

SERUM RESISTANCE AND OUTER MEMBRANE PROTEINS OF
HAEMOPHILUS DUCREYI

ERIC ELIAS

A dissertation submitted to the Faculty of Medicine,
University of the Witwatersrand, Johannesburg, in fulfilment
of the requirements for the degree of Master of Science.

December 1990

ABSTRACT

The pathogenicity of Haemophilus ducreyi, the etiological agent of chancroid is poorly understood. Although H. ducreyi appears not to invade the bloodstream, resistance to lysis by normal human serum may allow prolonged multiplication of the organism within tissues. The determinants of serum resistance, however, remain to be elucidated and could include a number of bacterial surface components including outer membrane proteins (OMPs) and lipopolysaccharide.

The present study was undertaken to determine the role of surface-exposed proteins and serum resistance in the pathogenesis of H. ducreyi infections. A possible connection between these factors was also sought. The level of resistance to 50% PNHS amongst selected clinical and reference strains of H. ducreyi was determined. The profile, antigenicity and surface exposure of their OMPs was characterised. In order to correlate these features with serum resistance as a virulence factor, attempts were made to generate an isogenic serum-resistant mutant of H. ducreyi. Four reference H. ducreyi strains (A75, A76, A77 ; Pasteur Institute, Paris, and ATCC 27722 ; American Type

Culture Collection), and six local clinical H. ducreyi strains (Y206, D29, FQ61, FQ33, Y137, Y56, Y155 and FQ24) were tested for serum resistance. Strains FQ33, FQ61, and Y206 were most resistant to complement-mediated killing, while reference strains were extremely susceptible. A77*, an isogenic strain of A77 was passaged in increasing concentrations of PNHS in order to generate an isogenic serum-resistant mutant. Although strain A77* was found to be more resistant than its parent strain, it was rapidly killed by serum.

OMP profiles of H. ducreyi were characterised by SDS-polyacrylamide gel electrophoresis (PAGE) and further studied by Western blotting using immune rabbit serum. A major OMP band of molecular weight 41kD was present in all the H. ducreyi strains and was found to be surface-exposed by radioiodination. No correlation could be confirmed between OMP composition, surface exposure, immunogenicity and the degree of serum resistance when comparing reference with clinical strains, and comparing parent strain A77 with its more serum-resistant isogenic progeny A77*. This does not exclude OMPs from contributing to the virulence of H. ducreyi.

Although serum resistance has been proposed as a marker of virulence, this too awaits definitive confirmation.

DECLARATION

I declare the following :

That this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the faculty of Medicine, University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other university.

That the Committee for Research on Human Subjects approved : "Serum Resistance and Outer Membrane Proteins of Haemophilus ducreyi" on the 31 October 1988 (Protocol number 19/10/88).

That the Animal Ethics Control Committee approved : "Serum Resistance and Outer Membrane Proteins of Haemophilus ducreyi" on 31 January 1989 (AEC number 88/195/2).

ERIC ELIAS

A handwritten signature in dark ink, appearing to read 'Eric Elias', written over a light blue horizontal line.

December 1990.

ACKNOWLEDGEMENTS

The following individuals are sincerely acknowledged :

Professor J. Metz, Director of the South African Institute for Medical Research (S.A.I.M.R.), Johannesburg.

Professor H.J. Koornhof, Head of the Department of Medical Microbiology, for his continued support.

Professor S.D. Miller, my supervisor, for his constructive criticism and guidance throughout the entire duration of this study. An enormous amount was gained from his endless knowledge.

The staff of the Sexually Transmitted Disease unit, especially Ms. Glenda Fehler for proof-reading my thesis, and Mr. Yusuf Dangor for the technical assistance given in culturing Haemophilus ducreyi, and supplying all the clinical isolates.

Ms. Gillian Jackson for assisting me on the computer.

The staff of the S.A.I.M.R. library.

Ms. H. Toxopeus, Media Department, S.A.I.M.R., for providing the bacteriological media required in this study.

Ms. A. Ferreira, Computer Centre, University of the Witwatersrand, for the statistical analysis of the results.

The staff of the Illustration Unit, S.A.I.M.R., for preparation of the graphs.

The staff of the Photographic Unit, S.A.I.M.R., for preparation of the photographs.

The S.A.I.M.R., Council for Scientific and Industrial Research and the University of the Witwatersrand for the bursaries awarded towards this study.

My family and friends for their continued support.

LIST OF ABBREVIATIONS

ATCC	American type culture collection
BHI	Brain heart infusion broth
CAT	Chloramphenicol acetyltransferase
CFU	Colony forming unit(s)
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis (aminoethyl ether) N N N' N-tetraacetic acid
FBS	Foetal bovine serum
FCS	Foetal calf serum
G + C	Guanine-plus-cytosine
GC	Gonococcal agar
HIV	Human immunodeficiency virus
Ig	Immunoglobulin
IS	Inactivated PNHS
kD	Kilodaltons
LPS	Lipopolysaccharide
L	Litre
MAC	Membrane attack complex
MIC	Minimum inhibitory concentration

Md	Megadalton
mg	Milligram
ml	Millilitre
mRNA	Messenger ribonucleic acid
NS	No PNHS
OMP	Outer membrane protein(s)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PNHS	Pooled normal human serum
SAIMR	South African Institute for Medical Research
SBA	Serum bactericidal assay
SDS	Sodium dodecyl sulphate
tris	Tris (hydroxymethyl) aminomethane
ul	Microlitre

TABLE OF CONTENTS

ABSTRACT.....	i
DECLARATION.....	iv
ACKNOWLEDGEMENTS.....	v
LIST OF ABBREVIATIONS.....	vii
TABLE OF CONTENTS.....	ix
LIST OF TABLES.....	xvi
LIST OF FIGURES.....	xvii
CHAPTER ONE.....	1
1.1 PART ONE - RECOGNITION AND TREATMENT OF CHANCROID.....	1
1.1.1 INTRODUCTION.....	1
1.1.2 ISOLATION OF THE CAUSATIVE AGENT.....	3
1.1.3 EPIDEMIOLOGY.....	7
1.1.4 TREATMENT OF CHANCROID.....	9
1.1.5 CHANGING ANTIMICROBIAL SUSCEPTIBILITY PATTERNS OF SOUTHERN AFRICAN ISOLATES OF <u>H. DUCREYI</u>	14
1.1.6 DIAGNOSIS OF CHANCROID.....	15
1.1.6.1 Clinical features.....	15
1.1.6.2 Laboratory diagnosis.....	20
1.1.6.2.1 Microscopy.....	20
1.1.6.2.1.1 Gram-stain.....	20

1.1.6.2.1.2 Immunofluorescence.....	23
1.1.6.2.1.3 Delayed hypersensitivity and serological tests	26
1.1.6.2.2 Culture and identification.....	28
1.1.7 PATHOGENESIS AND VIRULENCE TESTING.....	29
1.1.8 ANIMAL STUDIES.....	32
1.1.9 CHANCROID AND HUMAN IMMUNODEFICIENCY VIRUS (HIV)	
TRANSMISSION.....	34
1.2 PART TWO - HAEMOPHILUS DUCREYI - THE ETIOLOGICAL	
AGENT.....	37
1.2.1 STRUCTURE.....	37
1.2.1.1 Cellular morphology.....	37
1.2.1.2 Colonial appearance.....	39
1.2.1.3 Electron microscopy.....	40
1.2.1.4 Cell surface composition.....	42
1.2.2 METABOLISM.....	42
1.2.2.1 Biochemical characterisation.....	42
1.2.2.2 Growth requirements.....	46
1.2.2.2.1 Nutritional studies.....	46
1.2.2.2.2 Media.....	48
1.2.2.2.3 Cultural enviroment.....	56
1.2.2.2.4 Preservation of cultures.....	58
1.2.3 GENETICS.....	58
1.2.3.1 Taxonomy.....	58
1.2.3.1.1 Chromosomal elements.....	58

1.2.3.1.2 Plasmids.....	60
1.2.3.2 Antimicrobial resistance.....	61
1.2.3.2.1 Sulphonamides.....	61
1.2.3.2.2 Trimethoprim.....	62
1.2.3.2.3 Rifampicin.....	62
1.2.3.2.4 Chloramphenicol.....	63
1.2.3.2.5 Aminoglycosides.....	63
1.2.3.2.6 Tetracycline.....	64
1.2.3.2.7 Ampicillin.....	65
1.2.4 ANTIMICROBIAL SUSCEPTIBILITY TESTING.....	66
1.2.5 SPECIMEN COLLECTION.....	69
1.2.5.1 Swabs.....	70
1.2.5.2 Transport.....	70
CHAPTER TWO : THE GRAM-NEGATIVE CELL WALL.....	72
2.1 THE GRAM-NEGATIVE BACTERIAL CELL SURFACE.....	72
2.1.1 Introduction.....	72
2.1.2 Organisation of the intermediate layer and outer membrane.....	72
2.2 THE OUTER MEMBRANE PROTEINS.....	76
2.2.1 Nomenclature of the major proteins.....	76
2.2.1.1 Matrix proteins.....	77
2.2.1.2 TolG protein.....	79
2.2.1.3 Lipoprotein.....	80

2.2.1.4 Minor proteins.....	83
2.2.2 Structure and function.....	84
2.2.3 Factors affecting OMPs.....	88
2.2.4 Genetic determinants.....	88
 CHAPTER THREE : SERUM BACTERICIDAL ACTIVITY.....	93
3.1 INTRODUCTION.....	93
3.2 SERUM BACTERICIDAL ACTIVITY AS AN IMMUNE DEFENCE MECHANISM.....	94
3.3 CLINICAL RELEVANCE OF SERUM RESISTANCE AS A DETERMINANT OF BACTERIAL PATHOGENICITY.....	96
3.4 MECHANISM OF SERUM BACTERICIDAL ACTIVITY.....	100
3.4.1 Role of complement.....	100
3.4.2 Role of antibody.....	108
3.4.3 Sequence of events after exposure to serum.....	109
3.5 DEFINING RESISTANCE TO SERUM BACTERICIDAL ACTIVITY....	111
3.6 BACTERIAL COMPONENTS ASSOCIATED WITH SERUM RESISTANCE.	112
3.6.1 Lipopolysaccharides (LPS).....	112
3.6.2 Acidic polysaccharides.....	116
3.6.3 Outer membrane proteins (OMPs).....	120
3.6.3.1 Determinants of serum resistance in the outer membrane.....	123
3.6.4 Plasmid determined factors.....	125
3.7 DETERMINATION OF SERUM BACTERICIDAL ACTIVITY.....	127

CHAPTER FOUR : GENERAL MATERIALS AND METHODS.....	131
4.1 BACTERIAL STRAINS.....	131
4.1.1 Clinical isolates.....	131
4.1.2 Control strains.....	132
4.2 GROWTH CONDITIONS.....	132
4.2.1 Media.....	132
4.2.2 Incubation.....	133
4.2.3 Storage.....	133
4.3 RECONSTITUTION OF <u>H. DUCREYI</u> REFERENCE STRAINS A75, A76 AND A77.....	134
4.4 ANTIBODY PRODUCTION IN NEW ZEALAND WHITE RABBITS.....	136
4.5 INDIRECT IMMUNOFLUORESCENCE ASSAY OF <u>H. DUCREYI</u>	138
 CHAPTER FIVE : SERUM BACTERICIDAL ACTIVITY OF <u>H. DUCREYI</u> ..	141
5.1 INTRODUCTION.....	141
5.2 MATERIALS AND METHODS.....	143
5.2.1 Bacterial strains and media.....	143
5.2.2 Pooled normal human serum (PNHS) preparation.....	144
5.2.3 Serum bactericidal assay (SBA).....	145
5.2.4 Induction of serum resistance.....	146
5.3 RESULTS.....	147
5.3.1 Serum bactericidal assay and serum resistance.....	147
5.3.2 Induction of serum resistance.....	160
5.4 DISCUSSION.....	161

5.4.1 The serum bactericidal assay.....	161
5.4.1.1 Factors affecting the serum bactericidal assay....	161
5.4.1.1.1 Growth phase of the inoculum.....	162
5.4.1.1.2 Inoculum size.....	163
5.4.1.1.3 Insufficient contact between organism and human serum.....	164
5.4.1.1.4 Choice of media.....	165
5.4.2 Serum resistance.....	166
5.4.2.1 Measurement of serum resistance.....	166
5.4.2.2 Serum resistance and complement.....	167
5.4.2.3 Serum resistance and bacterial virulence.....	169
5.4.3 Induction of serum resistance.....	171
5.5 CONCLUSION.....	173

CHAPTER SIX : OUTER MEMBRANE PROTEINS AND ANTIGENICITY OF

<u>H. DUCREYI</u>	176
6.1 INTRODUCTION.....	176
6.2 MATERIALS AND METHODS.....	180
6.2.1 Strains and media.....	180
6.2.2 OMP isolation.....	182
6.2.3 SDS-PAGE.....	182
6.2.4 Western blot analysis.....	184
6.2.5 Radioiodonation of surface-exposed proteins.....	184
6.3 RESULTS.....	185

6.3.1 SDS-PAGE.....	185
6.3.2 Western blot analysis.....	189
6.3.2.1 Analysis of <u>H. ducreyi</u> by Western blotting.....	195
6.3.2.2 Antigenic cross-reactivity between OMPs of <u>H. ducreyi</u> and other <u>Haemophilus</u> species.....	196
6.3.3 Radioiodonation of surface-exposed proteins.....	196
6.4 DISCUSSION.....	199
6.4.1 OMP isolation.....	199
6.4.2 SDS-PAGE.....	202
6.4.3 Western blot analysis.....	205
6.4.4 Radioiodonation of surface-exposed proteins.....	208
6.5 CONCLUSION.....	209
 CHAPTER SEVEN : CONCLUSIONS AND FUTURE CONSIDERATIONS.....	212
7.1 CONCLUSIONS.....	212
7.2 FUTURE CONSIDERATIONS.....	215
 LIST OF APPENDICES.....	217
LIST OF REFERENCES.....	237

LIST OF TABLES

TABLE 1.1 Major biochemical characteristics of <u>H. ducreyi</u> .	
.....	44
TABLE 1.2 Solid media for primary <u>H. ducreyi</u> isolation	
.....	50

LIST OF FIGURES

FIGURE 1.1 Clinical appearance of multiple chancroid ulcers.	17
FIGURE 1.2 Multiple preputial and subpreputial ulcers in a patient with chancroid and phimosis.	19
FIGURE 1.3 Bubo formation in a patient with chancroid.	21
FIGURE 1.4 Indirect immunofluorescence of <u>H. ducreyi</u> X400 magnification.	24
FIGURE 1.5 Prolific bleeding of a chancroid ulcer.	36
FIGURE 1.6 Gram-stain of <u>H. ducreyi</u> grown on solid media.	38
FIGURE 1.7 <u>H. ducreyi</u> colonies on a solid agar medium.	40

FIGURE 2.1 Schematic model of the <u>E. coli</u> outer membrane.	73
FIGURE 2.2 Assembly model of the matrix protein and lipoprotein. Abbreviations used are : LP, lipoprotein ; MP, matrix protein ; and PG, peptidoglycan. The bound form lipoprotein molecules are darkened.	87
FIGURE 2.3 Chromosomal location of genes involved in the biosynthesis of OMPs. Genetic symbols are described in the text, except <u>pldA</u> for phospholipase A1. Parentheses around a gene symbol indicate that the location of that marker is not well defined.	90
FIGURE 3.1 Schematic representation of the mechanisms by which microorganisms activate C3 to C9 and generate biologically active products.	102

FIGURE 4.1 Indirect immunofluorescence of H. ducreyi strain A77 X400 magnification. Rabbit antiserum diluted 1:640.

.....140

FIGURE 5.1 Bactericidal effect of 50% PNHS on H. ducreyi reference strain A75 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

.....149

FIGURE 5.2 Bactericidal effect of 50% PNHS on H. ducreyi reference strain A76 at time intervals 0, 30, 60, and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

.....150

FIGURE 5.3 Bactericidal effect of 50% PNHS on H. ducreyi reference strain A77 at time intervals 0, 30, 60, and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

.....151

FIGURE 5.4 Bactericidal effect of 50% PNHS on H. ducreyi reference strain ATCC 27722 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

.....152

FIGURE 5.5 Bactericidal effect of 50% PNHS on H. ducreyi clinical strain D29 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

.....153

FIGURE 5.6 Bactericidal effect of 50% PNHS on H. ducreyi clinical strain Y56 at time intervals 0, 30, 60, and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

.....154

FIGURE 5.7 Bactericidal effect of 50% PNHS on H. ducreyi clinical strain Y206 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

.....155

FIGURE 5.8 Bactericidal effect of 50% PNHS on H. ducreyi clinical strain Y137 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

.....156

FIGURE 5.9 Bactericidal effect of 50% PNHS on H. ducreyi clinical strain FQ33 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

.....157

FIGURE 5.10 Bactericidal effect of 50% PNHS on H. ducreyi clinical strain FQ61 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

.....158

FIGURE 5.11 Bactericidal effect of 50% PNHS on H. ducreyi reference strain A77* at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Two controls included heat-inactivated PNHS (IS) and no PNHS (NS).

.....159

FIGURE 6.1 SDS - PAGE profiles of OMP preparations of H. ducreyi strains: Lane 1, low molecular weight standards ; lane 2, A75 ; lane 3, A76 ; lane 4, A77 ; lane 5, Y155 ; lane 6, Y137 ; lane 7, FQ24 ; lane 8, ATCC 27722 ; lane 9, D29.

.....186

FIGURE 6.2 SDS-PAGE profiles of OMP preparations of H. ducreyi and other Haemophilus species : Lane 1, H. aegyptius ; lane 2, H. influenzae ; lane 3, H. aphrophilus ; lane 4, H. parainfluenzae ; lane 5, ATCC 27722 ; lane 6, Y56 ; lane 7, Y137 ; lane 8, FQ33 ; lane 9, FQ61 ; lane 10, D29 ; lane 11, Y206 ; lane 12, A77 ; lane 13, A76 ; lane 14, A75 ; lane 15, low molecular weight standards.

.....187

FIGURE 6.3 Western blot analysis of H. ducreyi OMPs using anti-A75 rabbit immune serum (diluted 1:100) : Lane 1, A75 ; lane 2, A75 ; lane 3, A76 ; lane 4, A77 ; lane 5, Y155 ; lane 6, D29 ; lane 7, Y137 ; lane 8, FQ24 ; lane 9, ATCC 27722.

.....189

FIGURE 6.4 Western blot analysis of H. ducreyi OMPs using anti-A76 rabbit immune serum (diluted 1:100) :
 Lane 1, A75 ; lane 2, A75 ; lane 3, A76 ; lane 4, A77 ; lane 5, Y155 ; lane 6, D29 ; lane 7, Y137 ; lane 8, FQ24 ; lane 9, ATCC 27722.

.....190

FIGURE 6.5 Western blot analysis of H. ducreyi OMPs using anti-A77 rabbit immune serum (diluted 1:100) :
 Lane 1, D29 ; lane 2, ATCC 27722 ; lane 3, FQ24 ; lane 4, Y137 ; lane 5, Y155 ; lane 6, A77 ; lane 7, A76 ; lane 8, A75.

.....191

FIGURE 6.6 Western blot analysis of H. ducreyi and other Haemophilus species OMPs using anti-A75 rabbit immune serum (diluted 1:100) : Lane 1, H. aegyptius ; lane 2, H. influenzae ; lane 3, H. aphrophilus ; lane 4, H. parainfluenzae ; lane 5, ATCC 27722 ; lane 6, Y56 ; lane 7, Y137 ; lane 8, FQ33 ; lane 9, FQ61 ; lane 10, D29 ; lane 11, Y206 ; lane 12, A77 ; lane 13, A76 ; lane 14, A75

.....192

FIGURE 6.7 Western blot analysis of H. ducreyi and other Haemophilus species OMPs using anti-A76 rabbit immune serum (diluted 1:100) : Lane 1, H. aegyptius ; lane 2, H. influenzae ; lane 3, H. aphrophilus ; lane 4, H. parainfluenzae ; lane 5 ; ATCC 27722 ; lane 6, Y56 ; lane 7, Y137 ; lane 8, FQ33 ; lane 9, FQ61 ; lane 10, D29 ; lane 11, Y206 ; lane 12, A77 ; lane 13, A76 ; lane 14, A75
193

FIGURE 6.8 Western blot analysis of H. ducreyi and other Haemophilus species OMPs using anti-A77 rabbit immune serum (diluted 1:100) : Lane 1, H. aegyptius ; lane 2, H. influenzae ; lane 3, H. aphrophilis ; lane 4, H. parainfluenzae ; lane 5, ATCC 27722 ; lane 6, Y56 ; lane 7, Y137 ; lane 8, FQ33 ; lane 9, FQ61 ; lane 10, D29 ; lane 11, Y206 ; lane 12, A77 ; lane 13, A76 ; lane 14, A75
194

FIGURE 6.9 Autoradiograph of radioiodinated intact cells of H. ducreyi separated by SDS-PAGE : Lane 1, ATCC 27722 ; lane 2, Y56 ; lane 3, Y137 ; lane 4, FQ61 ; lane 5, FQ24 ; lane 6, FQ33 ; lane 7, D29 ; lane 8, Y206 ; lane 9, low molecular weight standards
197

FIGURE 6.10 Autoradiograph of radioiodinated intact cells of H. ducreyi reference strains separated by SDS-PAGE : Lane 1, A77 ; lane 2, A76 ; lane 3, A75 and lane 4, low molecular weight standards
198

CHAPTER ONE

(1.1) PART ONE - RECOGNITION AND TREATMENT OF CHANCROID

(1.1.1) Introduction

Chancroid, caused by Haemophilus ducreyi is a sexually transmitted disease characterised by painful ulcerations at the site of inoculation and enlargement of the regional lymph nodes. Lymph node suppuration, phimosis, paraphimosis and phagedemic tissue destruction are complications of the disease (Felman and Nikitas 1983).

Chancroid or "soft chancre" (ulcus molle), was differentiated clinically from syphilis, or "hard chancre" in 1852. Although credit is generally given to Bassereau for making this distinction, some degree of clinical differentiation of genital ulcers had been recognised much earlier (Kampmeier, 1982). Almost half a century passed before Auguste Ducrey, working at the University of Naples, saw the presumptive etiological agent. He followed up these observations by reporting that autoinoculation of the skin of the forearm of

three patients with purulent material from their own genital ulcers gave rise to clinically similar lesions (Ronald and Albritton, 1984). At weekly intervals he inoculated a new site with material from the most recent ulcer and was able to maintain serial ulcers through as many as fifteen generations. Following the fifth or sixth reinoculation he was able to observe a single micro-organism in the ulcer exudate. He described it as a short, compact, streptobacillary rod (1.5 x 0.5um), rounded at the ends and with an indentation at the sides. The bacilli were Gram-negative and were located both within and outside neutrophils. These observations were supported by the work of Krefting (1892) and Unna (1892), (cited by Ronald and Albritton, 1984), who described the histology of the chancroidal ulcer and visualised the clumps and chains of Gram-negative rods in the lesion. Ducrey could not culture the organism in vitro and was unable to detect the organism in pus from bubos. He concluded, however, that it was responsible for soft chancre, and further stated that others were incorrect in their attribution of a major role to the "common micro-organisms of suppuration" in the pathogenesis of the ulcer or the bubo. Repeated ulcer formation following autoinoculation were not observed in patients with syphilis. This observation led to the presumptive conclusion that there were different

etiological agents for syphilis and chancroid.

(1.1.2) Isolation of the Causative Agent

Between 1895 and 1900, several investigators succeeded in isolating the causative agent of chancroid on artificial media. Istamanoff (Istamanov) and Akspianz (Akopiantz) are credited with the first convincing culture in 1897 (cited by Ronald and Albritton, 1984). They performed their cultures using media consisting of macerated human skin and agar. Lenglet, (cited by Ronald and Albritton, 1984) reported isolations in 1898 of organisms that appeared to be H. ducreyi using similar human skin agar with blood. Benzancon etal, (cited by Ronald and Albritton, 1984) are frequently credited with the first isolation of H. ducreyi in 1900. Cultures were performed using nutrient agar supplied with 20 to 30% whole fresh rabbit blood (McEntegart et al, 1982 ; Sturm and Zanen, 1984). After serial passage these organisms were able to produce soft chancres when reinoculated into humans.

Surprisingly, the British Medical Research Committee in 1918 refused to accept this work. They concluded that no evidence existed to support the notion that "soft chancre" was a

specific disease induced by a single species of micro-organism (Pijper, 1920 ; cited by Ronald and Albritton, 1984). Thus, when the report of the Society of American Bacteriologists on characterisation and classification of bacterial types was presented in 1920, controversy arose regarding both the organism and the disease due to the inclusion of the Ducrey bacillus in the genus Haemophilus (Dienst, 1947).

Significant work continued during the 1920s and 1930s, despite the dispute arising from the early description of the organism. Teague and Deibert (1920), utilising clotted rabbit blood heated to 55⁰C for five minutes as the primary culture medium followed by subculture to blood agar plates at twenty-four hours, reported the isolation of H. ducreyi from up to 80% of patients with a clinical diagnosis of chancroid. In subsequent studies, Teague and Deibert demonstrated that both serum (rabbit or sheep), a red blood cell extract (rabbit), and casein digest or peptone were necessary to obtain good growth of H. ducreyi on agar media (Teague and Deibert, 1920).

In 1935 the United States Public Health Service, like the British Medical Committee two decades earlier, found that

chancroid "is a local disease of the external genital organs in which a sore develops. The cause of this sore is believed to be an infection with a germ, although some physicians question the part which this germ plays" (cited by Greenblat and Sanderson, 1938). Many clinical laboratories gave up attempts to isolate the organism and, with the exception of the confirmation of haemin requirement by Lwoff and Pirotsky (1937), it was not until chancroid re-emerged as an important clinical disease in the military forces in the 1940s that significant new work in this field was published (Satulsky, 1943 ; Sullivan, 1940).

Two series published in 1946 and 1956 were particularly notable in re-establishing the importance of earlier work on chancroid (cited by Ronald and Albritton, 1984). Sheldon and Heyman, and Beeson provided a number of articles in 1946 which confirmed the superiority of bacteriological culture over biopsy, stained smears, skin tests and autoinoculation. They discussed the in vitro growth requirements and inhibition of the organism by antibiotics. The second series (cited by Ronald and Albritton, 1984) published as the "V.D.R.L. Chancroid studies" in 1956, furthered an understanding of the nutritional requirements of H. ducreyi by comparing virulent and avirulent strains. It also reviewed

the results of experiments undertaken in the rabbit model developed by Reenstierna (1921). In addition to these two series, Mortara and Feiner (1945) published several papers in the mid-1940s confirming the findings of earlier studies using the rabbit model.

During the late 1960s and early 1970s, interest in chancroid and H. ducreyi waned once again. Renewed interest in characterising the organism arose in the later 1970s and early 1980s, when isolates became available from several urban outbreaks of chancroid in north America and sporadic cases were recognised in Europe, Asia and Africa. (Ronald and Albritton, 1984).

Chancroid is currently recognised as the most common cause of genital ulcers in southern Africa, particularly South Africa. Chancroid accounts for more than 40% of unselected cases of genital ulceration in studies undertaken in Johannesburg and Soweto (Duncan etal, 1981), Swaziland (Meheus etal, 1983) and amongst migrant mineworkers in Carletonville, Transvaal (Ballard, 1982). In addition, the disease was diagnosed in 38% of cases in Durban (Coovadia etal, 1985) and 44% of cases seen at GaRankuwa hospital near Pretoria, Transvaal (Crewe-Brown etal, 1982).

(1.1.3) Epidemiology

Genital ulcerative diseases are common worldwide. Chancroid is the leading cause of genital ulceration in developing countries. In the developed world chancroid is rarely seen or diagnosed (Mindel, 1989). Knowledge about the epidemiology of chancroid is limited due to insensitive culture methods and reliance on the clinical picture as a diagnostic standard (Morse, 1989).

