopy (SEM)

3.1 Hexamethyldisiloxane (HMDS)

This was obtained from Polysciences, Inc. (Warrington, Pa.) and kept tightly capped in a fume hood prior to use.

3.2 SEM procedure

Bacterial specimens were fixed in 3% glutaraldehyde in cacodylate buffer, washed three times in buffer and dehydrated in 50%, 70%, 90%, and absolute ethanol. Samples were then treated with two changes of HMDS and applied to specimen stubs with double sided sticky tape. Stubs were coated with 20 nm gold/palladium at a target to specimen distance of 5 cm with a current of 15 mA for 4 min. Specimens were viewed in a Hitachi H600 electron microscope in the scanning mode at an accelerating voltage of 40 kV.
<table>
<thead>
<tr>
<th>Procedure</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Wash in cacodylate buffer</td>
<td>15 min</td>
</tr>
<tr>
<td>2) Fix in 3% glutaraldehyde in cacodylate buffer</td>
<td>30 min</td>
</tr>
<tr>
<td>3) Wash in cacodylate buffer (ten changes)</td>
<td>30-40 min</td>
</tr>
<tr>
<td>4) Post-fix in 1% OsO₄</td>
<td>1 h</td>
</tr>
<tr>
<td>5) Wash in cacodylate buffer</td>
<td>5 min</td>
</tr>
<tr>
<td>6) Dehydrate in ethanol series in cacodylate buffer</td>
<td>20 min</td>
</tr>
<tr>
<td>7) Complete dehydration in absolute ethanol and propylene oxide</td>
<td>40 min</td>
</tr>
<tr>
<td>8) Resin / propylene oxide mixtures</td>
<td>120 min</td>
</tr>
<tr>
<td>9) Resin (two changes)</td>
<td>120 min</td>
</tr>
<tr>
<td>10) Polymerization</td>
<td>24 h</td>
</tr>
</tbody>
</table>

Total Time Required  
Approximately 30 h
Appendix 4

1. Production of polyclonal antisera against *L. pneumophila*.

1.1 Rabbits

White New Zealand rabbits were obtained locally and pre-bled to assess pre-immunization level of antibodies against *L. pneumophila*.

1.2 Antigen preparation

Whole cells of *L. pneumophila* (approximately 1 x 10^9 cfu/ml) were formalin fixed in 1% (v/v) formaldehyde (VWR) in PBS and a small volume of sterile egg yolk. Prior to immunization of rabbits, 1 ml of this solution was mixed with an equal volume of Freund's Incomplete adjuvant (VWR) and homogenized in a Sorval Omni Mixer.

1.3 Bleeding procedure

1.3.1 Ear bleed

Samples of blood obtained for antibody titer analysis following immunization were drawn aseptically from the central artery of one of the rabbit's ears. Vasodilation of this artery was achieved by warming the rabbit's ear with a 15 watt bulb of a portable lamp. When the artery was suitably dilated, the ear was swabbed with alcohol and a 21 gauge needle was inserted to release approximately 5 ml blood into a sterile test tube. The ear was then swabbed again with alcohol and treated appropriately to stop the bleeding.

1.3.2 Cardiac puncture

Once the antibody titer had reached a suitable level following immunization, the final volume of blood was collected by cardiac puncture and exsanguination of the rabbit. The rabbit was anesthetized with the addition of a
sodium solution of Nembutal into the peripheral vein of the ear and blood drawn through an 18 gauge needle and syringe inserted through the chest cavity into the heart. The rabbit was exsanguinated in a humane fashion with a final injection of Nembutal into the heart.

Blood obtained from ear bleeds and cardiac puncture was allowed to clot overnight at 4°C and the resultant serum decanted for titer analysis or stored frozen in 1 ml aliquots at -70°C.

1.4 Antibody titer assay

An indirect immunofluorescence assay (Materials and Methods, Chapter Four) was used to measure the titer of sera drawn from rabbits. Once obtained, doubling dilutions of the serum were made in PBS and used immediately in the analysis. Formalin fixed whole L. pneumophila cells were added to multiple spots on glass slides and allowed to dry. Dilutions of the serum were added to these areas as the primary antibody in an indirect immunofluorescence assay and allowed to incubate at 37°C for 1 hour. Three-fold washes were made with PBS whereupon goat anti-rabbit FITC conjugated serum was added to all spots on the glass slide and incubated for 1 hour at 37°C. These were washed three times and viewed with an BH-2 Olympus microscope in the immunofluorescence mode. Appropriate scores of immunofluorescence for each dilution were made and the most diluted spot which gave a positive score selected as the titer.

1.5 Immunization procedure

Rabbits were pre-bled and analyzed for pre-immunization antibody against L. pneumophila. A small area of the rabbits back was clean shaven,
swabbed with alcohol and the antigen-adjuvant mixture administered into 10 sub-cutaneous sites with a 21 gauge needle and syringe. These sites were swabbed with alcohol again and examined for unusual reactions. The rabbits were boosted with a similar antigen mass 2 and 4 weeks later. Antibody titer was checked every week during this time.
REPRINTS OF PUBLICATIONS