Chancroid is considered an uncommon sexually transmitted disease in the United States. In 1947 the reported number of chancroid cases peaked, with nine thousand five hundred and fifteen cases being reported (Morse, 1989). Between 1948 and 1981, generally decreasing numbers of cases were reported ; from 1967 to 1981 a mean of only nine hundred and twenty-three cases was reported annually (Morse, 1989). Between 1981 and 1983 an increase occurred with a large outbreak of nine hundred and twenty-three cases in Orange County, California (Blackmore etal, 1985). Since 1984, the number of reported cases of chancroid has increased more than six-fold involving nine large outbreaks (Schmid etal, 1987). These outbreaks shared many epidemiologic features. The disease occurred primarily amongst blacks and hispanics, and all the patients were heterosexual. The male / female ratio was

highest in outbreaks involving prostitutes, probably because a small number of women infected many men, and because the women were difficult to locate (Schmid etal, 1987).

In Britain, chancroid is exceptionally rare. In 1965 only seventy-four cases in men and five in women were reported from clinics for sexually transmitted diseases (Mindel, 1989). This represented 0.001% of all cases attending clinics. The number seen each year has remained virtually static (Mindel, 1989). Recently, outbreaks of chancroid have been seen in Greenland (Lykke-Olesen etal, 1979) and Rotterdam (Nayyar etal, 1979).

Chancroid is the most common cause of genital ulcers in South Africa and other African countries (Duncan etal, 1981 ; Nsanze etal, 1981 ; Crewe-Brown etal, 1982 ; Latif, 1982 ; Meheus etal, 1983 ; Plummer etal, 1983 ; Coovadia etal, 1985 ; Ndinya-Achola etal, 1986 ; Mabey etal, 1987). In Nairobi, Kenya, approximately five thousand patients with probable chancroid are seen annually in the one major clinic for sexually transmitted diseases (Ronald and Albritton, 1984).

Nsanze etal (1981) identified the following factors contributing to the dimensions of the problem in Kenya : a

large population of migrant labourers, of whom the majority are separated from their families ; a lack of control and treatment programmes for prostitutes ; and an uncircumcised male population. These factors are also particularly relevant to South Africa.

In a study of migrant mineworkers with chancroid in Carletonville, South Africa, the source of infection in most cases (49%) was verified as a short-term regular girlfriend, followed by casual "one-night stands" (33%), local prostitutes (15%) and spouses (4% ; Dangor etal, 1988(a)). In addition to migrant workers having multiple sexual partners, polygamy occurs more frequently in this population.

(1.1.4) Treatment of Chancroid

Prior to the advent of the sulphonamides, a variety of unsuccessful and poorly studied therapeutic approaches were used in the treatment of chancroid. Local therapies included application of either potassium permanganate, boric acid, gentian violet, silver nitrate or zinc peroxide (Sullivan, 1940). Intravenous tartar emetic was also employed (Jones, 1927). Other treatment providers cauterised the ulcer, or

immunised patients with chancroidal pus or with extracts of H. ducreyi and claimed excellent results (Watanabe, 1939).

Circumcision was standard therapy in many clinics; bubos apparently never developed after circumcision. The major complication of surgery was autoinoculation of the surgical margin (Rauschkolb, 1939). Some authors recommended a "dorsal slit" of the foreskin (Ronald and Albritton, 1984). Chancroid has been treated successfully with streptomycin (Asin, 1952 ; Anderson etal, 1984), kanamycin (Marmar, 1972) and gentamicin (Luders etal, 1975). Beta-lactams demonstrated in vitro activity against H. ducreyi (Reymann, 1949), and successful therapy has been described using these agents (Hammond etal, 1978). However, in 1979 Brunton etal described the first plasmid-mediated beta-lactam resistance in H. ducreyi. Since then, most studies have demonstrated beta-lactamase production in clinical isolates approaching 100% (Bilgeri etal, 1982 ; Plummer etal, 1983 ; Fast etal, 1983 ; Thomson and Bilgeri, 1982). This widespread resistance is assumed to be due to previous extensive use of penicillins in the treatment of ulcerative genital disease, irrespective of cause. Amoxicillin (500mg) with clavulanic acid (125mg) administered three times daily for seven days is also effective therapy (Schmid, 1986). Poor results have been

obtained using a single dose or a two-day regimen (Ndinya-Achola etal, 1986).

Sulphonamides originally appeared to be ideal agents for treatment of chancroid. They are inexpensive, safe, and generally well tolerated. By the early 1980s, however, a significant number of treatment failures were noticed in patients with chancroid who received sulphonamides alone (Hammond etal, 1979 ; Fast etal, 1983(a) ; Fast etal, 1983(b)). The addition of trimethoprim (cotrimoxazole) has been useful in circumventing this widespread resistance (Boyd, 1989). Sulphamethoxazole (800mg) and trimethoprim (160mg) administered twice a day orally for seven days is effective in areas where trimethoprim resistance is not prevalent (Schmid, 1986). Trimethoprim alone has been shown to be an effective antimicrobial in the treatment of chancroid (Plummer etal, 1983).

In southern Africa, most isolates remain susceptible to cotrimoxazole, and the doses mentioned above given for ten days, continue to be adequate treatment (Ballard etal, 1989). Recent studies have identified erythromycin as one of the drugs of choice for treating chancroid (Schmid, 1986 ; Ballard etal, 1989). Erythromycin was initially used in the

treatment of chancroid by Cordero in 1961 (Cordero, 1961). A dose of 500mg three times a day for at least five days is usually curative (Duncan etal, 1983), or alternatively four times a day for ten days (CDC, 1982). Potential disadvantages of the use of this antibiotic are the multiple daily doses, extended treatment periods, and high cost.

Since the first clinical use of a quinolone antibiotic (enoxacin) by Mensing in 1985 in the treatment of chancroid, H. ducreyi has been shown to be highly susceptible to the new fluoroquinolones (Meheus etal, 1983 ; Plummer etal, 1983 ; Wall etal, 1985). Ciprofloxacin (Naamara etal, 1987) administered as a single 1000mg oral dose (Fast etal, 1983 ; Ballard etal, 1989) has been shown to achieve cure rates similar to other standard regimens.

Ceftriaxone is extremely active against H. ducreyi. It offers the advantage of a relatively inexpensive, single intramuscular dose of 250mg, that appears to be as effective as erythromycin (Taylor etal, 1985 ; Schmid, 1986).

The majority of strains of H. ducreyi are resistant to oxytetracycline and tetracycline hydrochloride (Asin, 1952 ; Willcox, 1963). Minocycline (100mg) administered twice a day for 10 days, has been shown to have good activity against H.

ducreyi (Ballard etal, 1989). However, Dangor etal (1988) recently demonstrated the emergence of minocycline resistance in southern African isolates.

H. ducreyi has been reported to be very sensitive to rifampicin (Hammond etal, 1978 ; Dangor etal, 1988). This agent has the advantage of requiring minimal compliance (600mg once a day for three days), but concern exists about the use of this agent alone in areas of high tuberculosis endemicity (Miller S.D., personal communication).

Criteria for clinical responses to therapy are a decrease in ulcer pain, the disappearance of the purulent base of ulcers, the onset of epithelialisation, and failure to isolate H. ducreyi again within seventy-two hours (Ballard etal, 1989). The rate of healing appears not to depend solely on the antimicrobial agent or the duration of treatment, but is rather a function of the site and size of lesions, especially when in a sub-prepubial location (Ballard etal, 1989).

In contrast to the response to treatment of ulcers, changes in lymphadenopathy are variable. A few patients may develop large and fluctuant lymph nodes, despite the initiation of successful treatment. Repeated aspiration of these buboes may

be required to prevent inguinal or femoral ulcers developing (Ballard etal, 1989).

(1.1.5) Changing Antimicrobial Susceptibility Patterns of Southern African Isolates of H. ducreyi

In a study of one hundred and twenty-two strains of H. ducreyi isolated from migrant mineworkers, Dangor etal (1988(b)) confirmed beta-lactamase production in all of the strains which were resistant to amoxycillin. The MIC of amoxycillin was markedly reduced in the presence of clavulanic acid, suggesting the combination should be effective in the treatment of chancroid (Ndinya-Achola etal, 1986). Of the broader-spectrum cephalosporins, ceftriaxone was found to be the most active in vitro, followed by cefotaxime and ceftetrame. All the isolates were susceptible to macrolides, quinolones, spectinomycin and rifampin. Reduced susceptibility to minocycline, co-trimoxazole, and kanamycin was noted. Chloramphenicol and thiamphenicol resistance was noted for the first time among southern African isolates (Dangor etal, 1988). Since the previous study conducted by Bilgeri etal (1982), a changing antimicrobial susceptibility pattern among southern African

isolates of H. ducreyi has emerged.

(1.1.6) Diagnosis of Chancroid

(1.1.6.1) Clinical features

Most diagnoses of chancroid are made on a clinical basis (Boyd, 1989). The inaccuracy of this method was first highlighted by Chapel and co-workers (1977) who found that H. ducreyi was accurately identified by culture in only one third of suspected cases. This could have been due to atypical clinical manifestations of genital ulcers caused by the other classic aetiologic agents namely Treponema pallidum and Herpes simplex virus or failure to adequately isolate H. ducreyi from chancroid lesions.

The incubation period of chancroid is usually between four and seven days and is rarely less than three days or more than ten days (Ronald and Albritton, 1984). Unlike genital herpes, no prodromal symptoms are recognised. The portal of entry of H. ducreyi is believed to be a break in the integrity of the epithelium. Around this breach, the first pathological changes, in the form of edematous and swollen epithelial cells with an infiltration of polymorphonuclear leucocytes are observed (Sullivan, 1940). Externally, this

manifests as a small inflammatory papule surrounded by a narrow erythematous zone. Over the course of twenty to forty hours the lesion becomes pustular, eroded and ulcerated. Vesicles are not seen at any stage of the disease. Within seventy-two hours the pustule breaks down, resulting in a soft, sharply circumscribed ulcer with ragged, undetermined edges (FIGURE 1.1).

The base of the ulcer, in contrast to the smooth base of a syphilitic chancre, is irregular and has a granular surface which may bleed when scraped. A grey or yellow necrotic purulent exudate often covers the base of the ulcer (Ronald and Albritton, 1984 ; FIGURE 1.1). There is little inflammation of the surrounding skin. Ulcers are invariably painful and, depending upon their site, some are more painful than others. Several varieties of chancroidal ulcers have been described. Giant (>2cm) or serpiginous ulcers are formed when several smaller ulcers merge. Follicular ulcers (lechancre mou folliculaire) originate in hair follicles and resembles a folliculitis or pyogenic infection (Chapel etal, 1977). These occur most often on the vulva, on the labia majora and in hairy regions. Rarely multiple follicles may be involved with each pustule eventually forming a superficial ulcer.



FIGURE 1.1 Clinical appearance of multiple chancroid ulcers (Courtesy of Y. Dangor, SAIMR)

The so-called dwarf chancroid (*formes naives*) varies in size from 0.1 to 0.5 cm and is rounded and shallow (Gaisin and Heaton, 1975). It resembles a herpetic lesion, but can be distinguished by its irregular base and its sharp

haemorrhagic borders. In transient chancroid (chancre mou volant), the ulceration resolves rapidly in four to six days and is followed by acute regional lymphadenitis with suppuration ten to twenty days later (Gaisin and Heaton, 1975). Papular chancroid (ulcus molle elevatum) starts as a papule that becomes ulcerated. Lesions subsequently become raised, particularly around the edges and may resemble condylomata lata of secondary syphilis (Gaisin and Heaton, 1975).

In males the most common sites of involvement are the distal prepuce, the mucosal surface of the prepuce on the frenulum, or in the coronal sulcus. Lesions are seen less frequently on the glans, the shaft of the penis, or around the anus. Cicatrix formation with phimosis (FIGURE 1.2) is a late complication of chancroid that may require circumcision. Local extension may lead to involvement of the perineum, thigh, scrotum, or lower abdomen (Gaisin and Heaton, 1975). In females, the majority of lesions are at the entrance to the vagina including the fouchette, labia, vestibule and clitoris (Ronald and Albritton, 1984). Lesions of the cervix or vaginal wall occur less frequently and are often painless (Hammond etal, 1980 ; Ronald and Albritton, 1984). Extragenital lesions are less common but have been described

on the breasts, the fingers, the thighs and in the oropharynx (Ronald and Albritton, 1984).



FIGURE 1.2 Multiple preputial and subpreputial ulcers in a patient with chancroid and phimosis (courtesy of Y. Dangor, SAIMR).

Painful, tender inguinal lymphadenopathy is a characteristic feature of chancroid and may be present in up to 50% of patients (D'Costa etal, 1986 ; Ronald and Albritton, 1984 ; Sturm etal, 1987). The lymphadenopathy is usually unilateral (Plummer etal, 1983 ; Ronald and Albritton, 1984). Involved lymph nodes may rapidly become fluctuant (chancroidal bubo ; FIGURE 1.3) and spontaneously rupture leading to the formation of inguinal ulcers. Bubo pus is usually thick, creamy and viscous. Both lymphadenopathy and bubo formation are less common in females (Ronald and Albritton, 1984). Mild constitutional symptoms may accompany chancroid but H. ducreyi has not been shown to cause systemic infection or to spread to distant sites.

Patients with chancroid do not appear to develop long-lasting immunity and reinfection may occur immediately after cure (Felman and Nikitas 1983).

(1.1.6.2) Laboratory diagnosis

(1.1.6.2.1) Microscopy

(1.1.6.2.1.1) Gram-stain

Direct examination of clinical material in Gram-stained smears has been used with varying degrees of success as an

aid in the diagnosis of chancroid (Morse, 1989). In both industrialised and developing countries, careful darkfield microscopy and serology for syphilis are important additional laboratory investigations to exclude syphilis (Ronald, 1986).



FIGURE 1.3 Bubo formation in a patient with chancroid
(courtesy of Y. Dangor, SAIMR)

The identification of H. ducreyi is based on characteristics of cell morphology, staining and arrangement. When visualised in smears, H. ducreyi is usually observed extracellularly either singly or in small clusters (Heyman etal, 1945). Few Gram-negative organisms share the classical spatial or "school of fish" arrangement of H. ducreyi, in which bacilli are grouped in long parallel chains between cells and shreds of mucus. This was considered a most distinctive feature of H. ducreyi by early investigators. In practice, smears prepared from ulcer exudates rarely demonstrate this characteristic appearance (Heyman etal, 1945 ; Borchardt and Hoke, 1970 ; Nsanze etal, 1981 ; Choudhary etal, 1982).

Coovadia etal (1985) reported that direct Gram-staining of ulcer material did not help in identifying patients with chancroid. Only 27.5% of patients with culture-proven chancroid had characteristic Gram-negative bacilli present in direct smears from ulcers. Similarly, Sturm etal (1987) reported that the direct Gram-staining of ulcer material identified only 10% of patients who were culture-positive. Other investigators have reported that direct Gram-stain compares favourably with culture-positivity. Sensitivities ranging between 62 and 92.5% and specificities ranging between 51.4 and 98.7% have been reported (Nsanze etal, 1981

; Choudhary etal, 1982 ; Taylor etal, 1984). Direct Gram-staining of clinical material correlates poorly with a clinical diagnosis of chancroid. Sturm etal (1987) reported that only 5.3% of patients with a clinical diagnosis of chancroid had a positive smear. Choudhary etal (1982) found that only 36.4% of direct smears obtained from clinically diagnosed cases of chancroid showed the presence of H. ducreyi characteristic bacilli.

(1.1.6.2.1.2) Immunofluorescence

In view of the poor sensitivity of Gram-staining, alternative microscopical methods to rapidly identify H. ducreyi in ulcer or bubo exudate have been attempted. Immunofluorescence (FIGURE 1.4) is a relatively simple rapid technique that may be useful in situations where facilities for bacteriological culture are limited. Clinical smears can be easily fixed and transported to a diagnostic laboratory for examination.



FIGURE 1.4 Indirect immunofluorescence of H. ducreyi X400 magnification.

Denys etal (1978) developed an indirect immunofluorescence assay for H. ducreyi utilising antiserum prepared in rabbits. Although this polyvalent antiserum was extensively adsorbed with several Haemophilus species to eliminate cross-

reactivity, the persistence of cross-reactivity with various Haemophilus species made the interpretation of a positive smear difficult.

Direct and indirect immunofluorescent-antibody reagents for the identification of pathogenic organisms have been developed using hybridoma technology (Payne etal, 1988). Several hybridomas that secreted monoclonal antibodies which reacted with a panel of twelve strains of H. ducreyi from geographically diverse areas were obtained by Hansen and Loftus (1984). One of the monoclonal antibodies 8H4, did not react with selected strains of Treponema pallidum, Herpes simplex virus type 2, Neisseria gonorrhoeae, Haemophilus influenza or Haemophilus parainfluenzae. This monoclonal antibody was used to detect the presence of H. ducreyi in smears of lesion material from experimentally infected rabbits. The results suggested a potentially useful role for H. ducreyi-specific monoclonal antibodies as a diagnostic test for chancroid. Schalla etal (1986) used a different monoclonal antibody, B3, in an indirect immunofluorescence assay to detect H. ducreyi in smears prepared from genital lesion material. Monoclonal antibody B3 had a strong affinity for H. ducreyi, but only a slight affinity for some of the other Haemophilus species tested. To eliminate this cross-reactivity, the antibody was diluted. Fifty percent of the

smears of genital lesion material examined by this procedure exhibited intensely-stained rod-shaped organisms. H. ducreyi was isolated from 66% of the patients possessing the above positive smears suggesting that this antibody was sensitive but not specific. Recently Finn etal (1990) developed an immunofluorescence assay using murine monoclonal antibodies to H. ducreyi. Three monoclonal antibodies of isotype IgG2a demonstrated strong immunofluorescence titres against H. ducreyi, and did not cross-react with any other Haemophilus species tested. Immunoblotting revealed that the monoclonal antibodies reacted with a single polypeptide band of molecular weight 29kD in the outer membrane fraction of H. ducreyi. Further studies are needed to determine the sensitivity and specificity of this technique.

(1.1.6.2.1.3) Delayed hypersensitivity and serological tests

The Ito-Reenstierna skin test and the complement fixation test were formerly used as aids in the diagnosis of chancroid (Gaisin and Heaton, 1975). Both false-negative (Heyman etal, 1945 ; Strakosch etal, 1945) and false-positive (Heyman etal, 1945) reactions were reported with the skin test. Positive reactions may persist for years (Heyman etal, 1945). False-positive results have been reported with the

complement fixation test. These tests have been omitted from routine use due to their poor sensitivity, specificity and lack of standardised reagents. A lack of knowledge concerning the antigenic composition of H. ducreyi and the human immune response following infection has retarded the development of serological tests for the diagnosis of chancroid. Patients with chancroid demonstrate an elevation of both IgM (Schalla etal, 1986) and IgG (Museyi etal, 1988 ; Schalla etal, 1986). It is not known whether the duration and severity of illness may affect the antibody response. Use of dot-immunobinding (Schalla etal, 1986) and enzyme immunoassay (Museyi etal, 1988), to investigate culture-confirmed and clinically suspected cases of chancroid are difficult to evaluate as only a single antigen has been employed in these procedures. Since differences have been observed in the outer membrane protein (OMP) profiles of H. ducreyi from different geographical areas (Odumeru etal, 1983), it is likely that a broadly reacting antigen or a pool of different antigens is necessary to increase the sensitivity of any serological test (Morse, 1989).

A comparison of Western blots (immunoblots) of patient sera with their homologous strain and with the type strain of H. ducreyi, CIP542, have been carried out (Abeck etal, 1988).

Results indicated both qualitative and quantitative differences in the immune response to chancroid.

(1.1.6.2.2) Culture and identification

The laboratory diagnosis of chancroid depends on the isolation of H. ducreyi from a genital ulcer or bubo, and the exclusion of other agents associated with similar clinical findings, especially Herpes simplex virus, T. pallidum and Chlamydia trachomatis. In order to obtain isolated colonies for the definitive identification of H. ducreyi, subculturing on a solid medium is necessary.

A major advance in the ability to isolate H. ducreyi in pure culture was the development of a selective culture medium. An early formula consisting of enriched chocolate agar to which 3ug/ml of vancomycin was added (Hammond etal, 1978) had a sensitivity of only 56% when used to isolate H. ducreyi from patients with a clinical diagnosis of chancroid. Sottnek etal(1980) increased the sensitivity of culture to more than 70% by adding 10% foetal bovine serum (FBS) to the medium. Subsequent modification and refinement of formulae have led to a range of media being available which, in combination, yielded isolation rates of H. ducreyi of approximately 85% (Dangor, 1988(a)). A critical appraisal of these media is

contained in section 1.2.2.2.2 below.

A presumptive identification of H. ducreyi is based on typical colonial morphology and microscopic appearance of the organism using the Gram-stain procedure. Definitive identification is based on haemin (X-factor) requirement without the need for nicotinamide adenine dinucleotide (NAD, V factor), and other biochemical tests (Kilian, 1974). The major problems encountered in many studies are the difficulties in constantly isolating H. ducreyi by culture, the polymicrobial flora that may secondarily infect genital ulcers (Chapel etal, 1978), and the fact that the clinical appearance of genital ulcers may often be misleading (Chapel etal, 1977 ; Saltzman etal, 1984 ; Sturm etal, 1987).

(1.1.7) Pathogenesis and Virulence Testing.

Information regarding the pathogenicity of H. ducreyi is still limited. This is due in part to the paucity of appropriate biological assays for assessing the virulence of H. ducreyi (Johnson etal, 1988).

H. ducreyi is thought to penetrate the normal skin through minor trauma or abrasions, but factors mediating adherence

and the mechanisms by which the organism produces tissue necrosis are unknown. The skin must be scarified in order to induce experimental chancroid lesions (Ronald and Albritton, 1984). The inoculum size required for infection is not known, and no toxins or extracellular enzymes of H. ducreyi have been described.

In lesions the organism is present within both macrophages and neutrophils and is also free in clumps in the interstitium (Ronald and Albritton, 1984). The lymphadenitis associated with genital ulcer disease due to H. ducreyi is associated with a pyogenic inflammatory response. Suppuration is associated with vast numbers of neutrophils and a paucity of bacilli.

Both humans and other animals mount a humoral immune response to infection with H. ducreyi, but this response is non-protective since reinfection is common and repeated autoinoculations were observed in earlier human studies of chancroid. A cellular immune response to infection with H. ducreyi has not been reported (Albritton, 1989).

The classical test of virulence for H. ducreyi is the rabbit intradermal test (Feiner and Mortara, 1945 ; Dienst, 1948).

Strains have been defined as virulent if intradermal inoculation into a rabbit produces a typical lesion characterised by necrosis and eschar formation. Strains defined as avirulent fail to produce cutaneous lesions in rabbits (Morse, 1989).

The virulence of H. ducreyi may be an unstable trait. Thayer etal (1955) observed a loss in virulence after 44 passages in vitro, and Dienst (1948) demonstrated the loss of capacity to produce skin lesions after serial cultivation. This observation was, however, not confirmed by the later studies of Hammond etal (1980).

Virulence has been inconsistently associated with resistance to polymyxin B. Hammond etal (1980) observed that nineteen strains isolated during a chancroid epidemic in Winnipeg, Manitoba, Canada, were virulent, as defined by the rabbit intradermal test, and had MICs of polymyxin B of $\geq 32\mu\text{g/ml}$. Reference strains of H. ducreyi obtained from the Pasteur Institute in Paris, were avirulent and had polymyxin B MICs of $\leq 4\mu\text{g/ml}$. The association of virulence with resistance to polymyxin B is insubstantial as isogenic strains have not been compared. Recently, Odumeru etal (1984) reexamined this association using isogenic strains of H.

ducreyi, and found that polymyxin-susceptible mutants obtained from virulent polymyxin-resistant parent strains remained virulent for rabbits and that polymyxin -resistant mutants derived from avirulent polymyxin-sensitive parent strains remained avirulent. Polymyxin resistance appears thus not to be directly linked to virulence (Morse, 1989). Virulent strains of H. ducreyi which produce intradermal lesions have been forced to be resistant in vitro to the bactericidal action of both serum and polymorphonuclear leucocytes (Odumeru etal, 1984). Resistance to bactericidal action of serum has been associated with virulence in several Gram-negative bacteria. Although H. ducreyi does not produce bacteremia, resistance to lysis by normal human serum may allow successful extracellular multiplication of the organism within tissues (Odumeru etal, 1984).

(1.1.8) Animal Studies

The earliest biological model for studying chancroid was the infected patient. Bassereau (1852) demonstrated that patients with soft chancre could be reinfected at another skin site by autoinoculation of purulent material from their ulcer (cited by Ronald and Albritton, 1984). In 1889, Ducrey at the University of Naples, inoculated the skin of the forearm of

three patients with purulent material from their own genital ulcer (cited by Ronald and Albritton, 1984) and was able to maintain serial ulcers through as many as fifteen generations.

The first observation of experimental animal pathogenicity was that of Nimmel in 1901, who grew the organism and demonstrated its pathogenicity in rabbit and guinea-pig skin (McEntegart etal, 1982). Later investigators used rabbits and monkeys to produce lesions by direct intradermal inoculation of H. ducreyi (Morse, 1989). Cats, goats and sheep were refractory to inoculation (Morse, 1989).

Recently, Tuffrey etal (1988), reported the development of a mouse model of H. ducreyi infection. Six-to eight-week-old CBA mice inoculated intradermally or subcutaneously developed pustular nodules within twenty four hours. About half of all the sites developed ragged, full-thickness skin ulcers with rounded crater-like edges, typical of those occurring in chancroid. H. ducreyi could be recovered from the lesions for approximately one week after inoculation, suggesting that the organisms were multiplying in vivo. The lesions observed in mice were thought to resemble those of human disease more closely than those produced experimentally

in rabbits.

Thus, this model may be suitable for the study of the pathogenesis of chancroid and the virulence of H. ducreyi. A mouse model was utilised by Muller and Muller (1988) to investigate the humoral immune response to H. ducreyi infection. The kinetics of IgM and IgG antibody synthesis following intradermal infection were determined to be similar to that occurring in other bacterial infections. Saunders and Folds (1986) immunised rabbits intradermally with H. ducreyi. The animals responded with a vigorous immune response in which multiple antigenic polypeptides were detected by Western blotting techniques.

(1.1.9) Chancroid and Human Immunodeficiency Virus (HIV) **Transmission**

There is now considerable evidence of an association between infection with the human immunodeficiency virus (HIV) and genital ulceration of different causes (Kreiss etal, 1986 ; Stamm etal, 1988). Greenblatt etal (1988) found that a history of ulcerative genital disease was more prevalent in men with HIV infection than in uninfected men. Kreiss etal (1986) have described an increased incidence of genital

ulcers amongst HIV-seropositive female prostitutes in Kenya, with half of these ulcerations being due to H. ducreyi. In studies undertaken in Nairobi, Ronald and Plummer (1989) also demonstrated that chancroid is a major risk factor for acquisition of HIV. In addition, the host immune response to pathogens causing genital ulceration may bring activated lymphocytes and macrophages to areas of surface disruption (FIGURE 1.5). Recruitment of HIV-bearing cells to the ulcer bed may thus facilitate transmission. Lymphocyte infiltration has been prominent in biopsies from genital ulcers caused by H. ducreyi (Sheldon and Heyman, 1946).

As the number of people with HIV infection in Africa increases, chancroid, may assume greater significance both as a source of greater morbidity and a potential risk factor for spread of HIV (Boyd, 1989). In Africa, it may be said that chancroid would have remained a nasty, but inconsequential, venereological curiosity if HIV had not appeared (Ronald and Plummer, 1989).



FIGURE 1.5 Prolific bleeding of a chancroid ulcer
(courtesy of Y. Dangor, SAIMR).

(1.2) PART TWO - HAEMOPHILUS DUCREYI - THE ETIOLOGICAL
AGENT

(1.2.1) Structure

(1.2.1.1) Cellular morphology

H. ducreyi, the causative organism of chancroid is a small Gram-negative, microaerophilic, streptobacillary organism, 0.5 um in width and 1.5 to 2.0 um in length (Felman and Nikitas, 1983 ; Ronald and Albritton, 1984). It is non-motile, non-spore-forming and often appears in end-to-end chaining patterns consisting of three to twenty bacilli (Felman and Nikitas, 1983). Although few Gram-negative organisms share the classical spatial description of H. ducreyi, ulcer exudate rarely demonstrates this characteristic appearance (Nsanze etal, 1981).

Sng etal (1982) observed the tendency of H. ducreyi grown on solid media to aggregate into cohesive microcolonies (FIGURE 1.6). In twenty-four hour cultures organisms at the edge of

colonies tend to be arranged in parallel rows giving a wavy or "school of fish" appearance. Organisms in long chains described as "railroad tracks" are more evident in broth cultures (Albritton, 1989).

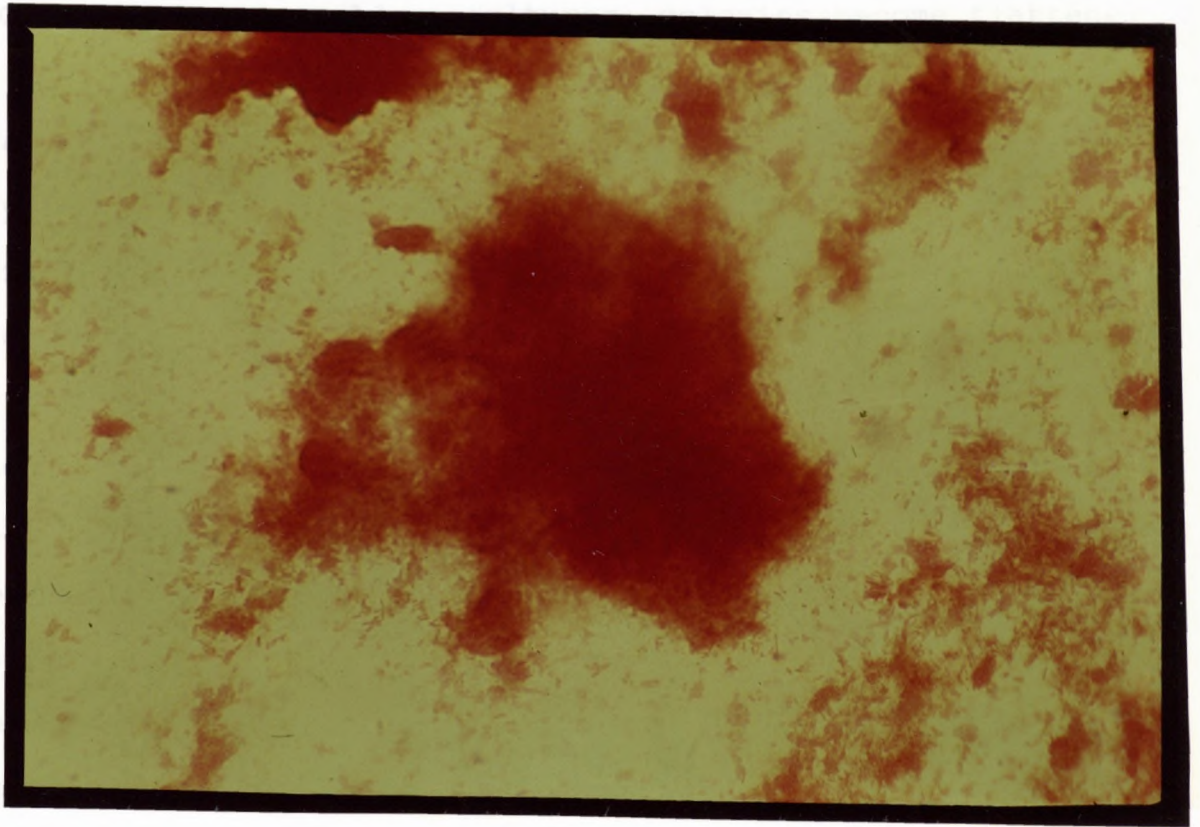


FIGURE 1.6 Gram-stain of H. ducreyi grown on solid media
(courtesy of Y. Dangor, SAIMR)

(1.2.1.2) Colonial appearance

Colonies of H. ducreyi are typically small, non-mucoid, yellow-grey, domed-shaped, semi-opaque, and adherent on most media. Occasionally, translucent colonies may be observed (Ronald, 1986 ; Albritton, 1989). Colonies of H. ducreyi tend to dissociate into multiple morphotypes even in pure culture. (FIGURE 1.7). In older cultures, colonies become flattened and their colour changes to yellowish-brown (Sturm and Zanen, 1984). Because of the cohesive nature of H. ducreyi colonies, they can be pushed intact across the agar surface and are difficult to remove with an inoculating needle; they are autoagglutinable in saline.

Colonies do not pit the agar surface and may be associated with zones of alpha-haemolysis on some blood agars, especially in areas of subsurface inoculation (Albritton, 1989). Aggregation of starch on some clear agars has also been reported (Hafiz etal, 1984). The adherence of cells within the colony and substantial loss of cell viability within the colony make single-cell colony isolations very difficult and this property has to some extent hindered the development of quantitative genetic studies.



FIGURE 1.7 H. ducreyi colonies on a solid agar medium
(courtesy of Y. Dangor, SAIMR)

(1.2.1.3) Electron microscopy

Killian and Theilade (1975) published the first English-language description of the cell wall ultrastructure of H. ducreyi. Electron microscopy of H. ducreyi CIP542,

demonstrated a typical Gram-negative cell wall with a cytoplasmic membrane surrounded by a rugose outer membrane. Marsch etal (1978) extended earlier cited work by Cazarre and Barreto (1974) and Ovchinnikov (1976) and suggested that electron-microscopic examination of tissue biopsies could be used to establish a presumptive diagnosis of chancroid. This was found to be unacceptable for routine use due to poor sensitivity and specificity. Ultrastructural studies of several H. ducreyi isolates by Bertram (MSc. Thesis, University of Manitoba, Winnipeg, 1980) confirmed the regular Gram-negative characteristics of the cell wall, as well as the discontinuous distribution of antibody-stabilised, extracellular capsular material. In contrast, recent studies by Johnson etal (1988) have failed to demonstrate the presence of a fine capsule and also reveal the absence of surface appendages such as pili or flagella.

Intercellular stranding of exocellular material has been implicated as the mechanism responsible for the characteristic appearance of H. ducreyi in short chains (Johnson etal, 1988). Bertram (1980) reported no difference in the amount of exocellular material in virulent and avirulent strains.

(1.2.1.4) Cell surface composition

Cell surface components of H. ducreyi have been fairly extensively studied. Indirect immunofluorescence has demonstrated common H. ducreyi-specific surface antigens (Denys etal, 1978 ; Slootmans etal, 1985). In vitro radio-iodination studies for H. ducreyi have demonstrated a variety of labelled surface-exposed proteins (Abeck and Johnson, 1987). Outer membrane protein (OMP) profiles vary amongst H. ducreyi strains from different geographical regions (Odumeru etal, 1983 ; Taylor etal, 1985). This characteristic has been useful in epidemiological studies of H. ducreyi isolated from different parts of the world. Details of the assembly and function of cell surface structures are presented in chapters two and three.

(1.2.2) Metabolism

(1.2.2.1) Biochemical characterisation

H. ducreyi has few distinguishing biochemical characteristics (TABLE 1.1). Little is known of the organisation and control of metabolic pathways in H. ducreyi. Results of studies involving several hundred strains suggest a reasonably consistent pattern of selected enzyme activity when using

standardised media and reagents (Albritton, 1989). These enzyme profiles may prove useful in the laboratory identification of H. ducreyi. Of note is the broad range of phosphatase activities exhibited by strains of H. ducreyi, including alkaline phosphatase, acid phosphatase and phosphoamidase. The presence of alkaline phosphatase is an important characteristic for the identification of H. ducreyi.

All strains are catalase-negative when hydrogen peroxide is dropped onto surface colonies. Some catalase activity may be demonstrated by the tube method (Sturm and Zanen, 1984). The oxidase reaction is variable, being generally negative when N,N-dimethyl-p-phenylenediamine oxalate is used (Sottnek etal, 1979), but positive when employing N,N,N,N-tetramethyl-p-phenylenediamine dihydrochloride (Nobre, 1982).

All strains fail to produce indole from tryptophan, and are H₂S-negative. Contradictory reports exist concerning urease production with some strains apparently demonstrating weak urease activity (Sottnek etal, 1980). Most strains of H. ducreyi are positive in the Voges-Proskauer reaction (Eddy, 1961).

TABLE 1.1 Major biochemical characteristics of H. ducreyi.

Characteristic	Reaction
Porphyrin biosynthesis.....	-
Nitrate reduction.....	+ ^v
Catalase.....	- ^v
Oxidase.....	+ ^v
Voges-Proskauer.....	+ ^v
Indole production.....	-
Urease.....	- ^v
Ornithine or lysine decarboxylase.....	-
Arginine dihydrolase.....	-
Alkaline or acid phosphatase.....	+
H ₂ S production.....	-
Deoxyribonuclease.....	-

Table adapted from Albritton (1989).

Refer to text for discussion of reported variable (v) reactions.

Although anaerobic electron acceptors have not been described amongst H. ducreyi, nitrate reductase is found in most

strains (Hollander etal, 1981). The requirement for haemin as a growth factor has been demonstrated by Pirotsky (1937 ; cited by Ronald and Albritton, 1984 ; Kilian, 1976 and Hammond etal, 1978). The latter two studies demonstrate the absence of enzymatic activity in the haemin biosynthetic pathway in the porphyrin test (Biberstein etal, 1963 ; Kilian, 1974). H.ducreyi also lacks the ferrochelatase or haeme synthetase enzymes which catalyse the insertion of iron into the protoporphyrin nucleus. H. ducreyi can be differentiated from other haemin-requiring species of Haemophilus by its lack of requirement for nicotinamide-adenine dinucleotide (NAD, V-factor).

Recently, Shawar etal (1990) investigated the use of sodium polyanetholesulphonate (SPS) disc susceptibility and the RapID-ANA system to identify and differentiate H ducreyi from other bacterial species. All H. ducreyi isolates were susceptible, evidenced by a zone of growth inhibition. Apart from Neisseria gonorrhoeae, Gardnerella vaginalis and Capnocytophaga species, no other strains showed any evidence of inhibition. These authors suggested that presumptively identified H. ducreyi be first subjected to an SPS disc susceptibility test, and the heavy lawn of bacterial growth away from the disc then be used in the RapID-ANA test.

(1.2.2.2) Growth requirements

(1.2.2.2.1) Nutritional studies

H. ducreyi has been described as a fastidious organism requiring blood for growth. It has been successfully cultivated on clotted blood (Teague and Diebert, 1920) and on various artificial media enriched with blood (Beeson and Heyman, 1945 ; Beeson, 1946 ; Deacon etal, 1956). Ajello etal (1956) provided additional evidence of the superiority of whole blood over serum or plasma in supporting the growth of H. ducreyi. They noted that H.ducreyi grew better and survived longer on whole-blood enriched media than in cultures containing serum. Ajello etal (1956) proposed that the superiority of whole blood over serum was due to the possibility of red blood cells being able to absorb toxic metabolic products. Current belief, however, is that erythrocytes supply haemin, an essential growth factor for members of the genus Haemophilus (Kilian and Biberstein, 1984). Quantitative haemin requirements of H. ducreyi are considerably higher than those reported for other haemin-requiring Haemophilus species (Hammond etal, 1978 ; Albritton etal, 1981). The minimum concentration of haemin required to initiate growth of H. ducreyi is 25 to 50ug/ml, much higher than the 1 to 10 ug/ml required by other haemin dependant Haemophilus species. The requirement for haemin can be

demonstrated in the porphyrin test using alpha-aminolevulinic acid as substrate (Hammond etal, 1978). Free haemin is not required as H. ducreyi is able to utilise haemoglobin, myoglobin, and certain other haeme proteins such as catalase, as a source of haemin (Albritton, 1984).

Initially, very little was known about the specific nutritional requirements of H. ducreyi. Several inorganic nitrogen compounds, single amino acids (L-aspartic-acid being the single most stimulatory), amino acid mixtures and acid-hydrolysed caseins were noted to support growth of H. ducreyi to a limited extent, but better growth was obtained in media containing partially hydrolysed proteins such as peptones. Ajello etal (1956), using a virulent H. ducreyi strain, demonstrated that in the presence of a small amount of rabbit serum, the nutritional requirements of H. ducreyi were satisfied by any one of a number of peptones prepared in a physiological sodium chloride solution. The need for sodium chloride at this concentration was specific and other salts failed to permit growth. No increase in the amount of growth occurred in the presence of glucose or other sugars. It was suggested that the more complex organic substances occurring naturally in peptones or serum, or possibly atmospheric carbon dioxide was utilised by this organism for its carbon

source.

While either serum or large quantities of washed erythrocytes supplied adequate nutrition, whole blood has been found to be more effective than either of these components.

Albumin has been shown to be an essential serum component for growth. Whether albumin serves as a nutritional source, adsorbes toxic metabolic products (Butler, 1962 ; Page, 1962), or provides a source for bound trace components or elements remains unknown.

An attempt to investigate H. ducreyi growth factors by simplifying one of the conventional media composed of Mueller-Hinton agar, 5% chocolatised horse blood, 5% serum and 1 or 2% IsoVitaleX was carried out by Van Den Bergh (1987). The horse blood was replaced by haemin (200ug/ml) and the serum by 0.2% albumin. The component in IsoVitaleX that is absolutely required by H. ducreyi is 0.01% L-glutamine. The above study also demonstrated a growth enhancing effect of selenium (Van Den Bergh, 1987).

(1.2.2.2.2) Media

The fastidious nature of H. ducreyi has been recognised since the first in vitro studies of the organism. Since Benzancon

first isolated H. ducreyi on a defined rabbit blood agar medium (cited by Albritton, 1989) from a genital ulcer of a patient with chancroid, various media and enviromental conditions for its isolation have been described (Sturm and Zanen, 1984). It was not until recently, however, that significant progress has been made towards a chemically defined medium similar to that described for H. influenzae (Herriot etal, 1970 ; Klein and Luginbuhl, 1979).

A variety of culture media have been proposed for the primary isolation of H. ducreyi. A number of these described since 1924 are summarised in TABLE 1.2.

TABLE 1.2 Solid media for primary *H. ducreyi* isolation

Medium		
Author	Agar Base	Supplements
Saelhof (1924)	phosphate ascitic fluid agar	clotted rabbit blood
Heyman <u>etal</u> (1945)	nutrient agar	defibrinated rabbit blood
Beeson (1946)	nutrient agar	whole serum, washed erythrocytes, whole rabbit blood
Daecon (1956)	nutrient agar	clotted rabbit blood
Hammond <u>etal</u> (1978)	gonococcal agar base	bovine haemoglobin 1% IsoVitaleX vancomycin

Sottnek <u>etal</u> (1979)	heart infusion agar	5% defibrinated rabbit blood, 10% FBS, vancomycin
Handsfield <u>etal</u> (1981)	gonococcal agar base	5% heated sheep blood 1% IsoVitalex vancomycin, 10% FCS
Hafiz <u>etal</u> (1984)	proteose peptone #3	rice starch, gelatin, glutamine, ferric nitrate, 5% equine blood, vancomycin
Oberhofer & Back (1982)	Mueller-Hinton	5% sheep blood, 1% IsoVitalex vancomycin
Hannah & Greenwood (1982)	chocolate (clinical standards)	vancomycin

Nobre (1982)	columbia agar base	10% heated defibrinated equine blood, 1% poly-ViteX, vancomycin
Sturm & Zanen (1984)	columbia agar base	30% haemolysed horse blood, 2.5% yeast dialysate, vancomycin
Nsanze <u>etal</u> (1984)	(a) Mueller-Hinton agar	5% chocolated horse blood, vancomycin
	(b) gonococcal agar base	2% bovine haemoglobin 5% FCS, 1% CVA enrichment, vancomycin

H. ducreyi grows well on nutritionally enriched media such as chocolatised blood agar or haemoglobin agar with complex supplements such as IsoVitaleX or CVA enrichment. Until 1978 the media most frequently used for primary isolation of H. ducreyi were 30% rabbit blood agar and whole blood agar (Sturm and Zanen, 1984). In 1978 Hammond etal described the isolation of H. ducreyi on gonococcal (GC) agar base supplemented with 1% bovine haemoglobin and 1% IsoVitaleX. The incorporation of 3mg/L vancomycin prevented overgrowth of Gram-positive cocci, without affecting recovery of the pathogen. In subsequent studies, Hammond etal (1978) confirmed that H. ducreyi requires haemin for growth with an optional concentration of between 25 to 50mg/L (Ronald, 1986). Excellent growth of laboratory strains can be obtained on a more defined agar medium containing peptides, glucose, glutamine, cysteine and haemin (Ronald, 1986). Subsequent studies compared several media for efficiency of primary isolation. Sottnek etal (1979) enriched media with a variety of animal and human sera. Of four sera tested, only foetal bovine serum (FBS) supported a heavy growth of all the H. ducreyi strains tested. Heart infusion agar proved to be the optimal agar base although Mueller-Hinton agar supported growth well. Columbia agar, proteose peptone #3 agar and GC agar base all performed slightly less favourably, and strains

grew poorly on trypticase soy agar.

Sottnek etal (1979) used 5% defibrinated rabbit blood as a source of haemin, and found it to be superior to bovine haemoglobin. Oberhofer and Back (1982) compared 5% sheep blood in Mueller-Hinton agar to human blood clot tubes. Isolates grew consistently only on the Mueller-Hinton sheep agar and Mueller-Hinton chocolate agar. Hafiz etal (1984) described the Sheffield medium, consisting of proteose peptone #3, rice starch, gelatin and agar with an added supplement consisting of glutamine, glucose and ferric nitrate. Equine haemin (0.02%) and 5% defibrinated horse blood were added as a source of haemin. The authors reported that this medium produced more luxuriant growth of H. ducreyi than previously described media (Ronald, 1986). In Kenya, however, the Sheffield medium failed to support H. ducreyi on primary isolation (Piot etal, 1983) and their formula is not recommended for isolation of African strains.

Nobre (1982) compared 20% defibrinated rabbit blood in Columbia agar base, with chocolate agar containing 10% heated, defibrinated horse blood in Columbia agar base, plus a vitamin supplement. The chocolate agar with horse blood was superior. Sturm and Zanen (1984) reported that "Beiling

agar", a mixture of two parts Columbia agar base and one part haemolised horse blood supplemented with 2.5% yeast dialysate, also gave excellent results. Taylor etal (1984) isolated H. ducreyi in Thailand on Mueller-Hinton agar base, supplemented with 5% chocolatised horse blood, 5% FCS and 1% IsoVitaleX. They compared this medium with heart infusion agar base with 5% rabbit blood and 5% FCS. Mueller-Hinton agar with equine blood was superior to heart infusion agar.

The above complex series of media have evolved due to the fastidious nature of H. ducreyi and the organism being unable to grow on simple media. Various media based on those mentioned in TABLE 1.2 have been utilised by a number of authors for primary isolation and growth of H. ducreyi (Sottnek etal, 1980 ; Nobre, 1982 ; Sng, 1982 ; Odumeru etal, 1983 ; Slootmans etal, 1983 ; Schalla etal, 1986 ; Abeck etal, 1988 ; Ballard etal, 1989).

Nsanze etal (1983 and 1984) have suggested that maximal isolation rates of H. ducreyi from clinical specimens requires the use of two media ; the authors propose gonococcal agar base with haemoglobin and serum, and Mueller-Hinton agar base with chocolatised horse blood as the

optimal regimen.

Clearly, the ideal medium for primary isolation of H. ducreyi from patients in all countries where chancroid is prevalent remains to be formulated. Apparent variation in nutritional requirements of local strains and in clinical laboratory preparations continue to demand different approaches.

Liquid culture of H. ducreyi is rarely employed because of the unsatisfactory growth of the organism in broth. Sng etal (1982) used a liquid medium consisting of Bacto proteose peptone #3, supplemented with 0.5% NaCl and 10% FBS. Granular growth was seen after twenty-four to forty-eight hours. After three to five days wooly colonies appeared consisting of long filaments. This type of non-dispersed growth in liquid media is a major constraint in experiments requiring accurate quantification of bacterial numbers (McEntegart etal, 1982).

(1.2.2.2.3) Cultural enviroment

Few studies have systematically evaluated enviromental conditions for optimal growth of H. ducreyi. It is recognised that strains grow best between 30 and 33⁰C, and many strains fail to grow on primary isolation if the temperature is higher than 36⁰C (Sturm and Zanen, 1984). Some isolates

have been found to grow at 22⁰C (Sturm and Zanen, 1984). The optimum incubation temperature has been suggested to be 33 to 35⁰C, with growth ceasing above 37.5⁰C (Sng etal, 1982 ; Nsanze etal, 1984 ; Ballard etal, 1989).

Sturm and Zanen (1984) clearly demonstrated that a water-saturated atmosphere with increased carbon dioxide was optimal for most strains but cautioned that this could be strain and passage variable. Isolates appear to be particularly carbon dioxide dependent on primary isolation (Sottnek etal, 1980 ; Nsanze etal, 1984).

Sturm and Zanen (1984) showed that the best growth was obtained in a microaerophilic atmosphere, achieved in a closed anaerobic jar without a catalyst, but with two carbon dioxide and hydrogen-generating envelopes : the so-called Campylobacter growth enviroment. In most situations, a candle extinction jar with a moistened paper towel provides an excellent enviroment for the culture of H. ducreyi (Nsanze etal, 1984). H. ducreyi also grows best at a neutral pH (Sturm and Zanen, 1984). Growth is scant after twenty-four hours incubation, and the typical colonies are usually only apparent at twenty-four to seventy-two hours. Culture plates should not be discarded as negative until the fifth

day (Ronald, 1986). Best growth is obtained when the agar plates are used within two weeks of preparation (Sottnek etal, 1980).

(1.2.2.2.4) Preservation of cultures

The optimal technique for freeze-drying H. ducreyi has not been reviewed. Skim milk or serum-inositol mixtures have been used for the lyophilisation of cultures of H. ducreyi (Odumeru etal, 1983 ; Albritton etal, 1985). Filter-sterilised heart infusion broth, tryptic soy broth, or bovine serum albumin, each containing 10% glycerol and 10% FCS, have also been successfully used, as have filter-sterilised skim milk, with or without 10% glycerol, and defibrinated rabbit blood. Frozen cultures have been preserved in liquid nitrogen or at minus 70⁰C (Morse, 1989).

(1.2.3) Genetics

(1.2.3.1) Taxonomy

(1.2.3.1.1) Chromosomal elements

Deoxyribonucleic acid (DNA) hybridisation studies have shown H. ducreyi strains to belong to a highly homogeneous group with homology values of 85 to 100% and thermal denaturation changes of <1⁰C (Casin etal, 1985). The guanine-plus-

cytosine (G + C) content is 38 to 39 mol% (Casin etal, 1985 ; Kilian, 1976). Albritton (1989) found similar results with clinical H. ducreyi isolates and the culture collection strains H. ducreyi ATCC 27722, NCTC 10945, and CIP A76. The type strain of H. ducreyi, CIP542, however, appeared unrelated to any of the current species of Haemophilus. In addition, Casin etal (1985) showed no hybridisation of DNA from H. ducreyi CIP542 and the related organisms H. agni, H. somnus, H. equigenitalis, and H. piscium, which historically were included in the genus Haemophilus but are not closely related to the type species H. influenzae or other members of the genus Haemophilus.

Three fragments of H. ducreyi chromosomal DNA coding for proteins expressed in E. coli and reactive with polyvalent rabbit antiserum have been isolated from a lambda gt11 library and shown to be specific for H. ducreyi (Parsons etal, 1989). In genetic transformation studies to determine relatedness within the family Pasteurellaceae, it was found that H. ducreyi, unlike some members of the family, showed no competition for homospecific transformation of H. influenzae (Albritton etal, 1984 ; Albritton etal, 1986). Transfer of chromosomal genes by transformation has not been demonstrated for H. ducreyi. It has thus been suggested that H. ducreyi

may not be genetically related to members of the genus Haemophilus or the family Pasteurellaceae despite their requirement for haemin as a growth factor and their sharing of common surface antigens with members of these genera (Albritton, 1989).

(1.2.3.1.2) Plasmids

Although apparently unrelated at the level of the chromosome, H. ducreyi shares a significant gene pool with members of the Pasteurellaceae and the Enterobacteriaceae families. The core plasmid for the several plasmids conferring ampicillin resistance in H. ducreyi is found in other species of Haemophilus and Neisseria (McNicol and Ronald, 1984).

Studies of various H. ducreyi plasmids shared by other genera show substantial homology in regions coding for antibiotic resistance and replicative function, although sequence divergence is also present (Willson etal, 1989). These studies suggest the presence of polypromoters as well as polyreplication sequences in these shared minireplicons. Few studies have reported susceptibilities to agents other than antibiotics (Mortara and Saito, 1946 ; Sloodmans etal, 1983), and no information is available regarding the genetics of susceptibility to non-antibiotic chemicals, including heavy

metals (Albritton, 1989).

Transfer of conjugative plasmids and mobilisation of non-conjugative plasmids have been demonstrated for H. ducreyi (Morse, 1989), although transfer of chromosomal genes by conjugation has not been demonstrated (Albritton, 1989).

Bacteriophages have not been demonstrated in H. ducreyi, and neither H. influenzae nor Enterobacteriaceae phages have been demonstrated to replicate in H. ducreyi.

(1.2.3.2) Antimicrobial resistance

(1.2.3.2.1) Sulphonamides

Sulphonamides have been used to treat chancroid since 1938 (Hanschell, 1938). Several reports have described clinical resistance to sulphonamides (Kerber etal, 1969 ; Marmar, 1972 ; Hammond etal, 1979). Sulphonamide resistance in H. ducreyi is now prevalent throughout the world (Ronald, 1986). Albritton etal (1982), demonstrated the presence of a 4.9-Md non-self-transferable plasmid in sulphonamide-resistant strains of H. ducreyi isolated from geographically diverse areas. These plasmids are similar to sulphonamide resistant plasmids present in Enterobacteriaceae (Albritton etal, 1984), and have a G + C content of 57 mol%. Moderate

resistance to sulphonamides (20-80mg/L) occurs in about one-third of strains from Kenya and appears not to be plasmid mediated (Plummer etal, 1983).

(1.2.3.2.2) Trimethoprim

Trimethoprim in combination with a sulphonamide preparation (Fast etal, 1983 ; Plummer etal, 1983) or with rifampicin (Plummer etal, 1983) has been effective for treating chancroid, in spite of the high prevalence of sulphonamide-resistant H. ducreyi strains. Sulphonamide-trimethoprim combinations continue to be quite effective in east Africa but are no longer a satisfactory treatment regimen in Thailand (Taylor etal, 1985 ; Ronald, 1986). The genetics and mechanism of trimethoprim resistance in these strains are unknown. The emerging trimethoprim resistance in H. ducreyi suggests that clinical resistance to the combination of trimethoprim and sulphonamides will occur more frequently (Morse, 1989).

(1.2.3.2.3) Rifampicin

Rifampicin is highly active against H. ducreyi, either alone (Bilgeri etal, 1982) or in combination with trimethoprim (Plummer etal, 1983). To date, rifampicin-resistant isolates have not been identified from clinical specimens

(Morse, 1989)

(1.2.3.2.4) Chloramphenicol

Only a few studies have examined the efficacy of chloramphenicol in the treatment of chancroid (Gaisen and Heaton, 1975). Sason-Le Pors etal (1982) and more recently Dangor etal (1988(c)) demonstrated the presence of chloramphenicol acetyltransferase (CAT) in chloramphenicol-resistant clinical isolates of H. ducreyi. After speculation that the genes involved with CAT synthesis were located on a plasmid, Roberts etal (1985) determined that the CAT determinant was located on a 34Md conjugative plasmid.

(1.2.3.2.5) Aminoglycosides

Treatment failures following streptomycin therapy have been observed in Singapore since 1980 (Rajan and Sng, 1982). Susceptibility studies showed a large percentage of these strains to be resistant to both streptomycin and kanamycin. A high frequency of kanamycin-resistant strains has been reported from Thailand (Taylor etal, 1985), and streptomycin-, kanamycin- and gentamicin-resistant strains reported from Amsterdam (Sturm and Zanen, 1984).

Sanson-Le Pors etal (1985) observed that streptomycin and kanamycin resistance was associated with a 3.07Md plasmid.

(1.2.3.2.6) Tetracycline

Recent studies have reported clinical and in vitro resistance of H. ducreyi to the tetracyclines (Kerber etal, 1969 ; Kraus etal, 1982). Tetracycline-resistant strains have been found in every geographic area surveyed, with the percentage of resistant strains varying from 16 to 100% (Albritton etal, 1984). Both plasmid-and chromosome-mediated tetracycline resistance mechanisms have been described in H. ducreyi. Plasmid-mediated tetracycline resistance is associated with conjugative plasmids of 34Md (Marshall etal, 1984 ; Roberts etal, 1985) and 30Md (Albritton etal, 1984).

Albritton etal (1984) observed a 30Md plasmid involved with the transfer of tetracycline resistance in conjugative matings with a laboratory-derived tetracycline-sensitive H. ducreyi strain. Tetracycline-resistant isolates of H. ducreyi have also been identified which do not transfer tetracycline resistance in matings with H. ducreyi or other species of Haemophilus (Albritton etal, 1984). This suggests that tetracycline resistance may be chromosomally mediated in these strains. Recently, studies carried out by Johnson etal (1989) and Roberts (1989) revealed that the tetracycline-resistance determinant, Tet M, of H. ducreyi was encoded by

chromosomal and plasmid-mediated genes respectively.

Chromosomal and plasmid mediated tetracycline resistance is inducible and is due mainly to a decrease in drug accumulation (Chopra and Howe, 1978).

(1.2.3.2.7) Ampicillin

Early in vitro studies indicated that H. ducreyi was penicillin susceptible with MICs in the range of 0.06 to 0.13 ug/ml (Mortara etal, 1944 ; Thayer etal, 1955 ; Singer and Beacon, 1956). Hammond etal (1978) observed that 16% of H. ducreyi strains isolated during an outbreak of chancroid in Winnipeg, Canada in 1975 were resistant to penicillin and ampicillin due to beta-lactamase production. Brunton etal (1979) established that these strains harboured a 5.7Md beta-lactamase plasmid. Two additional beta-lactamase plasmids have since been identified with sizes of 3.2Md and 7.0Md (Handsfield etal, 1981; Totten etal, 1982). Beta-lactamase producing H. ducreyi strains currently appear to be prevalent worldwide (Morse, 1989).

The beta-lactamase plasmids of H. ducreyi have been structurally compared with those of N. gonorrhoeae (Anderson etal, 1984 ; Brunton etal, 1981 ; Brunton etal, 1982). The

7.0Md plasmid of H. ducreyi carries the entire sequence of the 4.4Md "Asian" plasmid of N. gonorrhoeae. The 3.2Md H. ducreyi plasmid is identical to the 3.2Md "African" plasmid of N. gonorrhoeae (Anderson etal, 1984).

Laufs etal (1979) characterised a nonconjugative 4.4Md beta-lactamase plasmid from H. influenzae. The sequence of this plasmid was highly related to those of the 4.4Md and 3.2Md gonococcal plasmids and therefore to those of the plasmids from H. ducreyi

(1.2.4) Antimicrobial Susceptibility Testing

The guidelines of the National Committee for Clinical Laboratory Standards for antimicrobial susceptibility testing of fastidious organisms, including members of the genus Haemophilus, cannot be applied to H. ducreyi since this organism requires haemin and other supplemented nutrients for growth (Dangor etal, 1990). In addition, the cohesiveness of H. ducreyi colonies renders standardisation of inoculum size difficult and the slow and differing growth rates of isolates results in failure to consistently reproduce the logarithmic growth phase (Dangor etal, 1990).

Most published studies on in vitro susceptibility testing of H. ducreyi have used the agar dilution method (Hammond etal, 1978 ; Feltham etal, 1979 ; Bilgeri etal, 1981 ; Fast etal, 1983 ; Plummer etal, 1983 ; Slootmans etal, 1983 ; Taylor etal, 1985 ; Jones etal, 1986 ; Bowmer etal, 1987 ; Sturm, 1987). Disc diffusion (Feltham etal, 1979 ; Carpenter etal, 1981) and broth microdilution techniques (Kraus etal, 1982) have been less frequently used.

Homogenisation of the inoculum has been achieved by scraping colonies from the surface of the agar plates and suspending the organisms in Mueller-Hinton broth (Dangor etal, 1990). Either 10% FCS alone or 20% FCS together with 40% minimal essential medium has also been added to the Mueller-Hinton broth (Dangor etal, 1990). In Belgium and Thailand, H. ducreyi has been grown in haemin-containing Mueller-Hinton broth supplemented with glucose, glutamine, cysteine and bovine albumin fraction V or horse serum and IsoVitaleX. The bacterial suspensions were vortexed for three to thirty seconds or sonicated at six microns for ten to fifteen seconds and allowed to stand for fifteen minutes, after which they were compared with a 0.5 McFarland opacity standard (Dangor etal, 1990). The inoculum size has ranged from 10^4

to 10^7 CFU/ml.

There remains no standard medium for antimicrobial susceptibility testing of H. ducreyi. Enriched gonococcal (GC) agar (Hammond etal, 1978 ; Plummer etal, 1983 ; Bowmer etal, 1987) and enriched Mueller-Hinton agar (Carpenter etal, 1981 ; Fast etal, 1983 ; Slootmans etal, 1983 ; Taylor etal, 1985) have been used by most centres. Beiling medium with Mueller-Hinton agar instead of columbia agar (Sturm, 1987) and Sheffield medium (Jones etal, 1986) have also been employed. For this reason, it is often difficult to compare the MICs obtained in different studies. The incubation temperatures used by various centres have ranged from 33⁰C to 37⁰C, the percent atmospheric carbon dioxide has ranged from 5% to 10%, and the duration of incubation has ranged from twenty-four to ninety-six hours (Dangor etal, 1990).

With the exception of sulphonamides and trimethoprim, endpoint determinations should be based on the lowest concentration of antibiotic which yields no growth, two single colonies, or a fine, barely visible haze on the agar surface. Endpoint determinations of sulphonamide and trimethoprim susceptibility tests have usually been based on a significant decrease in growth (approxomately 80%) compared

with that seen on a control plate (Dangor etal, 1990).

The emergence of penicillin-resistant strains of H. ducreyi has necessitated the recognition of such strains. Beta-lactamase detection by either a chromogenic cephalosporin (O'Callaghan etal, 1972) or an iodometric paper strip (Jahn etal, 1979) test, or acidometric agar plate method (Miller S.D., personal communication) may all be simply and reliably used.

(1.2.5) Specimen Collection

Specimens for H. ducreyi culture should be obtained from the base (Duncan etal, 1981 ; Albritton etal, 1985) and undermined margins (Taylor etal, 1985) of the ulcer with a sterile swab. Recleaning the ulcer is not required (Albritton etal, 1985) although some investigators (Crewe-Brown etal, 1982 ; Taylor etal, 1984 ; Sturm etal, 1987) have recommended this. Swabs moistened with sterile saline (Taylor etal, 1985), as well as dry swabs (Sturm etal, 1987) have been used for specimen collection.

The isolation of H. ducreyi from inguinal bubo aspirates has been less successful than that from genital ulcer material

(Albritton etal, 1985).

(1.2.5.1) Swabs

Comparitive studies of swab material have not been reported. However, cotton (Borchardt and Hoke, 1970 ; Kinghorn etal, 1982 ; Taylor etal, 1985) -, rayon (Chapel etal, 1978) -, cotton-wool (Tan etal, 1977)-and calcium alginate (Duncan etal, 1981 ; Crewe-Brown etal, 1982 ; Coovadia etal, 1985 ; Kunitomo etal, 1986 ; Sturm etal, 1987)- tipped swabs have been used successfully. A bacteriological loop (Meheus etal, 1983) has also been used to obtain specimens, however, the amount of specimen obtained by this method may sometimes be inadequate.

(1.2.5.2) Transport

The maintenance of H. ducreyi viability in transport media has not been adequately studied. Three investigators (Borchardt and Hoke, 1970 ; Choudhary etal, 1982 ; Mallard etal, 1983) have used Amies medium for the transport of swabs to the laboratory ; one investigator has used Stuart transport medium (Sturm etal, 1987). Since primary isolation plates often yield few colonies of H. ducreyi after direct inoculation of specimens, it is conceivable that the dilution effect of transport media might cause some cultures to be

falsely negative. Current recommendations thus still require direct inoculation of swabs onto appropriate laboratory media.

Current evaluation of Amies transport medium by Dangor etal (Dangor Y., personal communication) has demonstrated bacterial growth to be supported for up to four days while stored at 4⁰C.

CHAPTER TWO

THE GRAM-NEGATIVE CELL WALL

(2.1) The Gram-Negative Bacterial Cell Surface

(2.1.1) Introduction

The cell wall of Gram-negative bacteria is multi-layered and comprises an inner cytoplasmic membrane, an intermediate rigid peptidoglycan layer, and an outer membrane layer (Slack and Snyder, 1978).

The outer membrane possesses a typical trilaminar structure and consists of proteins, phospholipids, lipoproteins, and a unique glycolipid called the lipopolysaccharide (LPS) (Slack and Snyder, 1978 ; Darveau etal, 1980).

(2.1.2) Organisation of the Intermediate Layer and Outer Membrane

Ultrastructural and biochemical data suggest that the organisation of the outer membrane is highly asymmetric

(DiRienzo etal, 1978 ; FIGURE 2.1).

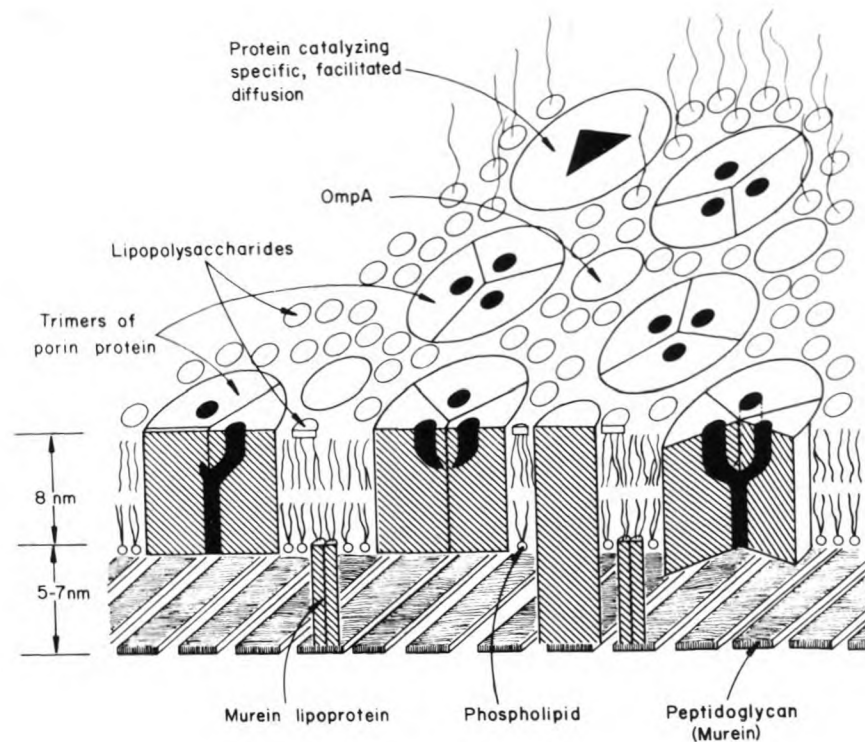


FIGURE 2.1 Schematic model of the *E. coli* outer membrane (Nikaido and Vaara, 1985)

Peptidoglycan (murein mucopeptide) is a heteropolymer composed of glycan strands linked to short peptides. The glycan consists of equimolar concentrations of alternating

N-acetylglucosamine and N-acetylmuramic acid residues, which are beta-1.4 linked. A peptide chain is bound to the carboxyl group of N-acetylmuramic acid and is composed of alternating L-and D-amino acids, which are usually L-alanine, D-glutamic acid, L-lysine and a terminal D-alanine.

In Escherichia coli, the peptide chains (bound to muramic acid) are cross-linked by a peptide bond between mesodiaminopimelic acid and D-alanine of the adjacent peptide side chains

The peptidoglycan forms a meshwork around the cell, and is responsible for the tensile strength and structural integrity of the bacterium. There are extensive protein-protein interactions over the entire surface of the outer membrane, and many outer membrane proteins (OMPs) penetrate the underlying peptidoglycan layer. The structure of the peptidoglycan is constant, but changes may occur under abnormal growth conditions.

The properties of LPS have been extensively investigated, and much is known about its structure, synthesis and biological characteristics. The LPS found in the outer membrane is a complex macromolecule containing three regions : a specific polysaccharide (region I) which determines serological

specificity, a core polysaccharide (region II), and an inner lipid component termed lipid A (region III). The fatty acid composition of lipid A varies between groups of bacteria and is responsible for endotoxin activity. The LPS is orientated so that the lipid A region is anchored in the lipid bilayer, and the polysaccharide chains extend towards the outside of the cell. Freeze-fracture studies on E. coli and Salmonella typhimurium demonstrate the asymmetric profile of the outer membrane fracture faces. These studies revealed that the external surface of the outer membrane, that is, the concave fracture face, was densely covered with particles 8-to-10nm in diameter. The convex fracture face showed only a few scattered particles. These particles probably represented outer membrane protein (OMP), judging from the following : (a) The deep rough LPS mutants of S. typhimurium and E. coli, which lost about 50% of the major OMPs, showed a decreased particle density. The degree of reduction in particle density was quantitatively correlated with the amount of the major OMPs that were lost (DiRienzo etal, 1978). (b) The decrease in particle density was also observed in mutant cells that contained normal LPS but lacked both the matrix and tolG proteins, but not in mutant cells that lacked one or the other of these proteins.

The ability of bacteria to avoid host defences and become invasive rests largely in cell surface components. The lipid A region alone can account for all the biological properties of endotoxin and the O-polysaccharide composition contributes to the virulence of the organism. Rough isogenic mutants are considerably less virulent than their smooth counterparts. Other surface components such as capsules, peptidoglycan, proteins and pili are additional well documented virulence factors (Aguero etal, 1984). Bacterial resistance to phagocytosis and the lytic activity of serum has been classically ascribed to the presence of polysaccharide capsules and O-antigens, respectively. Other cell surface structures, such as OMPs, may also contribute to serum resistance. Relatively little is known, however, about the detailed mechanisms by which the outer membrane contributes to serum resistance.

(2.2) The Outer Membrane Proteins

(2.2.1) Nomenclature of the Major Proteins

E. coli is the most intensively studied of all bacteria and serves as the basis for the numerous inferences concerning the behaviour of Gram-negative organisms. In 1970, Schnaitman reported that the E. coli outer membrane consisted of one

"major outer membrane protein" (MOMP) of a molecular weight of 44kD, which accounted for 77% of the total outer membrane protein. However, it was soon shown by Inouye and Yee (1972 ; 1973) that this component consisted of several distinct proteins. In addition to these major OMPs of higher molecular weight, another major protein, a lipoprotein, of lower molecular weight was present (Braun and Rehn, 1969). This protein was considered to be the most abundant protein in the E. coli cell based on molecular numbers.

The nomenclatures used for major proteins of the outer membrane of E. coli K-12 are as follows :

- (a) "Matrix proteins Ia and Ib" - proteins strongly associated with the peptidoglycan.
- (b) "TolG protein" - designates the heat-modifiable, trypsin-sensitive protein missing in tol G mutants.
- (c) Lipoprotein - designates the protein of the smallest molecular weight.
- (d) "Protein 3b" of the outer membrane, which has not yet been well characterised.

(2.2.1.1) Matrix proteins

Matrix proteins are characterised by their tight, but noncovalent association with peptidoglycan. When the

whole-cell envelope is solubilised in 2% SDS at 60⁰C, these proteins are found in the insoluble material complexed with peptidoglycan and bound lipoprotein. They vary in composition and electrophoretic mobility depending on the strain of origin.

In E. coli K12, matrix protein Ia is a single polypeptide, and seems to lack any non-protein moiety. It has been shown that matrix proteins Ia and Ib are different forms of a single polypeptide (Dirienzo etal, 1978). Matrix protein Ib is more acidic than Ia, and these proteins may differ by modification of amino acid residue(s) (Bassford etal, 1977). These matrix proteins are held to the peptidoglycan by ionic interactions. A striking feature of the matrix proteins Ia and Ib is their prominent beta-structure. This is in contrast to many other "intrinsic" membrane proteins which show high alpha-helix configuration. There is no doubt that the matrix proteins interact with peptidoglycan in vivo. Electron micrographs of the negative-stained matrix protein-peptidoglycan complex have shown the matrix protein molecules to be arranged as a hexagonal lattice layer (Rosenbusch, 1974). This lattice covers 60% or more of the outer surface of the peptidoglycan (Steven etal, 1977). Protein-protein interactions is a major force in maintaining

the hexagonal arrangement of the protein.

The most pronounced feature of the unit hexagonal cell is a triplet of indentations 2nm in diameter which are readily penetrated by stain. It has been suggested that these indentations represent channels or pores that span the protein monolayer as well as the outer membrane (DiRienzo etal, 1978). Additional evidence that the matrix proteins span the thickness of the outer membrane is provided by their ability to serve as receptors for phages (DiRienzo etal, 1978).

One function of the matrix proteins is to form pores that allow the rapid passive passage of hydrophilic molecules with small molecular weights through the outer membrane. This is further supported by the analysis of mutants lacking the matrix proteins; such mutants of S. typhimurium are defective in the diffusion of beta-lactam antibiotics, across the outer membrane. The structure and function of these proteins has led to their designation as "porins" (Nakae, 1976).

(2.2.1.2) TolG protein

The tolG protein is distinguishable from other major OMPs in that it is sensitive to digestion by trypsin and pronase

(DiRienzo etal, 1978). In addition, the amino acid composition shows only a moderate degree of hydrophobicity. This suggests that part of the protein is surface-exposed, which is supported by its role as a receptor for certain phages (Datta etal, 1977 ; Van Alphen etal, 1977).

Analysis of mutants lacking tolG protein (tolG⁻) revealed it to have an important function in F-pilus mediated conjugation (Van Alphen etal, 1977). TolG⁻ mutants are defective as recipients in conjugation due to a failure in pair formation. Inhibition of conjugation and inactivation of phages by the tolG protein were greatly enhanced by the presence of LPS; this suggests an interaction of the tolG protein and LPS in vivo.

(2.2.1.3) Lipoprotein

Lipoproteins occur as free forms in the E. coli membrane or are covalently bound to the peptidoglycan. Both forms exist almost exclusively in the outer membrane and differ only in their affinity for peptidoglycan. Collectively, the free and bound lipoprotein molecules are numerically the most abundant proteins in the cell.

The amino acid sequence of the lipoprotein shows several

striking features. Firstly, it is strongly repetitive. At the N-terminal portion, there are three almost identical adjacent sequences. The C-terminal part of the polypeptide chain is more variable but still shows striking homology when certain sequence gaps are introduced. Secondly, the hydrophobic amino acid residues are regularly arranged in an alternating three-to-four pattern.

Based on the above considerations, Inouye (1974) proposed a three-dimensional model for assembly of the lipoprotein. In this model, the lipoprotein occurs as an alpha-helix with six such helices arranged to form a superhelical tubular assembly possessing a hydrophilic interior and a hydrophobic exterior. The assembly is stabilised by ionic interactions between adjacent alpha-helices.

In another model proposed by Braun et al (1976), it was suggested that two helical rods were arranged such that they were stabilised intramolecularly either by hydrophobic or hydrophilic interactions. Three fatty acid residues extended out from the protein moiety and penetrated into the inner layer of the lipid bilayer of the outer membrane. In this model, the bound form of the lipoprotein could serve as an anchor to connect the outer membrane with the peptidoglycan

layer. However, it is not clear what the possible functions of the more plentiful free form of the lipoprotein might be.

The free form of the lipoprotein has been extensively purified and paracrystallised and its ultrastructure examined by electron microscopy (De Martini etal, 1976). The paracrystals are needle-shaped and show a highly ordered ultrastructure. The basic unit of the paracrystals has been proposed to consist of a number of lipoprotein molecules arranged with their 2.3nm-long lipid hydrocarbon chains side by side and their 8.7nm-long protein moieties projecting from the lipid region. In this unit the lipoprotein molecules may be assembled as a superhelical structure or as a simple coiled-coil structure (DiRienzo etal, 1978). It is not clear whether the lipoprotein is exposed to the outside surface of the outer membrane, although experiments carried out with antilipoprotein serum have suggested that a part of the lipoprotein is exposed to the surface of the outer membrane (Inouye, 1974).

Although the function of the lipoprotein is at present not well established, several physiological and morphological defects have been observed in mutants lacking this protein.

(2.2.1.4) Minor proteins

Between ten and twenty "minor proteins" are present in the outer membrane. The term "minor proteins" may be misleading, since under certain growth conditions some of these proteins are produced in quantities almost as great as a "major protein".

Many other minor proteins, in addition to the proteins described above, have been identified as receptors for phages and colicins. Most of them, as well as additional proteins with no known receptor functions, are now known to have vital roles for the growth of the cell, such as the uptake of nutritional substrates through the outer membrane (DiRienzo etal, 1978). For example, the uptake of vitamin B₁₂ by E. coli requires a specific receptor located in the outer membrane that is identical to the receptor for the E-type colicins as well as for phage BF23.

OMPs apparently participate in the process of DNA replication and cell division (DiRienzo etal, 1978). Gudas etal (1976) reported that an OMP designated protein D was produced at a specific time in the cell cycle shortly before DNA synthesis began, and that its synthesis was inhibited by naladixic acid.

Furthermore, protein D preferentially bound to double-stranded DNA in vitro. These results suggested the involvement of this protein in initiation of DNA synthesis.

(2.2.2) Structure and Function

The protein composition of the outer membrane appears to be flexible. When one or several proteins are lost or drastically reduced due to mutation there is a compensatory increase in one or several other proteins (DiRienzo etal, 1978). For example, in E. coli when matrix protein Ia is absent, the level of matrix protein Ib or both Ib and tol G protein increases so that the total amount of OMP remains constant (Chai and Folds, 1977). In a mutant strain lacking the matrix proteins Ia and Ib, the amount of the tolG protein is approximately equal to the total amount of all three proteins in the parental strain. Conversely, mutants lacking the tolG protein partially compensate for this loss by synthesising increased amounts of the matrix proteins.

The reason for this flexibility in composition is unknown. It is possible that the compensation of some proteins for others may help to maintain a certain degree of rigidity of the outer membrane. Interestingly, mutants that have defective LPS possess a decreased amount of major OMPs, suggesting that

LPS may play an important role in the assembly of the OMPs (DiRienzo etal, 1978).

The OMPs appear to subserve many of the functions of the outer membrane. Some of the major OMPs of a number of organisms have been shown to traverse the outer membrane, and to function as porins, permitting the flow of small molecular weight compounds from the external milieu to the periplasmic space, and vice versa (Darveau etal, 1980). When outer membrane vesicles were reconstituted from combinations of phospholipids, LPS, and OMPs isolated from S. typhimurium, they were permeable to sucrose as well as to small molecular weight oligosaccharides only when the membrane proteins were included in the reconstitution system. This supported the finding that closed membrane vesicles similar to the native outer membrane were reassembled only when OMPs were included in the reconstitution mixture (Nakamura and Mizushima, 1975). Cytoplasmic membrane protein, when substituted in the mixture, was unable to reform membrane vesicles.

Additional reconstitution studies with selected OMPs indicated that the matrix protein produced diffusion channels in the reconstituted vesicles. Electron microscopy reveals that three of these protein molecules appear to be arranged

in a unit cell that shows a triplet of indentations each approximately 2nm in diameter. These indentations probably represent lipoprotein. Lipoprotein is required to reconstitute membranous vesicles that resemble the outer membrane structure (DiRienzo etal, 1978). Mutants of S. typhimurium that lack the matrix protein were deficient in the diffusion of the beta-lactam antibiotic, cephaloridine, across the outer membrane (DiRienzo etal, 1978). Confirmatory studies have shown that mutants lacking the matrix protein demonstrated a greatly reduced ability to utilise minimal concentrations of several low molecular weight metabolites. These results indicate strongly that the matrix protein is required for the formation of the passive diffusion pores.

Pores in the outer membrane may be assembled as illustrated in Figure 2.2

Although the matrix protein pores are nonspecific for small molecular weight, hydrophilic substrates, they also allow the rapid transmembrane diffusion of hydrophilic substances with molecular weights smaller than 650 daltons. They do not permit the passage of saccharides with molecular weights higher than 900-to-1000 daltons. Therefore low molecular weight nutrients such as amino acids, sugars, and salts

probably diffuse across the outer membrane through the matrix protein pores into the periplasmic space and subsequently are actively transported into the cytoplasm (DiRienzo etal, 1978).

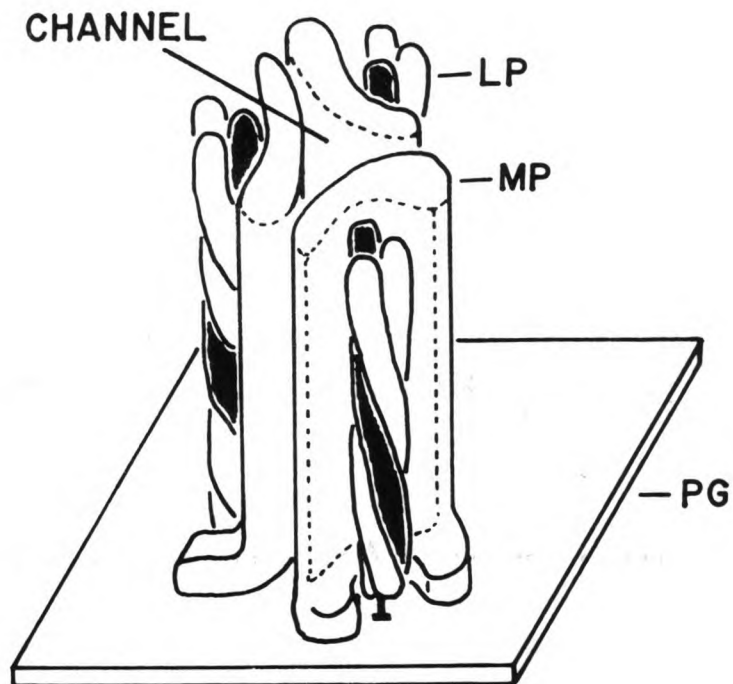


FIGURE 2.2 Assembly model of the matrix protein and lipoprotein. Abbreviations used are : LP, lipoprotein ; MP, matrix protein ; and PG, peptidoglycan. The bound form lipoprotein molecules are darkened. (DiRienzo etal, 1978).

(2.2.3) Factors Affecting OMPs

Environmental factors such as carbon source, incubation temperature, and medium osmolarity affect the protein composition of the outer membrane of E. coli. Based on the limited information available, it is not yet possible to predict how other bacteria might develop altered OMP composition with regard to environmental change (DiRienzo etal, 1978). Hancock and Braun (1976) observed that the production of three OMPs of E. coli K-12 were repressed by the presence of iron in the growth medium.

(2.2.4) Genetic Determinants

One of the great advantages of using E. coli for the study of membrane proteins is the extent to which its genetics are well-characterised and understood. Many mutants with defective or altered OMPs are resistant to phages and colicins, resistant or supersensitive to inhibitory reagents such as antibiotics, detergents and dyes, and show impaired utilisation of growth substrates or altered cellular shape. Isolation and characterisation of such mutants are extremely important for the study of biosynthesis, assembly, and function of these proteins and of the outer membrane itself.

Genetic studies of matrix proteins Ia and Ib have revealed

the complex aspects of their biosynthesis and assembly (FIGURE 2.2). E. coli K12 mutants lacking only protein Ib have been isolated among the strains that are resistant to phages PA-2 (par), TuIb, or Mel (meo). These mutants were probably identical and mapped at about 47.5 minutes on the E. coli linkage map (FIGURE 2.3). TolF mutants, which are tolerant to colicins A, E2, E3, K, and L, were found to be specifically lacking protein Ia. The tolF locus was closely related to or identical with the cmlB locus which determines increased resistance to antibiotics chloramphenicol and tetracycline, and maps at 20.8 minutes on the E. coli K12 chromosome linkage map (DiRienzo etal, 1978 ; FIGURE 2.3). In contrast to these mutants, the tolIV and tolXIV mutants, which were tolerant to colicins E2, E3, K, and L were missing both proteins Ia and Ib. These mutations were mapped at the same locus, designated as ompB, which is located at 73.8 minutes on the E. coli K12 chromosome linkage map (DiRienzo etal, 1978 ; FIGURE 2.3). Mutants lacking the matrix protein are defective in the passive diffusion of small molecular weight substances, such as sugars and amino acids. The gene locus of the "pleiotropic transport mutants" (kmt) of E. coli B/r, with a deficiency in the matrix protein, has been mapped at 73.7 minutes. Thus the kmt gene is probably identical with

the ompB gene (DiRienzo etal, 1978).

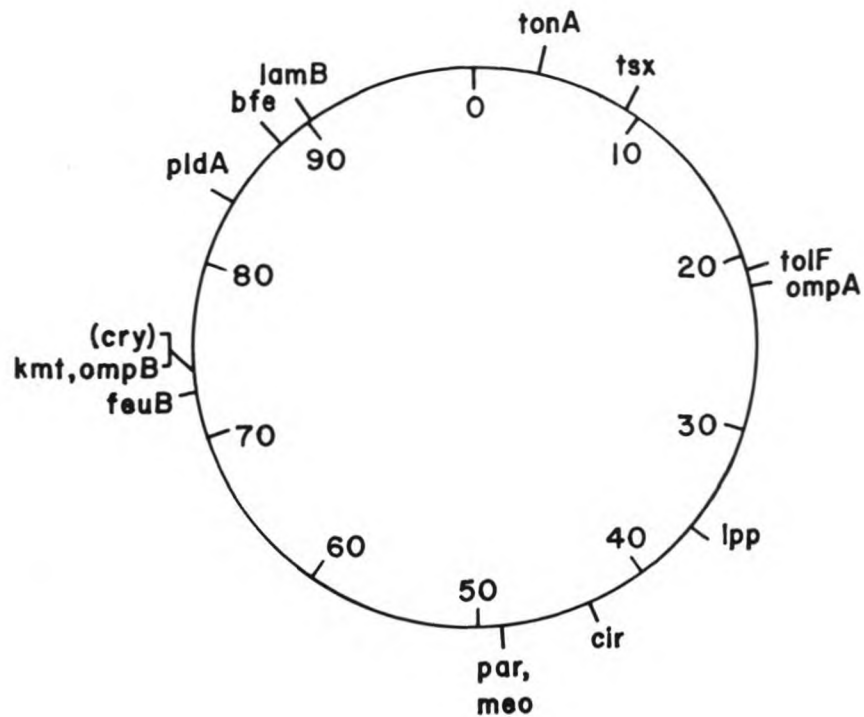


FIGURE 2.3 Chromosomal location of *E. coli* genes involved in the biosynthesis of OMPs. Genetic symbols are described in the text, except *pldA* for phospholipase A1. Parentheses around a gene symbol indicate that the location of that marker is not well defined (Adapted from DiRienzo etal, 1978).

These findings together with the fact that the matrix proteins Ia and Ib are chemically quite similar, suggests that they are coded for by the same structural gene but undergo different post-transcriptional modifications. Probably the ompB gene is the structural gene for a precursor polypeptide and other genetic loci are involved in the modification process. However, other possibilities, such as the existence of more than one structural gene cannot be excluded (DiRienzo etal, 1978).

Mutants missing the tolG protein were among those specifically tolerant to colicin L-JF 246 (tolG) or resistant to phage K3 (con) or phage TuII (tut). Later these mutants were shown to be identical and the gene symbols were renamed ompA (DiRienzo etal, 1978). This gene locus was mapped at about 21.5 minutes on the E. coli K12 chromosome linkage map. The ompA gene is the structural gene for the tolG protein.

A lipoprotein mutant of E. coli among a collection of temperature-sensitive cell division mutants was found to be due to a single-base change from U to C in the structural gene for the lipoprotein. The mutation (lpp-1) was found to map at 36.5 minutes on the E. coli chromosome.

Another lipoprotein mutant designated epo, in which both the free and bound forms of the lipoprotein were totally absent, was as a result of an inactive mRNA being produced for the lipoprotein. Chromosomal DNA from the lpo mutant did not have the restriction fragment that could hybridise with the purified radioactive mRNA for the lipoprotein. This suggests the lpo mutation is most likely a deletion of the structural gene (lpp) for the lipoprotein. The mutation is also mapped at 36.5 minutes on the E. coli chromosome.

The lpo mutant seemed to grow and divide normally. However, it leaked considerable amounts of periplasmic enzymes into the medium (DiRienzo etal, 1978)

Molecular cloning of genes would appear to be the most logical approach to more accurately study biosynthesis, regulation and assembly of OMPs.

CHAPTER THREE

SERUM BACTERICIDAL ACTIVITY

(3.1) Introduction

For a variety of Gram-negative pathogens, susceptibility of strains to the complement-mediated bactericidal activity in normal mammalian serum is associated with infection or colonisation confined to mucosal surfaces, whereas serum resistance is usually associated with systemic spread (Blaser *et al*, 1987). Susceptibility to the serum bactericidal system is a wide-spread characteristic of Gram-negative bacteria. Serum is known to possess bactericidal activity against susceptible representatives of practically every Gram - negative genus so far examined (Taylor, 1983).

Exposure of many strains of Gram-negative bacteria to suitable concentrations of human or animal serum results in loss of viability, and sometimes dissolution of the bacterial cells (Taylor, 1983). The majority of investigators of bactericidal phenomena have utilised serum from humans

(Wardlaw, 1962 ; Bjorksten etal, 1976 ; Olling, 1977), or from domestic (Tachibana, 1965 ; Mittal and Ingram, 1975) and laboratory animals (Amano etal, 1958 ; Collins, 1967) as sources of complement. The capacity to kill Gram-negative bacteria is associated, however, with serum from a wide variety of warm-blooded and cold-blooded animals, including fish, and probably reflects the distribution of immunological responsiveness and the complement system in the animal kingdom.

In addition to susceptible strains, there are Gram-negative bacteria that appear refractory to the bactericidal action of serum. These resistant strains are frequently isolated as causative agents of invasive infections. Serum resistance has therefore been proposed as an important determinant of bacterial virulence in at least some infections due to Gram-negative bacteria.

(3.2) Serum Bactericidal Activity As An Immune Defence Mechanism

Precise in vivo evaluation of the role of bactericidal mechanisms against Gram-negative bacteria has been difficult to achieve. This is mainly due to difficulties in

distinguishing complement-mediated bactericidal phenomena from other specific and non-specific host defence systems that may also modify the interaction between host and invading parasite. In addition, other host systems, such as immune phagocytosis by blood leukocytes may also require at least some of the components of complement for efficient processing and elimination of invading microorganisms (Taylor, 1983).

The importance of the complement system in host defence is suggested by the observation that the acute stage of many Gram-negative infections is characterised by increased biosynthesis and turnover of complement proteins (Atkinson and Frank, 1980) and a reduction in the serum level of natural complement inhibitors (Whaley etal, 1980). Thus, in these situations, complement behaves as an acute phase reactant. In some infections the primary mechanism is provided by immune phagocytosis, and many serum-resistant strains can be efficiently phagocytosed after opsonisation. Nevertheless, an extensive body of literature has steadily accumulated implicating resistance to the bactericidal action of serum as an important determinant of virulence in certain infections (Taylor, 1983).

(3.3) Clinical Relevance of Serum Resistance as a Determinant of Bacterial Pathogenicity

Roantree and Rantz (1960) and Fierer etal (1972) found a much higher proportion of strains isolated from the blood of bacteremic patients than of those isolated from faeces or urine to be resistant to killing by human serum. Similarly, Vosti and Randall (1970) found significantly fewer serum - susceptible E. coli strains isolated from blood cultures than from urine and faeces. There thus appears to be a strong correlation between serum resistance and the ability of a variety of Gram-negative bacteria to invade and survive in the human bloodstream.

The survival of apparently serum-susceptible bacilli in the blood of bacteremic patients might be related to a number of factors. Serum from some bacteremic patients has been reported to be ineffective in bactericidal assays utilising the infecting homologous organism, even though the strain is fully susceptible to serum from healthy individuals (Roantree and Rantz, 1960). Bacteria may circumvent the serum bactericidal system by adopting an intracellular location in the blood or reticuloendothelial system (Roantree and Pappas, 1960). For example, viable bacteria may be found within

leukocytes of patients with infections of the blood system (Smith, 1966). Bacteria dividing at submaximal rates may be less susceptible to serum than more rapidly dividing cells (Taylor, 1978), and this may assist survival of apparently susceptible isolates in vivo. Also, the genetic basis for virulence may be fully expressed only during growth in vivo ; gonococci adapted to growth in chambers implanted subcutaneously into guinea-pigs were found to be resistant to human serum, but loss of this phenotypically acquired trait occurred after a few generations of in vitro growth (Rittenberg etal, 1977).

A most important factor is the immune status of the infected host. Pre-existing disease is of major importance in the acquisition of bacteremia and in its outcome (McCabe and Jackson, 1962 ; Zinner and McCabe, 1976 ; Olling, 1977) and may therefore reduce the necessity for invading bacteria to express virulence determinants. For example, patients with sickle-cell anemia frequently contract systemic salmonellosis, and sera from such individuals display deficient bactericidal function against Salmonella typhimurium, probably as a result of abnormal alternative pathway activity (Hand and King, 1977). Clearly, it is important to consider the possibility that the serum of patients under investigation may have a

reduced capacity to kill normally susceptible organisms when evaluating serum bactericidal systems.

There is evidence to suggest that cell-mediated host defences may be impaired within the urinary tract (Chernew and Braude, 1962 ; Miller and North, 1974). It is possible that the serum bactericidal system may constitute an important defence mechanism in those parts of the urinary tract in contact with antibody-complement-containing-fluids. Gower and co-workers (1972) examined the serum susceptibility of the infecting Gram-negative organisms from patients with infection that had been localised to either the upper (ureters and kidneys) or lower (bladder) urinary tract. A very high proportion of lower infections was due to serum-susceptible organisms. In contrast, the majority of upper urinary tract isolates were not susceptible to serum obtained from the infected patient. In some cases, the infecting strains were inherently serum-resistant, but others were efficiently killed by normal human serum.

Complement-mediated bactericidal mechanisms may be important in other types of infection. The overwhelming majority of enterobacteria causing bovine mastitis were found to be serum-resistant (Carrol and Jasper, 1977). Strains of Bacteroides

fragilis, the predominant species found in serious intra abdominal infections, formed a group characterised by a high degree of serum resistance (Casciato etal, 1979). Waisbren and Brown (1966) recorded specific defects in the ability of patients sera to kill the infecting organism in a variety of clinical situations that included Pseudomonas pneumonia, chronic pyelonephritis, endocarditis, peritonitis, and septicemia. Susceptibility to the serum bactericidal system seems to be of primary importance in determining the pathogenesis of infections due to Neisseria species. Gonococci causing localised genital infections are generally susceptible to human serum (Eisenstein etal, 1977). Serum-resistant strains are usually associated with invasion of the blood system and dissemination (Schoolnik etal, 1976 ; Eisenstein etal, 1977).

The most convincing evidence in favour of a protective role for bactericidal activity in infections is afforded by observations on patients with total or near-total congenital deficiencies of a single complement protein. Patients with genetic deficiencies of the proteins of the membrane attack complex (MAC) have a propensity to recurrent infection with bacteria of the Neisseria group, such as gonococcal or meningococcal bacteremia and meningitis (Brooks etal, 1978 ;

Petersen etal, 1979). This implies that the late-acting complement components are necessary for normal host defence against pathogenic Neisseria species, particularly with regard to impeding dissemination of serum-susceptible bacteria. Individuals deficient in C1, C4, and C2 do not appear to be unduly susceptible to infections with Gram-negative organisms. It is likely that in such individuals the alternative pathway is responsible for preventing serious recurrent infections (Atkinson and Frank, 1980).

(3.4) Mechanism of Serum Bactericidal Activity

(3.4.1) Role of complement

Complement was first described by Ehrlich and Mogenroth in 1899 as a serum substance which completes the action of antibody (Chan and Atkinson, 1985). Over the past eighty-five years, the complement system has been demonstrated to be a complex self-assembling cascade composed of at least twenty serum glycoproteins. When functioning in an ordered and integrated fashion, these serum glycoproteins serve as important mediators of inflammation and host defence (Winkelstein, 1983).

The terminal components (C3-C9) are responsible for the

majority of the biologically significant actions of the complement system. In order to subserve these biological functions, however, C3-C9 must first be activated. Microorganisms may activate the terminal components by at least two different pathways, the classical and alternative pathways. (FIGURE 3.1)

Activation of the classical pathway is usually initiated by an antigen-antibody complex. Antibodies of the appropriate class or subclass (IgG1, IgG2, IgG3 and IgM in humans) combine with an antigen to form an immune complex, which is able to bind and activate the first component of complement (C1). Activated C1 then activates C4 and C2. The major cleavage products of C4 (C4b) and C2 (C2a) then act as an enzymatically active bimolecular complex, the classical pathway C3 convertase (C4b,2a), to cleave C3 into two fragments of unequal size, C3a and C3b. In this enzyme, the C4b functions to bind native C3 while the C2a carries the active site. Some of the nascent C3b can then combine with C4b,2a to create a new enzyme, the classical pathway C5 convertase (C4b,2a,3b), which cleaves C5 into two fragments of unequal size, C5a and C5b. In this enzyme, the C3b acts to bind native C5 while the C2a carries the active site. The activation of C5 and creation of C5b subsequently leads to

the assembly of C5b, C6, C7, C8 and C9 into the MAC
(Winkelstein, 1983)

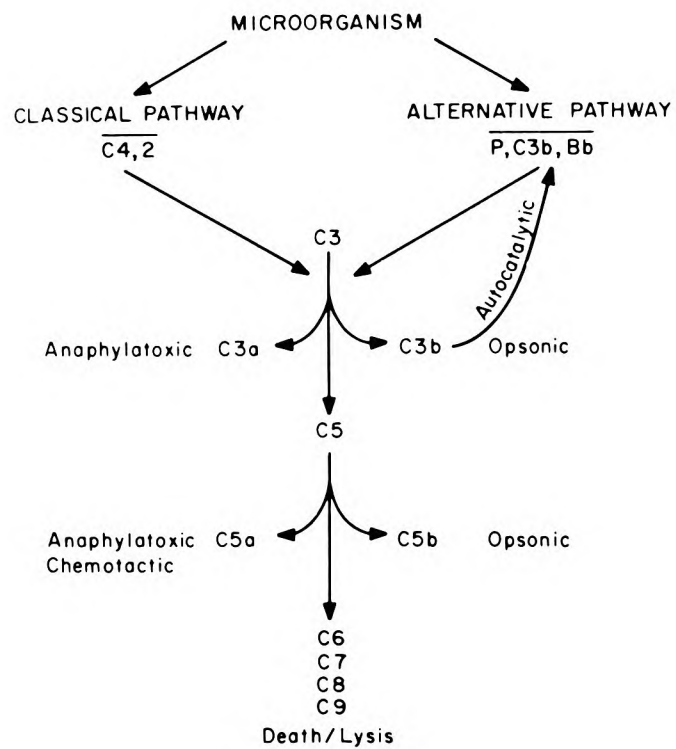


FIGURE 3.1 Schematic representation of the mechanisms by which microorganisms activate C3 to C9 and generate biologically active products (Winkelstein, 1983).

Operation of the alternative pathway may be activated in total absence of specific antibodies or immunoglobulins. In the alternative pathway, the activation of C3 to C9 is initiated by a C3-cleaving enzyme that is distinct from C4b,2a. Like the classical pathway C3 convertase, this enzyme is also a bimolecular complex (C3b,Bb). It is composed of the larger cleavage product of C3 (C3b) and the larger cleavage product of alternative pathway factor B (Bb). The activated Bb of this alternative pathway C3 convertase is generated by the enzymatic cleavage of native factor B by factor D. The C3b portion of the alternative pathway C3 convertase may be generated through at least two different mechanisms. First, it may be generated from native C3 by the action of the classical pathway C3 convertase. Second, and independent of the classical pathway, the C3b may be generated by a relatively inefficient C3 convertase produced in the fluid phase by the interaction of native C3, native factor B and D. Whether generated by one or the other of these mechanisms, if nascent C3b then fixes to an appropriate surface (such as the surface of a bacterium) and combines with Bb, it has the potential to form an efficient particle-bound C3 cleaving enzyme, the alternative pathway C3 convertase (C3b,Bb). However, a number of opposing factors influence the activity of this enzyme. The enzyme is quite labile and at

physiological temperatures rapidly undergoes intrinsic decay. One of the proteins of the alternative pathway, properdin, functions to stabilise the enzyme and retards its intrinsic decay. Two other proteins, C3b inactivator (I) and beta1H (H), act to inhibit the generation and / or expression of the C3b,Bb enzyme. Factor H not only competes with B for binding to C3b in the assembly of the alternative pathway C3 convertase but it can also displace Bb from the C3b,Bb complex once the C3-convertase has formed. Factor I inhibits the alternative pathway C3 convertase by inactivating the cell-bound C3b through proteolytic cleavage ; its rate of inactivation of C3b is markedly accelerated by factor H (Winkelstein, 1983).

Recently, evidence has accumulated that particles capable of activating the alternative pathway do so, at least in part, by virtue of their ability to bind nascent C3b and to protect the C3b,Bb enzyme from the actions of beta1H and C3b inactivator (Fearon and Austen, 1977). Furthermore, at least one molecular mechanism by which a particle protects the alternative pathway C3 convertase from these two control proteins has also been elucidated. The binding of beta1H to a particle, and thus its inhibitory effects on the alternative pathway C3-cleaving enzyme, is favoured by the presence of

sialic acid residues on the particle (Fearon, 1978). Thus, particles with high levels of sialic acid on their surface are relatively poor activators of the alternative pathway, whereas particles with low levels of sialic acid are relatively good activators of the alternative pathway (Winkelstein, 1983).

Although the two pathways utilise different sets of early components, they both share the terminal components (C5 to C9 or the MAC). The deposition of the assembled proteins of the MAC onto the surfaces of susceptible bacteria is responsible for serum mediated killing (Taylor, 1983).

Activation of the classical pathway, by creating nascent C3b, can lead to activation of the alternative pathway. Since C3b is both the product of the alternative pathway C3-cleaving enzyme and also forms part of the C3 convertase, the activation of C3 via the alternative pathway has the potential to be autocatalytic in nature (Winkelstein, 1983). Since microbial activation of the classical pathway usually requires the participation of antibody, this pathway has traditionally been viewed as being more significant in acquired immunity than in natural immunity. Conversely, since the alternative pathway can be activated by microbes without

a requirement for antibody, this pathway has traditionally been viewed as being more significant in natural immunity than in acquired immunity. A number of diverse microorganisms are capable of activating the classical pathway in the absence of antibody. In each case, the microorganism binds and activates C1 directly. This mechanism of antibody-independent classical pathway activation has been described for some species and strains of Gram-negative bacteria (Loos etal, 1978) and Gram-positive bacteria (Eads etal, 1982), for some enveloped RNA viruses (Hirsch, 1982), and for some mycoplasma species (Bredt etal, 1977). In the case of Gram-negative bacteria, C1 apparently binds directly to the endotoxic LPS in the cell wall, leading to activation of the classical pathway and a complement-mediated bactericidal reaction (Loos etal, 1974 ; Clas and Loos, 1981).

In addition to antibody-independent activation of the classical pathway, the non-immune host is also able to activate the classical pathway through antibody-dependent mechanisms. Low levels of natural antibodies to microbial cell walls are present in most individuals and thus the potential for the "non-immune" individual to utilise these naturally occurring antibodies in activation of the classical

pathway also exists (Winkelstein, 1983).

A wide variety of microorganisms are able to activate the alternative pathway. These include both Gram-negative and Gram-positive bacteria, most fungi, and some enveloped viruses. In the case of Gram-negative bacteria, the endotoxic LPS is the active constituent responsible for activating the alternative pathway (Marcus etal, 1971).

Although a wide variety of microorganisms is capable of activating the alternative pathway, some are relatively poor activators while others are relatively good activators. The reasons for this variation are largely unknown. As suggested above sialic content of the bacterium seems to play a role in this regard (Winkelstein, 1983).

Serum bactericidal activities require the participation of the full terminal complement sequence (Mayer, 1978). Full expression of bactericidal activity depends on how much C5b-9 is activated, how much of the MAC is assembled on the bacterial cell wall, and how susceptible the structure is to the lytic action of C5b-9 (Winkelstein, 1983). For example, in studies of isogeneic strains of Salmonella, the strain that was serum-resistant was able to activate the terminal

components and generate the MAC, equally as well as the serum-sensitive strain (Joiner etal, 1982). However, once generated the C5b-9 did not attach as well to the surface of the serum-resistant strain as it did to the serum-sensitive strain (Joiner etal, 1982).

Assembly of the MAC involves the transformation of hydrophilic molecules into a macromolecular amphiphilic complex capable of integration into lipid containing bilayers (Taylor, 1983).

(3.4.2) Role of antibody

In the serum bactericidal reaction, initiation of the classical pathway generally results from formation of an antigen-antibody complex at or near sites on the bacterial surface that can interact with activated molecules of the complement cascade. Extremely small amounts of antibody are required for complement activation (Taylor, 1983). There is wide variation in the efficiency with which different antibody classes can function in complement-mediated killing. Although natural antibodies in serum may belong to the IgM, IgA or IgG classes (Cohen and Norins, 1966), the predominant natural antibodies to representative enterobacterial species are contained in the IgM fraction of human plasma (Michael

and Rosen, 1963). Landy etal (1965) found that, after a single injection of Salmonella enteritidis polysaccharide, more than 99% of the complement - activating activity was associated with the IgM fraction of rabbit serum.

(3.4.3) Sequence of events after exposure to serum

Exposure of susceptible Gram-negative cells to serum is generally followed by a period during which there is little apparent change in viability. The length of this period depends upon the bacterial strain used, the test system, the serum concentration, and the metabolic state of the bacterial cells (Taylor, 1983). Although direct evidence is lacking, it would be reasonable to assume that during this latent period the components of the MAC are under assembly at their target sites (Taylor, 1983). Once functional lesions have been induced, loss of viability rapidly ensues, and killing proceeds in a logarithmic fashion (Davis and Wedgewood, 1965 ; Ogata and Levine, 1980 ; Taylor and Kroll, 1983). Rapid killing of bacteria suggests that the bactericidal mechanism has a high degree of efficiency. The Gram-negative cell presents a difficult target for complement attack due to a complex envelope structure, the ability to respond rapidly to changes in the environment, and the capacity for repair of

damaged sites (Taylor, 1983).

Wright and Levine (1981) examined the dose-response kinetics for the killing of E. coli. They found that the killing of E. coli, as well as complement-mediated inner and outer membrane damage, required more than one hit, and that the lethal process closely approximated two-hit killing kinetics. The authors suggested that this reflected the dimeric nature of the MAC, but did not preclude the possibility that damage to inner and outer membranes were independent events.

It is evident that the precise nature of the lethal event after interaction of MAC proteins with fluid bacterial membranes still remains relatively unclear (Kato and Bito, 1978 ; Taylor, 1983). Integration of the MAC into phospholipid bilayer areas of the outer membrane disrupts outer membrane integrity and removes the barrier to hydrolysis of peptidoglycan by lysozyme (Inoue etal, 1968 ; Glynn, 1969 ; Martinez and Carrol, 1980). It is thought to be unlikely, however, that damage confined to the outer membrane is responsible for the lethal activity of complement. A possibility considered by Feingold etal (1968) was that effective lesions occurred only when terminal complexes were deposited on the bacterial surface at points of contact

between the cytoplasmic and outer membranes. Simultaneous damage of both membranes would then occur.

(3.5) Defining Resistance To Serum Bactericidal Activity

Under optimal conditions, many strains of Gram-negative bacteria are rapidly killed by human and animal sera. Some strains appear to be completely refractory to the serum bactericidal system. A considerable increase in viable cell numbers occurs despite prolonged incubation in the presence of adequate amounts of sensitising antibody and an excess of complement (Taylor, 1978 ; Hughes etal, 1982). Many strains, however, that fall between these two extremes, and significant reduction in survival may be apparent only after lengthy periods of incubation (Taylor, 1974 ; Traub and Kleber, 1976 ; Hughes etal, 1982). Taylor (1983) suggested that these strains should be regarded as susceptible to serum, or at least significantly different from strains that are capable of replicating in serum, because decrease in viable cell numbers implies insertion of functional MACs into sites on the surface of the cell envelope from which they can effect killing, albeit slowly. On the other hand many studies have described as resistant those strains which have undergone significant reduction in viability after incubation

in serum (Roantree and Rantz, 1960 ; Kimball etal, 1964 ; Simberkoff etal, 1976 ; Ogata and Levine, 1980). It thus remains extremely difficult to provide a reference definition of serum resistance. It has been suggested that the term "serum resistance" be restricted to the description of strains that are totally refractory to serum when tested in early logarithmic phase in assays containing excess amounts of the components of the bactericidal system (Taylor, 1983).

(3.6) Bacterial Components Associated With Serum Resistance

(3.6.1) Lipopolysaccharides (LPS)

The earliest attempt to systematically define cell envelope components involved in the determination of serum resistance can be attributed to Wardlaw (1963 ; 1964), who compared the composition of purified envelopes from a smooth serum - resistant E. coli strain with those from the rough, extremely susceptible strain. The author found a nine-fold difference in the amount of LPS that could be extracted from the envelopes by the phenol water method (Westphal and Jann, 1965). However, this technique removes only a small proportion of R polysaccharide from rough strains, thus explaining the disparity in yields of this macromolecule from

the two strains.

Both Rowley (1956), and Michael and Landy (1961), using only smooth enterobacteriaceae, showed that serum-susceptible organisms possessed less intrinsic endotoxic activity than resistant ones. No significant differences, however, could be found in the amount of LPS extractable from twenty-eight smooth, urinary E. coli strains of differing serum susceptibilities (Taylor, 1976). Similarly, mutations to serum resistance occurred independantly of quantitative changes in E. coli LPS content (Taylor, 1975), and phenotypically induced transitions from serum resistance to susceptibility were not accompanied by differences in LPS yields (Taylor etal, 1981). Thus, although strains with a low LPS content may generally be susceptible to serum, it is unlikely that the absolute amount of polymer in the envelope is the major factor determining resistance. Furthermore, comparisons between smooth and rough strains with regard to serum reactivity may not be valid (Taylor, 1983).

LPS may, in other ways, play a central role in determining the susceptibility of enterobacteria to serum (Taylor, 1983). For example, mutations from the smooth to the rough colonial form, are usually, but not invariably, associated with loss

of the ability to synthesise LPS O-side chains (Jann and Westphal, 1975 ; Orskov etal, 1977 ; Wilkinson, 1977), and are accompanied by drastic increases in serum susceptibility (Nelson and Roantree, 1967 ; Rowley, 1968 ; Inoue, 1972 ; Taylor, 1975 ; Madonna and Allen, 1981). Evidence of the influence of the O-side chain moiety of LPS on serum susceptibility was obtained by Roantree and co-workers (Nelson and Roantree, 1967), who derived serum-susceptible mutants from resistant, smooth Salmonella strains. The majority of such mutants were rough or part rough as a result of partial or total loss of the O-side chain. A small number, however, possessed LPS with an O-side chain sugar-to-core sugar ratio identical to that found in the parent strains, indicating that other factors must be involved in the determination of resistance of the smooth parental strains. In general, (Collins, 1964 ; Tempest and Ellwood, 1969 ; Chester and Meadow, 1975 ; Goldman and Leive, 1980), more O-side chain material was associated with LPS from serum-resistant than serum-susceptible urinary strains of E. coli, but this relationship did not reach levels of statistical significance (Taylor, 1976). Similarly, no quantitative differences in LPS sugar composition could be found between serum-resistant mutants and their respective E. coli parent strains (Taylor, 1975). It appears unlikely,

therefore, that the serum resistance of Gram-negative bacteria is determined solely by the length and number of O-antigenic polysaccharide chains associated with LPS, although the possibility that a subtle rearrangement of LPS components within the molecule might account for the observed differences in serum reactivity (Taylor, 1983).

Many strains carrying a full complement of LPS O-side chains may be susceptible to serum although, unlike rough strains, they usually exhibit a delayed sensitive response (Taylor, 1974). The mechanism by which O-side chains may do this is unclear; the attractive possibility that low membrane fluidity resulting from synthesis of a complete LPS reduces the rate of insertion of functional MACs into the cell envelope has so far not been investigated. It has been frequently suggested that long O-side chains cause the antibody-mediated activation of complement at some distance from the site of lesion formation, and intermediates therefore decay before they can be incorporated into functional MACs (Muschel and Jackson, 1966 ; Rowley and Turner, 1968 ; Muschel and Larson, 1970 ; Rowley, 1973). Such a concept could also be extended to include antibody-independent activation of the alternative pathway, as the polysaccharide moiety of LPS is known to activate complement

(Morrison and Kline, 1977).

(3.6.2) Acidic polysaccharides

It has been suggested that acidic polysaccharides may exert an influence on the extent of bacterial killing by serum due to an ability to impede antibody binding and subsequent attachment of complement components to the bacterial surface (Glynn and Howard, 1970). Current evidence would tend to suggest, however, that polysaccharide capsules do not represent a diffusion, permeability or adsorption barrier to macromolecules such as IgG (King and Wilkinson, 1981) or other proteins (King etal, 1980), and that O-inagglutinability frequently associated with the presence of acidic polysaccharides superficial to the bacterial surface is most likely due to surface protein components (Jann etal, 1970) or to inhibition of lattice formation between adjacent bacteria (Ceppellini and Landy, 1963).

No obvious relationships were found when acidic exopolysaccharides were prepared by two methods from a large number of urinary E. coli isolates of various serum susceptibilities (Taylor, 1976). Some susceptible and resistant strains produced large amounts of K-antigen with high haemagglutination-inhibiting activity, but both groups

also contained strains that produced small quantities of polymer with no detectable activity.

McCabe etal (1978) found a similar lack of correlation for E. coli bacteremia strains. Mutations to serum resistance did not result in increased exopolysaccharide production. Inheritance of the his-linked genes for K27 antigen production by E. coli recipients did not result in an altered serum response, regardless of whether recombinants produced rough LPS or had simultaneously inherited O-side chains (Taylor and Robinson, 1980).

Klebsiella strains have frequently been found to be susceptible to serum (Fierer etal, 1972 ; Gower etal, 1972), even though most members of this genus produce copious amounts of hexuronic acid-containing acidic polysaccharide (Sutherland, 1979).

E. coli strains implicated in neonatal meningitis (Robbins etal, 1974) and renal infection in infancy (Kaijser etal, 1977) synthesise the K1 antigen. It has been suggested that the K1 antigen or the presence of closely linked traits may be a virulence factor related to the pathogenesis of these diseases. Several groups have considered the possibility that

the K1-mediated pathogenicity of these strains is related to a capacity to resist the bactericidal activity of serum (Taylor, 1983).

Investigators have assigned a role for the K1 antigen on the basis of genetic studies of serum resistance. K1-negative mutants of E. coli K1 clinical isolates were found to be more susceptible to high concentrations of serum than their parent strains (Cabello, 1979). Rough K1-positive E. coli bacteremic isolates were resistant to 10 to 20% human serum, whereas single-isogenic K1-negative derivatives, with an identical OMP profile, were found to be extremely serum-susceptible (Gemski etal, 1980). Opal etal (1982) found the presence of K1 capsular antigens afforded protection for some rough E. coli strains against serum bactericidal activity, whereas K27 failed to do so. These studies suggest a possible direct role for the K1 antigen in the protection of E. coli against serum killing.

In view of the above findings, there is still no clear picture regarding the relationship between K1 carriage and serum resistance. Bjorksten etal (1976) found no consistency in the way faecal, blood or cerebrospinal fluid isolates of E. coli K1 reacted in serum bactericidal systems. No

significant relationship could be found between the amounts of acidic polysaccharide produced by urinary and faecal K1 strains, and their reactivity in human serum (Olling, 1977).

There appears to be evidence both for and against a major role for acidic polysaccharides as mediators of serum resistance. These polymers form a distinctly heterogeneous group of bacterial products with regard to chemical composition, antigenicity, molecular size, biophysical properties, and the intimacy of their association with the cell surface (Taylor, 1983). These factors are likely to distort any such complex relationship as that between serum resistance and the interaction of the bacterial surface with activated complement components unless steps are taken to minimise these effects (Taylor, 1983). Any increase in serum resistance directly attributable to the presence of acidic polysaccharides cannot form part of a general mechanism for resistance of Gram-negative bacteria, as many groups, such as Salmonella species, possess a high level of intrinsic serum resistance and yet do not produce acidic polysaccharides (Nelson and Roantree, 1967 ; Dlabac, 1968 ; Myers etal, 1980).

(3.6.3) Outer membrane proteins (OMPs)

Lambden etal (1979) examining a series of opacity variants of N. gonorrhoeae P9, the causal agent of disseminated gonococcal infection, reported changes in surface proteins which may have been of importance in serum resistance. They observed that production of OMP in addition to protein I conferred on the variants an increased resistance to serum killing. Despite the gonococcal resistance to serum killing being multifactorial, the most resistant variants were distinguished from the others in that they each produced protein IIa, of molecular weight 28.5kD. McBride etal (1981) also working with gonococcal strain P9, found the opaque colonial types produced one or more of a range of additional OMPs (II ; IIa ; IIb ; IIc ; and IId), in the molecular weight range 27.5kD to 29.0kD. They demonstrated that survival of gonococci inoculated into guinea-pig subcutaneous chambers were variants which produced opacity related proteins (IId ; IIe ; and IIf) not present in the initial inoculum, thus suggesting that OMP composition was an important survival factor in vivo. Studies carried out by De Hormaeche etal (1983) suggested that change in the LPS molecular configuration of encapsulated in vitro grown gonococcal cells, may have rendered the cells resistant to

bactericidal antibodies.

A most recent study of disseminating gonococci was carried out by Patel etal (1988). Serum-susceptible *N. gonorrhoeae* were induced to resistance to complement-mediated killing by fresh human serum by incubation in guinea-pig blood for three hours at 37⁰C. Since induction did not occur in the presence of bacteriostatic concentrations of chloramphenicol or neomycin, it was deduced that proteins mediated the acquisition of resistance. Only two qualitative differences, both in minor components, were shown between serum-resistant and serum-susceptible organisms. These were a protein A of about 205kD in the serum-resistant organisms and a protein B of about 16kD in the serum-susceptible organisms. They also indicated that many proteins were present in larger amounts in the serum-resistant organisms, including the three principle OMPs. However, when immunoblotting was carried out, no marked differences between the profiles for serum-resistant and serum-susceptible organisms were detected, irrespective of whether the immunoblotting was carried out with anti-serum-resistant or anti-serum-susceptible sera. When anti-serum-resistant serum was adsorbed with serum-susceptible organisms, and then used in immunoblotting, there were no reactions with any of the

antigens that had immunoblotted in the profile of serum-resistant organisms with unadsorbed antiserum. As expected, no reactions were obtained when the adsorbed anti-serum-resistant serum was reacted with the serum-susceptible profile, thereby confirming the lack of major changes in proteins when resistance was acquired.

Thus, one major question remained unanswered. Did an LPS-OMP interaction play a role in the acquisition of serum resistance?

Taylor and Parton (1977) observed that a serum-resistant mutant derived from a smooth delayed-susceptible urinary isolate of E. coli, produced more envelope protein of molecular weight 46kD than the parent strain. The amount of this protein was subject to variation as a result of alterations in growth conditions and was well correlated with environmentally induced modification of the bacterial response to serum (Taylor etal, 1981). The authors suggested that the protein factor was involved in the determination of serum resistance, but that it was only functional when superimposed on a full complement of LPS O-side chains.

OMPs have been implicated to play a role in the resistance of

Aeromonas salmonicida (Munn etal, 1982), Campylobacter fetus (Blaser etal, 1987) and smooth E. coli mutants (Taylor and Parton, 1977) to the bactericidal action of serum.

(3.6.3.1) Determinants of serum resistance in the outer membrane

There is no evidence suggesting that resistant Gram-negative bacteria circumvent the bactericidal action of serum by producing proteases or other extracellular products that neutralise or destroy the functional integrity of complement components in the fluid phase (Joiner etal, 1982(a)). It appears that the outer membrane constitutes the main barrier to the serum killing of Gram-negative bacteria (Taylor, 1983). Serum resistance does not result from a block in the activation of the complement cascade. Rather, MACs formed on the surface of serum-resistant strains are not effectively inserted into bacterial membranes and are released without causing lethal damage (Joiner etal, 1982(a) ; 1982(b)).

Studies by Joiner etal (1982(a) ; 1982(b)) using the smooth Salmonella minnesota strain S218 and the deep rough mutant (Re595) of S218, demonstrated that the serum-susceptible Re595 strain consumed relatively small amounts of C5, C7, and C9, with the components being efficiently and stably

deposited on the bacterial surface. Much larger amounts of the terminal components were deposited onto S218 cells. Binding reached a peak after about ten minutes, followed by a progressive loss of the bound components with prolonged incubation. The likely mechanism of serum resistance of S. minnesota S218 was failure of the MAC to integrate into the outer membrane. Furthermore, MACs could be eluted from the surfaces of S218 cells, but not Re595 cells, indicating that MACs on the surface of the serum -resistant strain did not behave as integral membrane proteins (Joiner etal, 1982(b)).

The fluidity of artificial and biological membranes is intimately related to the ability of MACs to integrate into these structures. Consequently, factors reducing the fluidity of either the outer or cytoplasmic membranes of Gram-negative bacteria could contribute to their serum resistance. Increased membrane fluidity might enable the more efficient assembly and integration of MACs on phospholipid membranes (Mayer, 1981). Thus, those macromolecular components of the outer membrane that are thought to confer serum resistance may do so by virtue of the fact that they reduce membrane fluidity and prevent integration of MACs (Taylor, 1983).

(3.6.4) Plasmid determined factors

The earliest report of an altered serum response after the acquisition of foreign DNA is that of Muschel etal (1966, 1968), who found increased serum resistance after lysogenisation of E. coli K12 with lambda phage.

Williams Smith (1974) noted that E. coli strains invasive for humans and domestic animals harboured col V plasmids which, when transferred to K12 and other E. coli strains, enabled the host bacteria to survive in blood and serum. They do not, however, play an important role in determining the serum susceptibility of E. coli populations in urinary tract infections (Hughes etal, 1982). Reynard and Beck (1976) and Hughes etal (1982) reported that the plasmids R1 and R100 (NR1), were able to confer relatively high degrees of resistance to rabbit serum on a number of E. coli K12 strains. This ability appeared to be restricted to F-like plasmids. Reynard and co-workers reported, however, that plasmid R1 did not provide K12 strains with protection against human serum. Subsequent work on the related plasmid R6-5 demonstrated resistance was determined by the tra T gene (Moll etal, 1980). A further factor contributing to E. coli serum resistance was encoded by the iss gene of the col

V.I-K94 plasmid (Binns etal, 1979).

Much of this confused situation was clarified by Ogata and Levine (1980), who found that differences in the serum reactivity of pairs of K12 strains with and without R100 could be detected provided a high bacterial cell density and relatively low cell concentrations of rabbit serum were used. Thus, what is being measured is a reduction in the rate of killing, manifested under certain experimental conditions. One could imagine that insertion of novel plasmid-encoded proteins with unrelated cell functions into the outer membrane might fortuitously reduce membrane fluidity and hence the rate at which MACs are inserted into the membrane, especially when concentrations of serum are limiting (Taylor, 1983).

Binns etal (1979) suggested that more readily detectable effects of plasmids on bacterial survival in serum could be achieved if strains with a certain intrinsic level of resistance were used as hosts. In addition, the LPS O side chains, the surface components responsible for the delay in serum killing, were considered essential for expression of plasmid factors that modify susceptibility to serum, at least in systems containing adequate concentrations of fresh human

serum (Taylor and Hughes, 1978).

(3.7) Determination of Serum Bactericidal Activity

In its most simple form, determination of serum bactericidal activity involves exposure of a suspension of viable organisms to a suitable concentration of antibody and complement, incubation at the optimum temperature for complement activity, and measurement, after suitable periods of time, of the absolute concentration of surviving organisms by some form of viable counting (Taylor, 1983). Due to the variation of assays performed, determination of a standard endpoint for the number of bacteria killed is difficult.

The majority of studies of serum bactericidal activity have used a number of viable counting procedures to determine bacterial survival rates after exposure to serum-buffer mixtures. It has been common practice to select only one exposure time, usually in the one-to two-hour range, at which to evaluate survival. The reliability of this approach is questionable as it has been frequently observed that killing of some strains does not proceed at a constant rate. The true nature of the interaction between bacterial cells and activated complement components can probably only be

evaluated when the number of bacterial survivors is estimated at frequent time intervals during the reaction (Taylor, 1974 ; Taylor, 1983).

Although measurement of the serum activity in the presence of a large excess of serum components would appear to be the most direct way of determining the degree of serum killing, some workers have preferred to estimate the survival of bacteria over a range of serum concentrations. Bactericidal action would be expressed as the reciprocal of the highest serum dilution showing killing activity (Rowley and Wardlaw, 1958 ; Tachibana, 1965 ; Schwab and Reeves, 1966 ; Tramont etal, 1974 ; Mittal and Ingram, 1975).

An alternative assay based on the ability of surviving bacteria to remultiply when removed from the action of antibody and complement, was developed by Muschel and Treffers (1956). This assay involved exposure of bacteria to suitable serum concentrations for a reaction period of one hour, followed by the addition of a standardised volume of growth medium, such as brain infusion broth. The anticomplementary activity of the medium terminates the bactericidal action of the serum, and the density of the cultures after a second incubation period is proportional to

the number of organisms surviving the initial exposure to serum. The optical densities obtained are converted to percentages of the control values and plotted on a probit scale against the log of the serum volume. The resulting linear relationship enables the 50% lethal dose of serum to be determined. The limitation of this assay is that it is only valid when antibody concentration is varied against a background of constant complement concentration, or vice versa (Taylor, 1983). It also contains the assumption that surviving bacteria that have been exposed to potentially damaging amounts of complement will multiply at an identical rate to bacteria that have not been so exposed after transfer to nutrient media. Michael and Braun (1964) compared a photometric assay with the conventional direct plating method, and consistently found a greater number of survivors with the latter procedure. This suggested a continuation of complement-mediated bactericidal or bacteriostatic activity after addition of broth.

The need to screen large numbers of bacterial cultures or clones has led to the development of a number of rapid assays for determining serum bactericidal activity. An example of this approach is the plaque assay (Nakano etal, 1968 ; Fierer etal, 1972 ; Silver etal, 1981), which involves spotting

serum dilutions onto the surface of seeded agar plates. This method is difficult to control, and some batches of agar possess high anticomplementary activity.

Techniques that do not rely on determination of cell viability have also been used to quantify serum bactericidal activity. Kato and Bito (1978) studied irreversible loss of protein (beta-galactosidase) synthesis as an index of bacterial killing, because serum treated cells may be active in both respiration and macromolecular synthesis but inactive in cell division. Fierer et al (1974) measured the release of ^{51}Cr -labelled LPS from serum-susceptible and serum-resistant bacteria and found a correlation between serum susceptibility and release of label. However, even heat-inactivated serum released approximately 20% of ^{51}Cr from serum-susceptible E. coli cells.

In spite of a number of parameters having been defined that significantly influence the outcome of the serum bactericidal reaction, it is surprising that no definitive standards exist for tests of bactericidal activity. As a result, no single technique has emerged as a readily acceptable and widely applicable reference assay for estimation of serum activity.

CHAPTER FOUR

GENERAL MATERIALS AND METHODS

(4.1) Bacterial Strains

(4.1.1) Clinical isolates

Clinical isolates of H. ducreyi used in this study were designated Y206, D29, FQ61, FQ33, FQ24, Y137, Y56, and Y155, and were isolated from migrant mineworkers attending a sexually transmitted disease clinic at the Leslie Williams Memorial Hospital in Carletonville, South Africa. Following a clinical examination, material from the base of the target ulcer was collected with a calcium alginate swab, and inoculated directly onto solid media. The two types of media used for primary isolation of H. ducreyi were Mueller-Hinton base enriched with heated sterile horse blood, IsoVitaleX and vancomycin (Bilgeri, etal, 1982), and gonococcal agar base containing bovine haemoglobin, foetal calf serum (FCS), IsoVitaleX and vancomycin (Nsanze etal, 1981). Culture media were immediately placed into moist candle extinction jars prior to them being transported to the

laboratory. Bacterial growth was documented after forty-eight hours incubation at 36⁰C in 5 to 10% carbon dioxide. Typical H. ducreyi colonies that could be pushed intact across the surface of the agar were identified according to the criteria of Kilian (1976).

In addition, four other clinical Haemophilus species namely, H. parainfluenzae, H. aphrophilus, H. influenzae, and H. aegyptius. isolated in the greater Johannesburg region were used.

(4.1.2) Control strains

A number of serum-sensitive reference strains of H. ducreyi were included as controls. These comprised strains ATCC 27722 (obtained from the American Type Culture Collection), A75, A76, and A77 (obtained from the Pasteur Institute, Paris).

(4.2) Growth Conditions

(4.2.1) Media

All the H. ducreyi strains used were grown on gonococcal (GC) agar base (Gibco) supplemented with 1% haemoglobin (Difco), 1% IsoVitaleX (BBL, Microbiological), 5% FCS (Highveld Biological) and 3mg/L vancomycin (Lilly). This medium is

referred to as Nsanze agar medium (Nsanze etal, 1981 ; APPENDIX 1).

The four additional Haemophilus species used were grown on Columbia agar base enriched with citrated horse blood (APPENDIX 2).

(4.2.2) Incubation

All H.ducreyi strains were incubated at 36⁰C under 5% carbon dioxide and high humidity for forty-eight to seventy - two hours. Experiments were performed with low-passaged, clinical H. ducreyi strains, namely FQ24 ; FQ33 ; FQ61 and Y206, and high-passaged clinical strains, namely D29, Y137, Y56 and Y155.

The most suitable diluent used for all experimental manipulations was brain heart infusion (BHI) broth (APPENDIX 3).

(4.2.3) Storage

H. ducreyi strains were initially stored in 20% skim milk containing 20% glycerol, but this was subsequently replaced by BHI broth containing 25% defibrinated rabbit blood. Storage was at minus 70⁰C.

(4.3) Reconstitution of H. ducreyi Reference Strains A75, A76 and A77

H. ducreyi strains A75, A76 and A77 were initially received in a lyophilised state from the Pasteur Institute in Paris, France.

The lyophilised cultures were reconstituted using the following media :

- (1) GC agar base (Gibco), 1% IsoVitaleX (BBL, Microbiological), 1% haemoglobin (Difco), 5% FCS (Highveld Biological), and 3mg/L vancomycin (Lilly).
- (2) GC agar base, 1% IsoVitaleX, 1% haemoglobin, 1% haemin (Sigma), and 5% FCS.
- (3) GC agar base, 1% IsoVitaleX, 1% haemoglobin, 5% heat - inactivated (heating for thirty minutes at 56⁰C) FCS, and 3mg/L vancomycin.
- (4) GC agar base, 1% IsoVitaleX, 1% haemin, and 5% FCS.
- (5) Mueller-Hinton agar base (BBL, Microbiological), 10% citrated horse blood, 1% IsoVitaleX, and 3mg/L vancomycin.
- (6) BHI agar base (Difco), 1% IsoVitaleX, 1% haemin, 5%

FCS and 3mg/L vancomycin.

- (7) BHI agar base, 1% IsoVitaleX, 1% haemin, and 3mg/L vancomycin.
- (8) BHI agar base, 1% IsoVitaleX, 1% haemin, and 5% FCS.
- (9) BHI broth (Difco), 1% IsoVitaleX, and 0.5% haemin.

Failure of the lyophilised strains to grow on the above media necessitated a duplicate set of reference strains being sent from the Pasteur Institute on blood agar slopes. The following media were used to propagate the strains :

- (1) GC agar base, 1% IsoVitaleX, 1% haemoglobin, 5% FCS, and 3mg/L vancomycin (Nsanze agar medium).
- (2) GC agar base, 1% IsoVitaleX, 1% haemoglobin, 5% inactivated FCS, and 3mg/L vancomycin.
- (3) GC agar base, 1% CVA enrichment (Gibco), 1% haemoglobin, 5% FCS, and 3mg/L vancomycin.
- (4) BHI agar base, 15% horse blood.
- (5) BHI agar base, 25% rabbit blood.
- (6) Chocolated agar.
- (7) BHI broth, 1% IsoVitaleX, and 0.5% haemin.

All of the above media had been inoculated with H. ducreyi strain ATCC 27722 prior to this in order to determine their

efficiency in supporting bacterial growth of H. ducreyi. Medium number (5) above supported growth of the lyophilised strains most successfully, but the high cost involved in preparation of this formula prevented its continued use. Media numbers (1) and (2) above were the alternative choices, with both supporting adequate growth of H. ducreyi reference strains. Nsanze's medium was preferred since it did not require prior inactivation of the FCS.

(4.4) Antibody Production in New Zealand White Rabbits

The outer membrane proteins of H. ducreyi reference strains A75, A76, and A77 were used as inoculum for antiserum production in New Zealand white rabbits. A 1ml volume of purified OMPs, at a concentration of 100 to 200ug/ml was added to 1ml of Freund's complete adjuvant (Sigma). When combined, there was a distinct zone of separation between protein and adjuvant which disappeared upon repeated passage of the suspension through a syringe needle. Further homogenisation was required by sonication on ice for five ten second bursts at a level of fifteen microns (SoniPrep, 150MSE). The resultant suspension was used for the initial vaccination. A suspension of OMPs in incomplete Freund's adjuvant was prepared by the same method and used for

subsequent inoculations.

The vaccination protocol consisted of five subcutaneous inoculations fourteen days apart and a booster inoculation three weeks later. At the time of each inoculation, three volumes of 0.5ml antigen suspension were injected subcutaneously at three different sites on each rabbit. Each vaccination was administered to two rabbits. A test bleed to ensure the absence of cross-reacting antibodies was performed prior to the initial inoculation. This procedure was repeated before each subsequent inoculation to confirm the development of antibody. Each blood sample was allowed to clot at room temperature followed by refrigeration at 4⁰C overnight. The blood was then centrifuged at 5000g for fifteen minutes following which the serum was decanted and stored at minus 70⁰C until use. Three weeks after the sixth and final inoculation, the rabbits were anaesthetised with ether and exsanguinated by cardiac puncture. Approximately 50ml of blood was collected from each rabbit, and the serum separated and stored until required.

The antibody titre of each bleed was determined using an indirect immunofluoresence assay. Serum from the first test

bleed served as a control.

(4.5) Indirect Immunofluorescence Assay of *H. ducreyi*

Approximately ten *H. ducreyi* colonies were removed from the surface of the culture medium for use as an antigen. The colonies were suspended in 2ml PBS (pH7.2 ; APPENDIX 8) which was repeatedly aspirated through a thin syringe and 0.45mm gauge needle (Microfine). The antigen suspension was washed three times in PBS and the pellet finally resuspended in 4ml PBS. Twenty ul of antigen was then spread over duplicate wells of a multitest microscope slide (Highveld Biological) that had previously been soaked in methanol for five minutes. The slide was air dried at room temperature, fixed for ten minutes in acetone, washed for ten minutes in PBS and five minutes in distilled water, and finally air dried. Forty microlitres of anti-A75,-A76 and-A77 rabbit serum, (APPENDIX 8), was then added to each pair of wells. Serum was tested neat and at doubling-dilutions in PBS up to a final dilution of 1:2560. Rabbit serum from the first test bleed was used as a negative control. Each slide was incubated for thirty minutes at 37⁰C in a humid chamber, washed for five minutes in PBS, and rinsed for five minutes in distilled water. Twenty microlitres of fluorescein-conjugated donkey

anti-rabbit immunoglobulin (Amersham International) diluted in rhodamine counterstain (Difco, APPENDIX 8) was then added to each well and the slide incubated in a humid chamber for a further thirty minutes. Thereafter the slide was washed for five minutes in PBS, and rinsed for five minutes in distilled water. A coverslip was then mounted on the slide with 10% glycerol (APPENDIX 8), examined under X400 magnification using ultraviolet illumination.

Positive results were graded from + to +++ according to the degree of fluorescence. The titre of rabbit antiserum was regarded as the reciprocal of the highest dilution at which a bright apple-green staining of the entire bacterial cell contour was retained (FIGURE 4.1).



FIGURE 4.1 Indirect immunofluorescence assay of H. ducreyi strain A77, X400 magnification. Rabbit antiserum diluted 1:640.

CHAPTER FIVE

SERUM BACTERICIDAL ACTIVITY OF H. DUCREYI(5.1) Introduction

It has long been recognised that normal human serum possesses bactericidal activity against a wide range of Gram-negative bacteria (Wedgewood, 1965 ; Johnson etal, 1984). A correlation between microbial virulence and resistance to the bactericidal action of human serum has also been observed for several Gram-negative organisms (Cannon etal, 1981). These include bacteremic strains of Escherichia coli (Aguero and Cabello, 1983), Gardnerella vaginalis (Boustouller and Johnson, 1986), Campylobacter fetus (Blaser etal, 1985), Pseudomonas aeruginosa and Neisseria gonorrhoeae (Roantree and Pappas, 1960 ; Young and Armstrong, 1972 ; Schoolnik etal, 1976), Bacteroides fragilis (Casciato etal, 1979), Salmonella minnesota (Joiner etal, 1982), Aeromonas salmonicida (Munn etal, 1982) and certain strains of Klebsiella pneumoniae (Benge, 1988).

Whereas bacterial strains isolated from the bloodstream are usually serum-resistant, isolates from stool, urine or mucosal surfaces are often sensitive (Cannon etal, 1981). Serum-sensitive E. coli strains have, however, occasionally been isolated from the blood of infected individuals (Elgefors and Olling, 1978). Serum-resistant Gram-negative strains are frequently isolated as causative agents of infections involving tissue damage (Taylor, 1983).

H. ducreyi, the aetiological agent of chancroid, does not invade the bloodstream, but resistance to lysis by normal human serum may allow prolonged multiplication of the organism within tissues. This process is probably as reflected in a positive rabbit intradermal test which is one of the criteria for assessing virulence of H. ducreyi (Odumeru etal, 1984).

The bactericidal action of serum complement proteins is an important component of the host defence against infection with Gram-negative bacteria (Wright and Levine, 1981(a)). Complement activation by Gram-negative bacteria can occur via the classical or alternative pathway. The classical pathway can be activated by the interaction of antibody with bacterial surface antigens, or directly by the lipid A moiety

of LPS (Gotz and Muller-Eberhard, 1971 ; Morrison and Kline, 1977). The killing of Gram-negative bacteria by serum requires the participation of terminal components (C5 to C9) of the complement system (Joiner etal, 1982). In a study carried out by Odumeru etal (1985), it was ascertained that the classical complement pathway was involved in the serum killing of serum-sensitive H. ducreyi strains. These isolates were designated as avirulent due to their negative reaction in the rabbit intradermal model.

In the present study, six clinical H. ducreyi strains isolated in South Africa and four reference H. ducreyi strains (Pasteur Institute, France) were investigated for serum susceptibility. An attempt was made to induce reference strains A75, A76 and A77 to serum resistance by continued in vitro passage in PNHS. The results are discussed in relation to the accuracy of serum bactericidal activity determination.

(5.2) Materials and Methods

(5.2.1) Bacterial Strains and Media

H. ducreyi reference strains A75, A76 and A77 were obtained from the Pasteur Institute, Paris. H. ducreyi reference strain ATCC 27722 was obtained from the American Type Culture Collection. Clinical H. ducreyi isolates Y206, D29, FQ61,

FQ33, Y137, Y56, Y155 and FQ24 were isolated from patients with chancroid at the Leslie Williams Memorial Hospital in Carletonville, South Africa. These strains were supplied by Mr. Y. Dangor, SAIMR.

An attempt was made to induce H. ducreyi reference strain A77 to serum resistance. The resultant strain is denoted as A77*. H. ducreyi strains were grown on Nsanze agar medium (APPENDIX 1) and incubated in carbon dioxide candle extinction jars under high humidity at 35⁰C. The diluent used in subsequent experiments was BHI broth.

(5.2.2) Pooled Normal Human Serum (PNHS) Preparation

Approximately 50ml of blood was obtained by venepuncture from six to eight healthy individuals who had no previous history of H. ducreyi infection. The blood was allowed to clot, refrigerated overnight at 4⁰C, centrifuged at 2000g for ten minutes, and the supernatant serum aseptically removed. Sera were pooled, filter sterilised, dispensed into 1ml aliquots, and stored at minus 70⁰C until required. Heat-inactivated PNHS was prepared by heating aliquotes of pooled sera at 56⁰C for thirty minutes. Inactivated samples were stored in the same manner as mentioned above.

(5.2.3) Serum Bactericidal Assay (SBA)

Colonies of H. ducreyi growing on Nsanze agar for thirty-six hours were resuspended in 2ml of BHI broth and passed repeatedly through a 2ml syringe with a 0.45mm gauge needle (Microfine), until a visibly turbid suspension was obtained. One hundred microlitres of the above suspension was inoculated into 2ml BHI broth supplemented with haemin and IsoVitaleX (APPENDIX 3), and incubated overnight. The broth culture usually appeared slightly granular. This was once again passed through a 2ml syringe with a 0.45mm gauge needle in order to produce a uniformly turbid suspension. A 100ul volume of this bacterial suspension was added to 500ul PNHS and 400ul diluent (BHI broth). The mixture was incubated at 35⁰C. At time intervals 0, 30, 60 and 120 minutes, 10ul was removed and serially diluted in BHI broth. Volumes of 10ul of each dilution were spotted onto duplicate Nsanze agar plates, and the viable counts determined according to the Miles and Misra (1938) method. Two growth controls were used, one consisting of bacteria in BHI broth, and the other of bacteria in BHI broth plus heat-inactivated PNHS. Each assay was performed three times. The survival of H. ducreyi following exposure to normal and heat-inactivated PNHS was expressed as a percentage of the initial inoculum. Serum resistance was defined as a <50% reduction of the original

bacterial inoculum within two hours incubation in 50% PNHS. The results were statistically analysed using analysis of variance and a Duncan t-test.

(5.2.4) Induction of Serum Resistance

Two different methods were used in an attempt to induce H. ducreyi reference strains A75, A76 and A77 to serum resistance.

In the first method, bacterial cells which survived exposure to 50% PNHS for sixty minutes, were inoculated into a 2ml volume of supplemented BHI broth. After incubation in a candle extinction jar at 35⁰C overnight, bacterial cells were homogenised using the needle and syringe method, and the bactericidal assay then carried out as mentioned previously. Bacteria surviving the longest following exposure to PNHS were included in subsequent tests. A total of ten successive SBAs were undertaken to select increasingly serum-resistant bacterial clones.

The second method involved the serial passage of bacterial colonies in increasing concentrations of PNHS. Bacterial cells which survived previous exposure to 50% PNHS for sixty minutes, were inoculated into duplicate 1ml volumes of

supplemented BHI broth containing a final concentration of 10% PNHS, homogenised with a needle and syringe, and incubated at 35⁰C for twenty-four hours. After two 50ul samples were removed and inoculated onto duplicate Nsanze agar plates to determine bacterial viability, two 200ul samples were inoculated into duplicate 1ml volumes of supplemented BHI broth containing a final concentration of 20% PNHS. These suspensions were then homogenised and incubated for twenty-four hours. This procedure was repeated until the PNHS concentration reached 60%, with the bacterial viability being tested at each percentage increase of PNHS. At this point, the degree of induced serum resistance of the bacterial strain was determined using the serum bactericidal assay.

(5.3) Results

(5.3.1) Serum Bactericidal Assay and Serum Resistance

The serum bactericidal assay for each bacterial strain was repeated three times, and the mean values expressed as a percentage survival rate using the following formula (Ross etal, 1985) :

$$\text{Percentage bacterial survival} = \frac{\text{CFU}_{t=120/60/30}}{\text{CFU}_{t=0}} \times 100$$

The mean percentage survival rates are depicted in FIGURES 5.1 to 5.11.

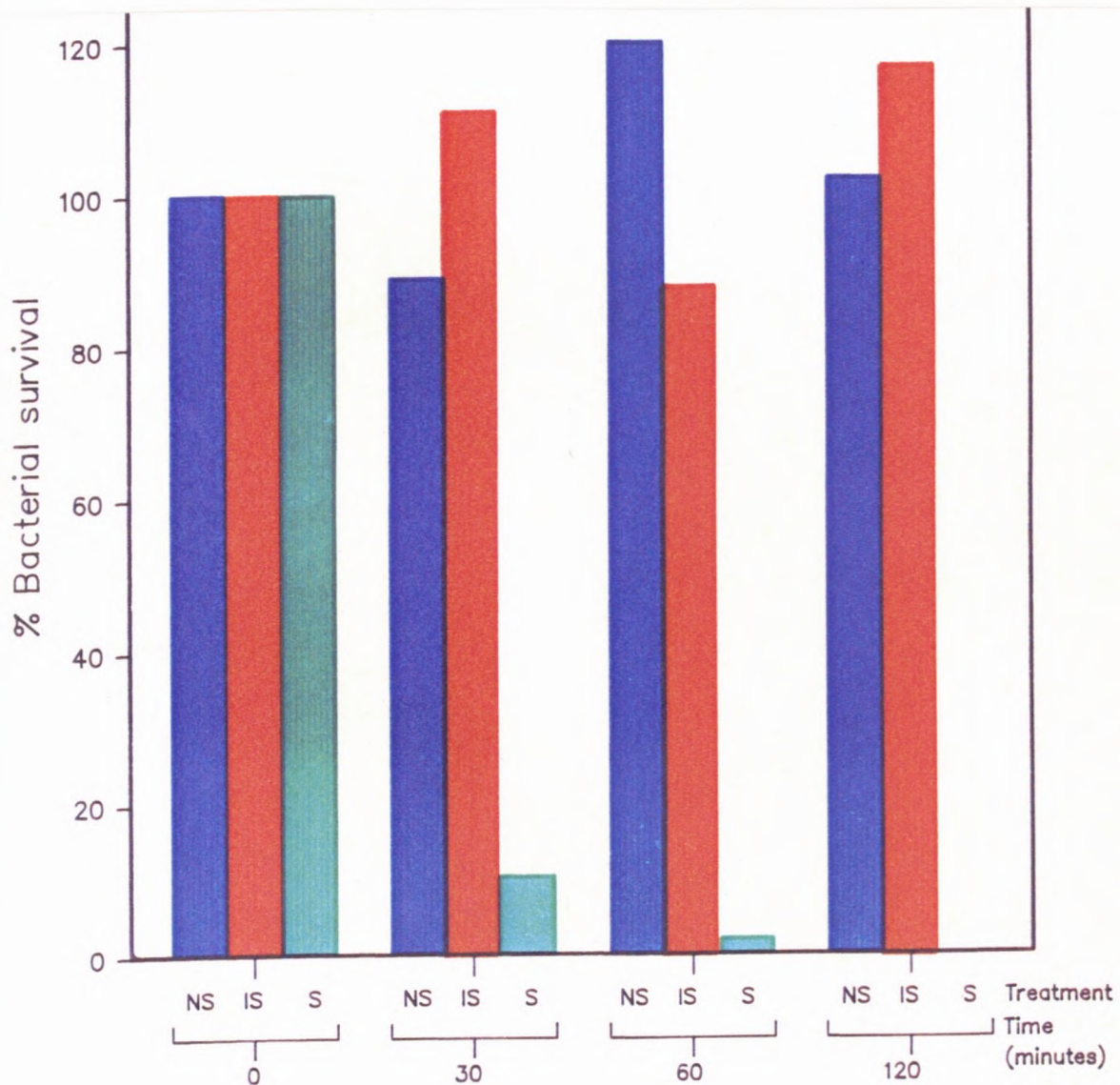


FIGURE 5.1 Bactericidal effect of 50% PNHS on *H. ducreyi* reference strain A75 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

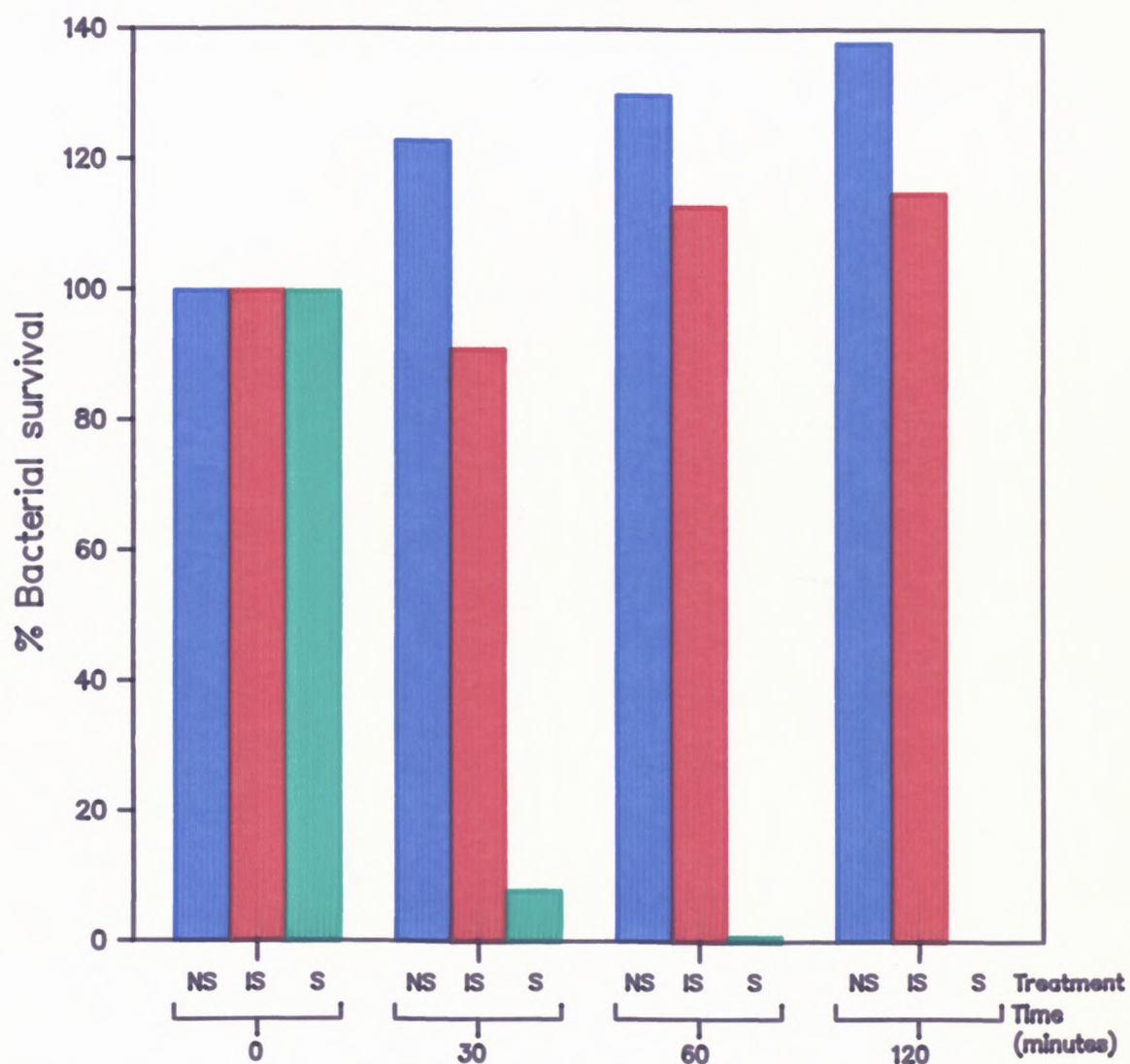


FIGURE 5.2 Bactericidal effect of 50% PNHS on *H. ducreyi* reference strain A76 at time intervals 0, 30, 60, and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

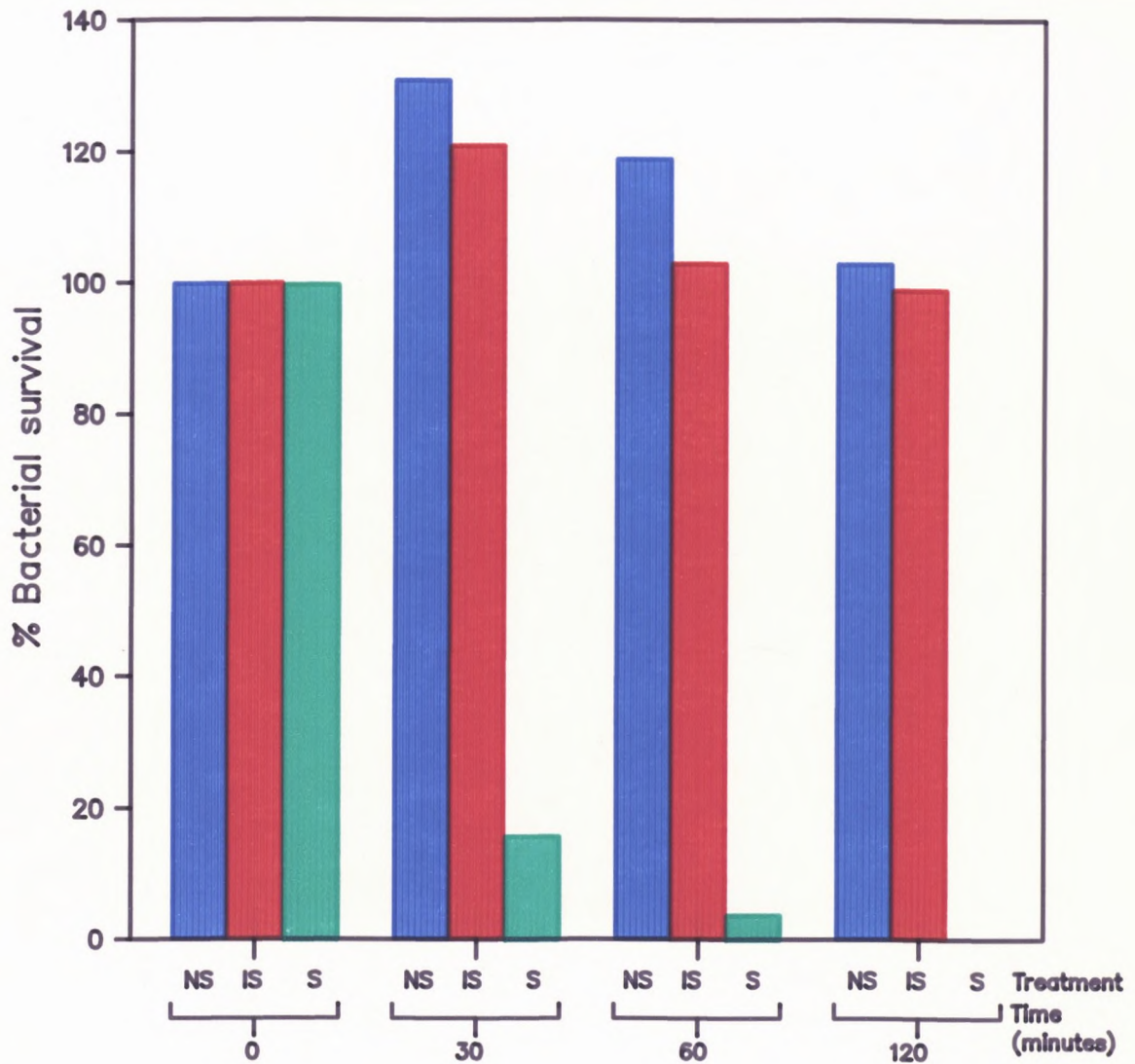


FIGURE 5.3 Bactericidal effect of 50% PNHS on *H. ducreyi* reference strain A77 at time intervals 0, 30, 60, and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

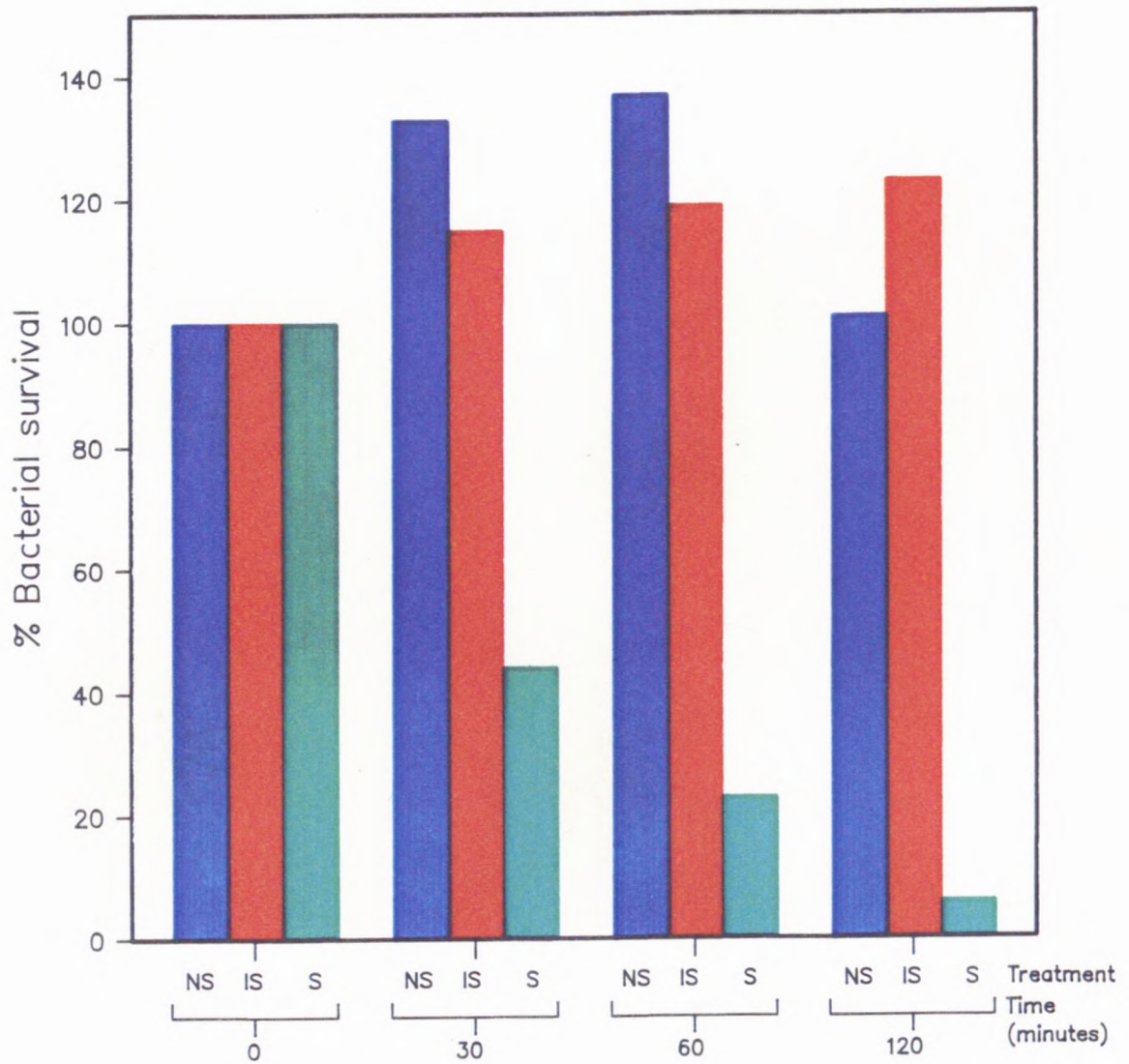


FIGURE 5.4 Bactericidal effect of 50% PNHS on *H. ducreyi* reference strain ATCC 27722 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

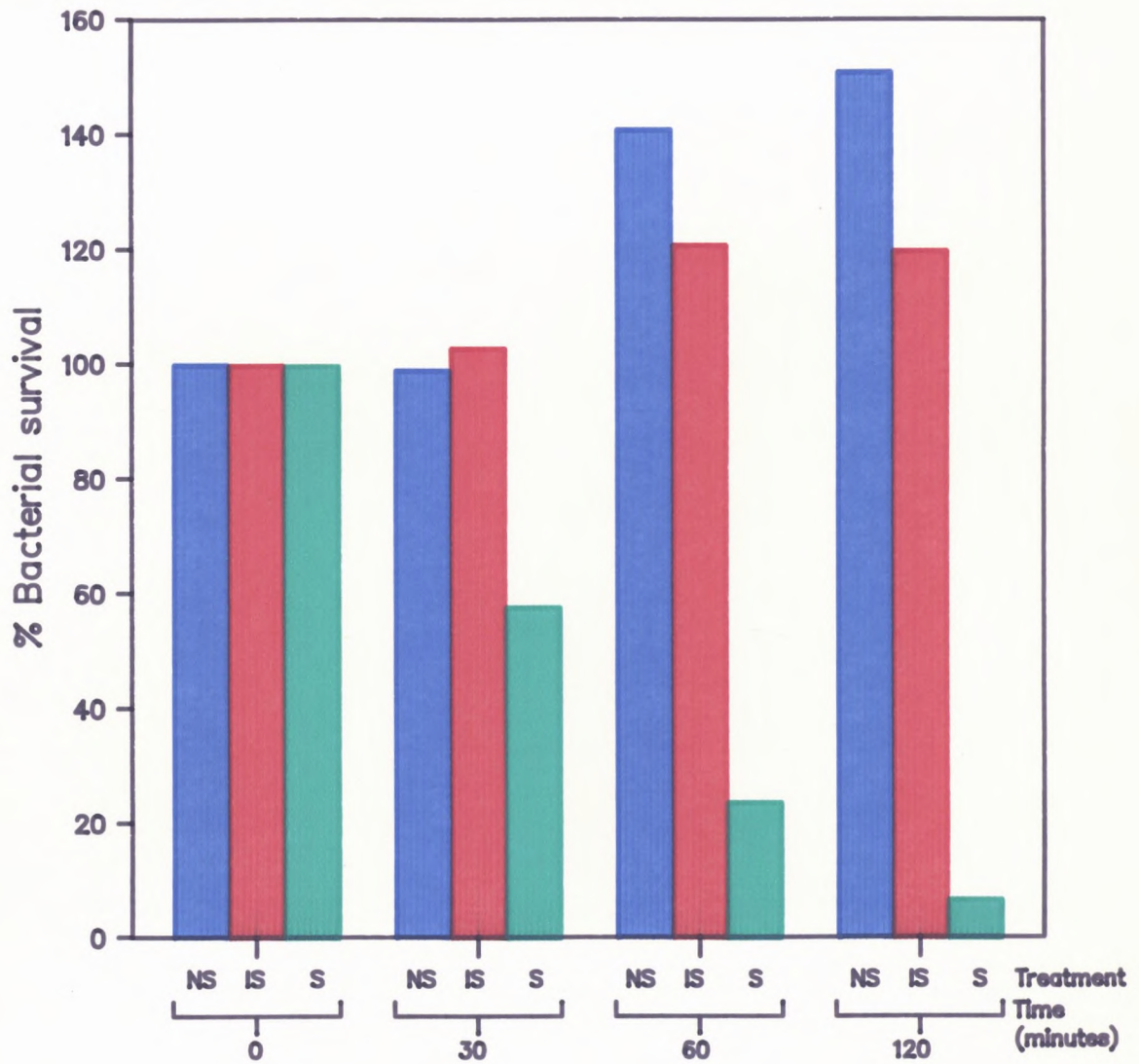


FIGURE 5.5 Bactericidal effect of 50% PNHS on *H. ducreyi* clinical strain D29 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

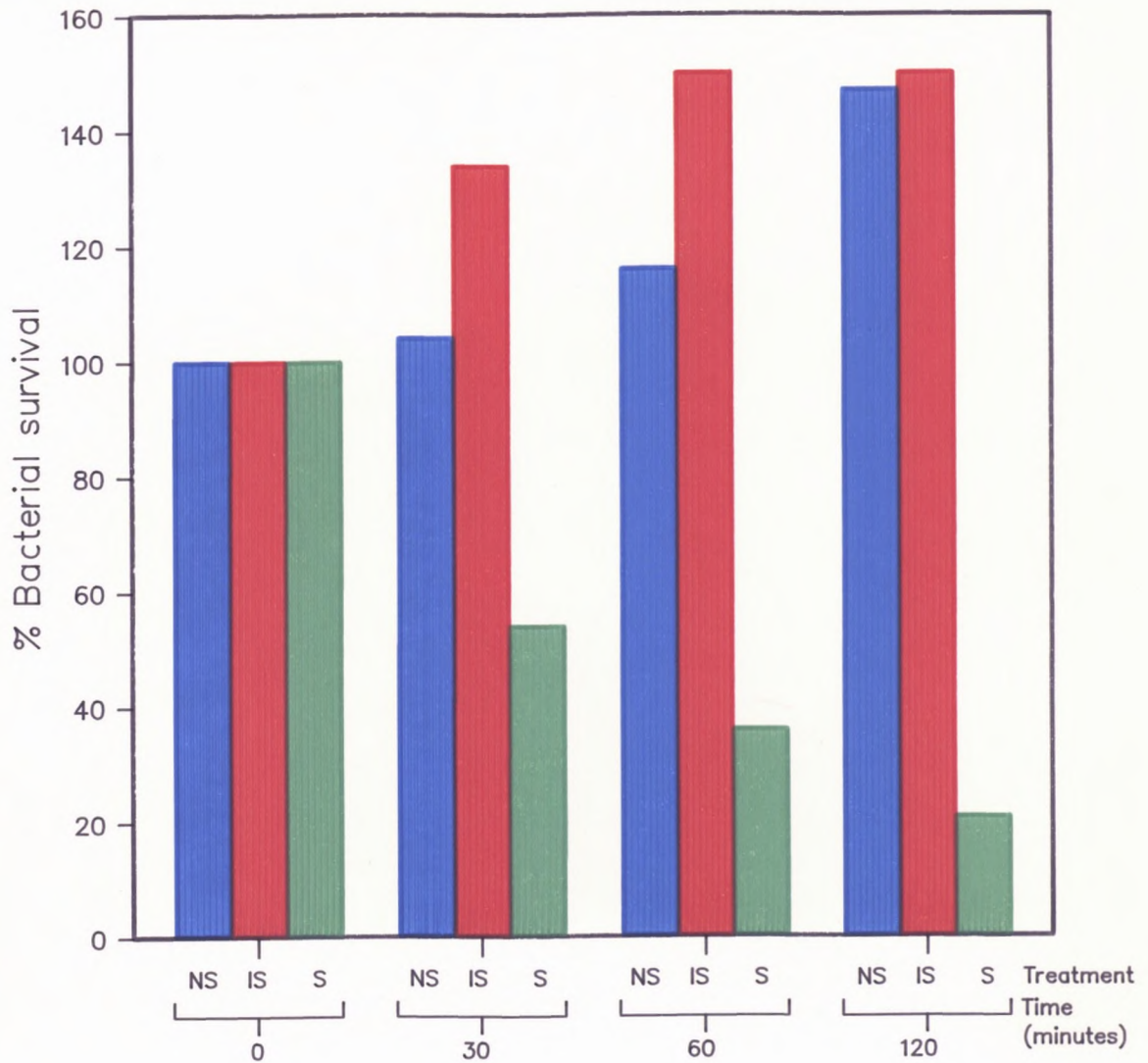


FIGURE 5.6 Bactericidal effect of 50% PNHS on *H. ducreyi* clinical strain Y56 at time intervals 0, 30, 60, and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

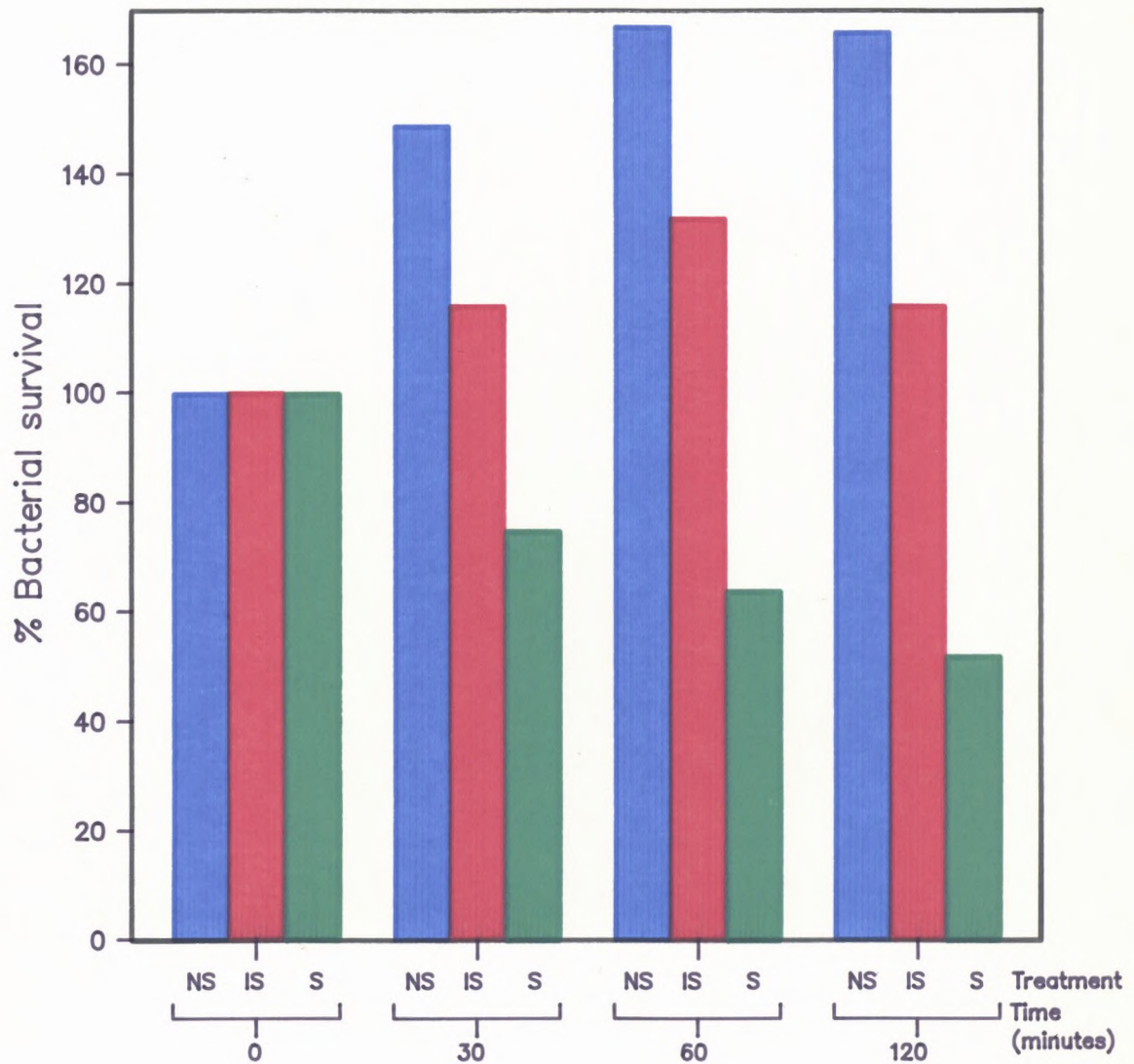


FIGURE 5.7 Bactericidal effect of 50% PNHS on *H. ducreyi* clinical strain Y206 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS)

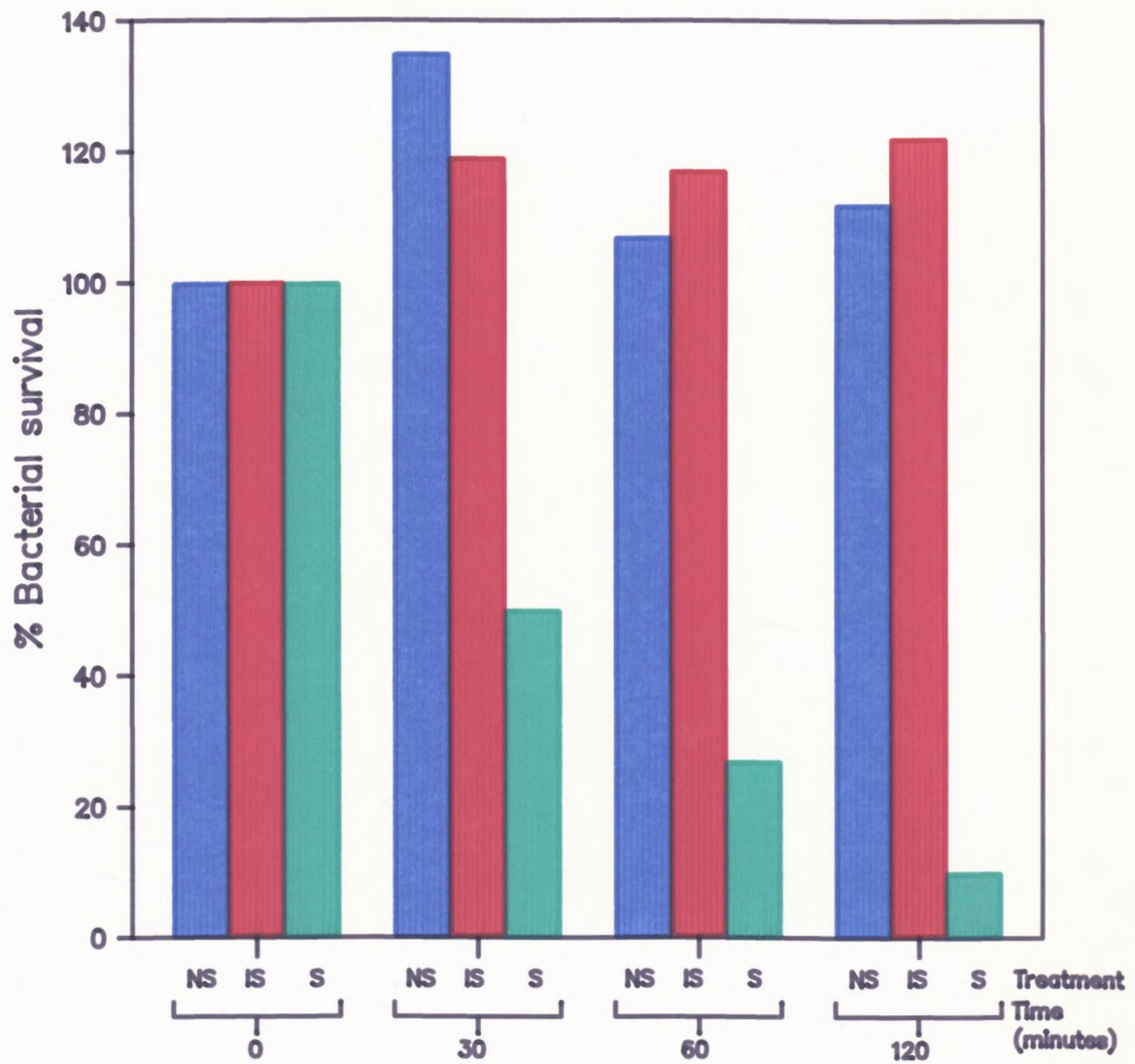


FIGURE 5.8 Bactericidal effect of 50% PNHS on *H. ducreyi* clinical strain Y137 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

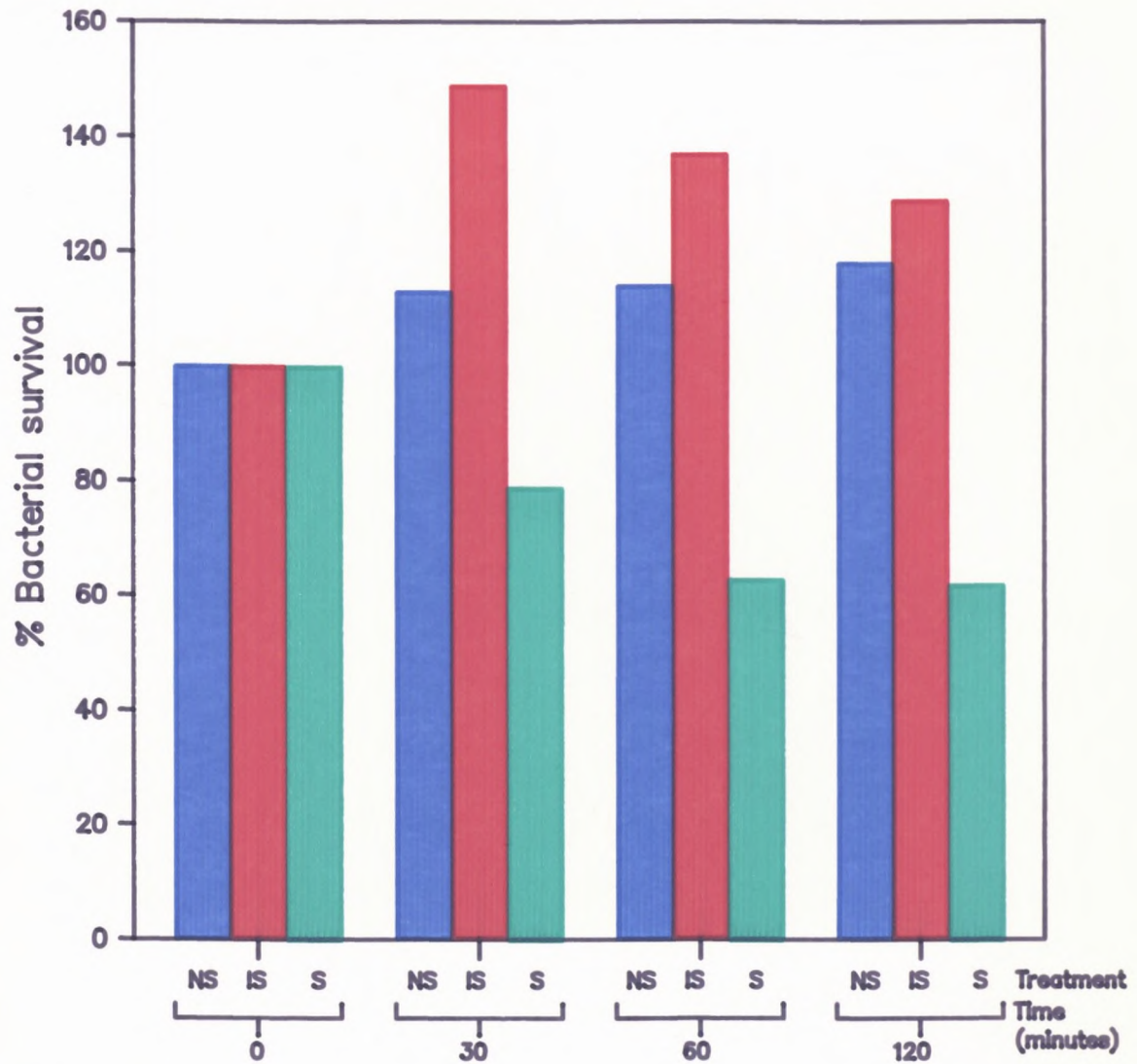


FIGURE 5.9 Bactericidal effect of 50% PNHS on *H. ducreyi* clinical strain FQ33 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

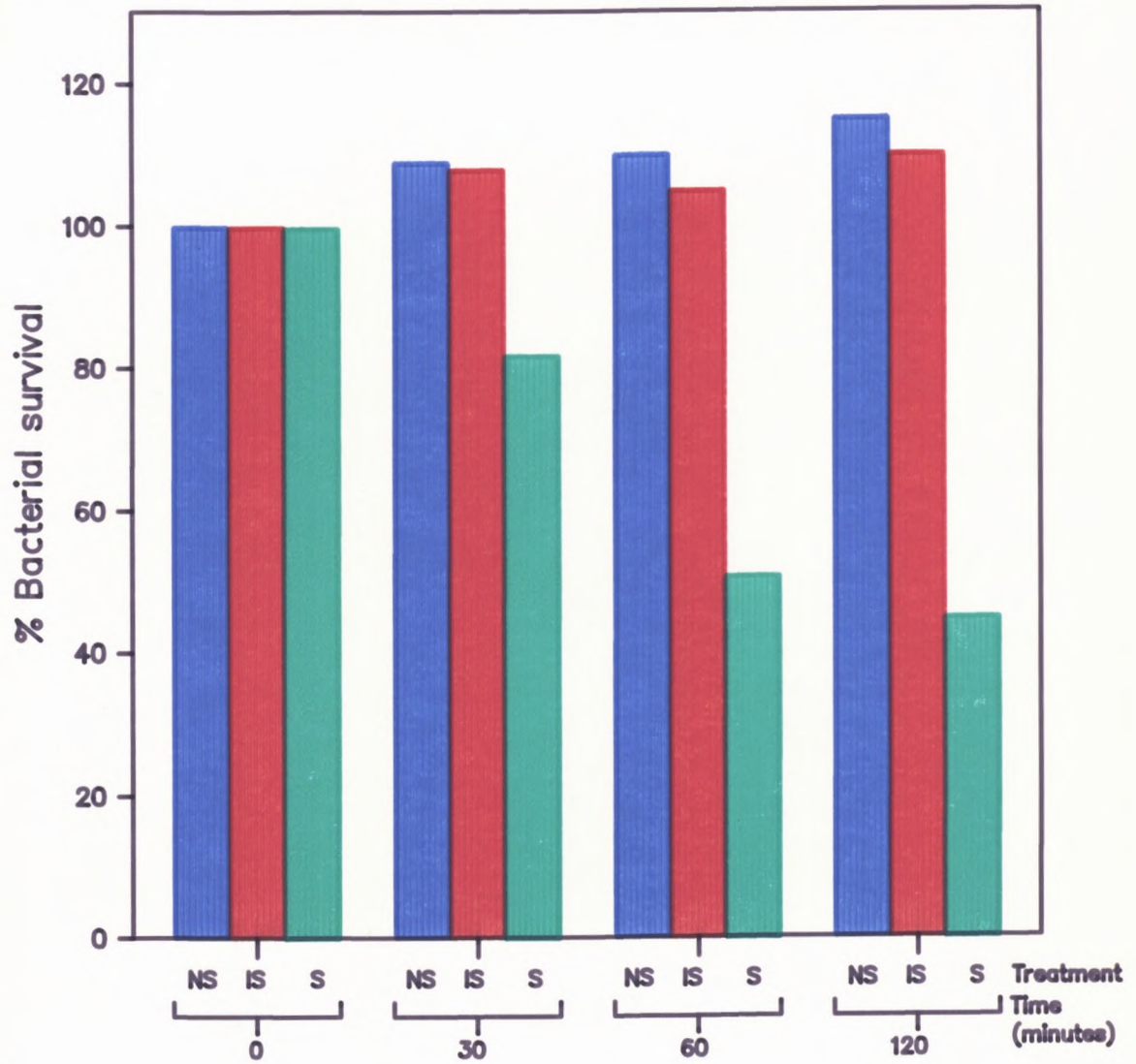


FIGURE 5.10 Bactericidal effect of 50% PNHS on *H. ducreyi* clinical strain FQ61 at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

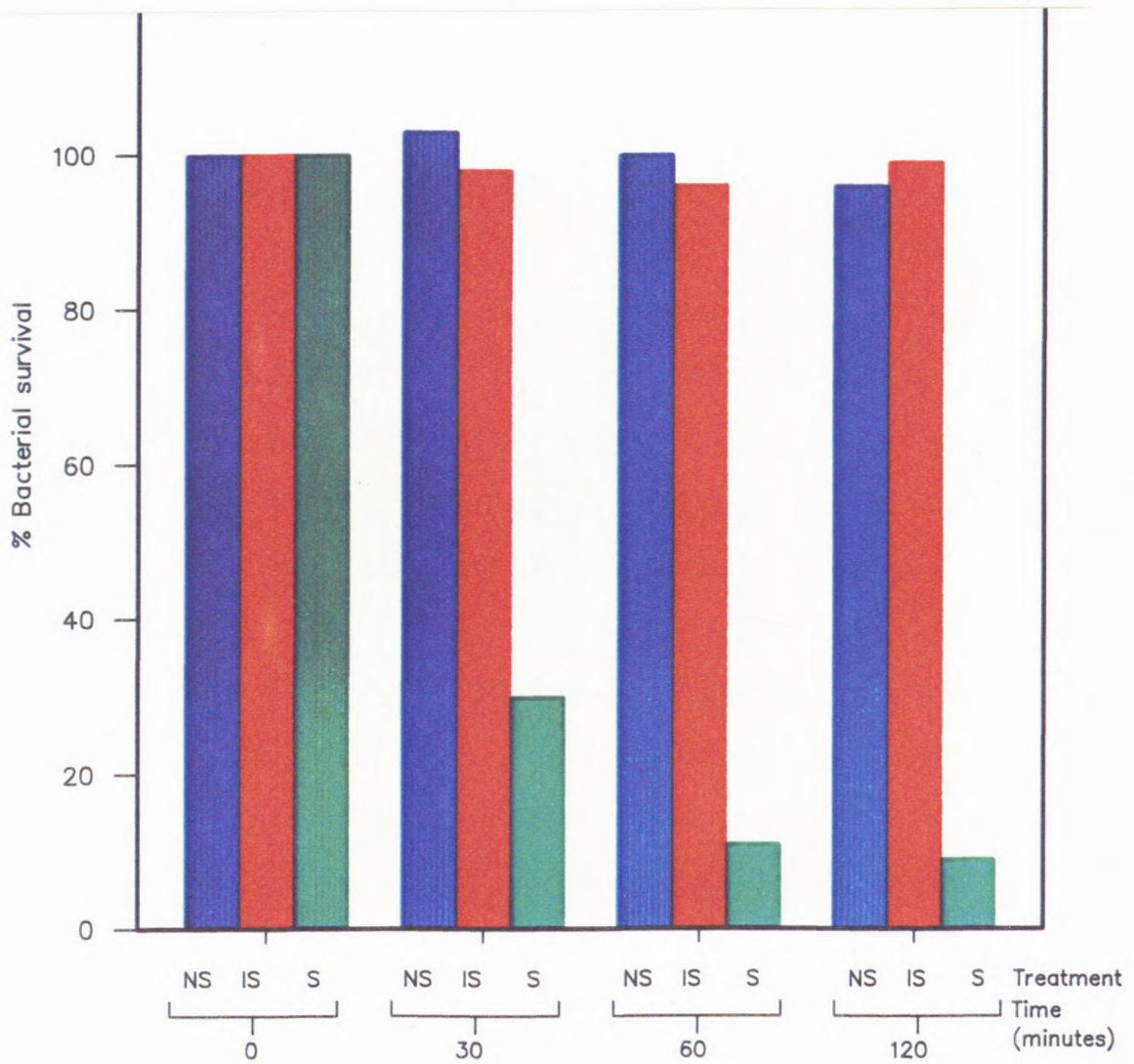


FIGURE 5.11 Bactericidal effect of 50% PNHS on *H. ducreyi* reference strain A77* at time intervals 0, 30, 60 and 120 minutes, expressed as a percentage survival of the initial bacterial inoculum at time zero (S). Controls included heat-inactivated PNHS (IS) and no PNHS (NS).

H. ducreyi reference strains A75, A76, A77 and ATCC 27722 (FIGURES 5.1 to 5.4) were extremely sensitive to 50% PNHS. Killing occurred rapidly, although strain ATCC 27722 survived somewhat longer.

Clinical strains were more serum-resistant than reference strains, especially strains FQ33, FQ61 and Y206 (FIGURES 5.5, 5.6 and 5.7, respectively ; $p < 0.05$; Duncan t-test). Clinical strains Y137, D29 and Y56 (FIGURES 5.8, 5.9 and 5.10 respectively) appeared to be more serum-sensitive.

Although strains FQ33, FQ61 and Y206 appeared serum-resistant by the criterion of percentage bacterial survival, killing continued following prolonged incubation in PNHS. The killing rate was, however, considerably less than that of strains Y137, D29 and Y56.

The two controls displayed active growth during the two hour incubation period. All strains exhibited active growth from time zero.

(5.3.2) Induction of Serum Resistance

Both methods employed to induce serum resistance of strains A75, A76 and A77 were unsuccessful. They proved to be

extremely cumbersome and time consuming. Following numerous passages survival rates remained less than ten percent at an incubation period of thirty minutes.

Strain A77* (passaged in increasing concentrations of PNHS) appeared to be slightly more serum-resistant, with an 11% survival at sixty minutes. However, following more prolonged exposure to PNHS, the survival rate decreased to below 10%.

(5.4) DISCUSSION

(5.4.1) The Serum Bactericidal Assay

There is no reference method for assessing the in vitro antibacterial activity of serum against Gram-negative bacteria. Many techniques are described in the literature that vary in important aspects such as the age of the culture used as the inoculum, the medium in which the inoculum is suspended, the proportion of serum used and the time interval over which activity is assessed (Taylor et al, 1972). These variations in technique make it impossible to accurately compare results obtained in different studies.

(5.4.1.1) Factors affecting the serum bactericidal assay

A number of factors have been shown to affect the in vitro

killing of human serum on bacterial cells :

(5.4.1.1.1) Growth phase of the inoculum

It has long been observed that the serum susceptibility of Gram-negative bacteria was influenced by the growth conditions employed for preparation of the bacterial inoculum. This may be due to environmental influences on the biosynthesis of cell-surface structures that affect the degree of serum susceptibility (Taylor, 1978), and to effects on the metabolic state of the target cell (Griffiths, 1974).

Cells in rapidly growing cultures (mid-logarithmic phase) are killed more effectively than cells in slowly growing cultures (stationary phase ; Tuomanen etal, 1986). The growth phase of the inoculum may thus have a marked effect on the results of bactericidal testing (Kim and Anthony, 1981). Davis and Wedgewood (1965) showed that resting E. coli organisms were resistant to the bactericidal action of serum over a twenty minute reaction period. Organisms in the rapid growth phase were more susceptible to serum, and those tested at different times during the growth cycle yielded variable results. Organisms just entering the logarithmic growth phase were more sensitive than organisms in the rapid growth phase. DeMatteo etal (1981) found that Pseudomonas aeruginosa

isolates grown in broth for preparation of inocula demonstrated significantly greater sensitivity to SBA than those grown on agar. Again the use of organisms in early logarithmic growth phase augmented the serum sensitivity of these isolates. In contrast, Blaser etal (1985) in a study of serum-sensitive and serum-resistant Campylobacter strains found bacterial growth phase to be of no significance when prolonged incubation times of eighteen to forty-two hours were used. This suggests that for many bacteria the initiation of cell division may uncover a cell wall site particularly sensitive to serum. Test organisms should therefore be used at a defined point in their growth cycle to obtain reproducible and reliable results (Davis and Wedgewood, 1965).

(5.4.1.1.2) Inoculum size

Inoculum size is recognised as the single most important variable in bacterial susceptibility testing (Pien and Vosti, 1974 ; Stratton, 1988). The inoculum size should be thought of as both the concentration of microorganisms per millilitre and the absolute number of microorganisms tested. The absolute number of organisms appears to be important in terms of the selection of resistant mutants. The recommended inoculum size is 5×10^5 CFU/ml (Stratton, 1988).

Interestingly, various authors suggest that inoculum size has little effect on the results of the serum bactericidal reaction. Taylor etal (1972) tested six isolates of E. coli at inocula of 10^5 , 10^6 and 10^7 CFU/ml, and in serum concentrations ranging from 8.3% to 75%, found no evidence of an inoculum effect. Likewise, Young and Armstrong (1972) tested isolates of P. aeruginosa at inocula of 10^3 , 10^5 and 10^6 CFU/ml in 10% and 50% serum without an observable effect on survival.

In the present study, H. ducreyi cells were grown on solid media for thirty-six hours and were then inoculated into supplemented BHI broth. After overnight incubation the cells were demonstrated to be in the early logarithmic growth phase (J. Jardine, unpublished results). The broth culture was repeatedly passed through a syringe and needle so as to reduce clumping of bacterial cells. This resulted in a fairly uniform inoculum size and generally consistent SBA results for all the H. ducreyi strains tested.

(5.4.1.1.3) Insufficient contact between organism and human serum

Adherence of viable microorganisms to the surface of containers above the meniscus can attribute to insufficient

contact between test microorganisms and serum resulting in fairly low rates of killing (Taylor etal, 1983). Because of the tendency of H. ducreyi cells to form clumps, it is possible that there could be diminished contact between individual bacterial cells and serum during the SBA. The repeated aspiration of the bacterial inoculum through a fine gauge needle presumably tended to minimised this problem. Performing SBAs using continuous shaking, might allow for better contact between bacterial cells and serum (Stratton, 1988).

(5.4.1.1.4) Choice of media

Culture media may have a profound effect on the results of antibiotic susceptibility testing and evaluation of serum activity (Peterson etal, 1978 ; Stratton, 1983). The concentration of protein, pH, phosphates, osmolarity, salts and divalent cations (Stratton, 1988) in broth used as diluent for the SBA may all affect the activity of PNHS.

Selection of a physiological diluent (Stratton and Reller, 1977) is thus critical for obtaining reliable results. The diluent must also support the viability of bacterial cells over the entire incubation period of the SBA. Although Mueller-Hinton broth is usually recommended for dilution

testing (Ericsson and Sherris, 1971), brain heart infusion broth was used as the diluent in this study. Odumeru etal (1984 ; 1985) used proteose peptone saline as a diluent in their investigation of the serum bactericidal activity against H. ducreyi.

(5.4.2) Serum Resistance

(5.4.2.1) Measurement of serum resistance

There is no generally accepted reference method for determining serum bactericidal activity. Taylor (1983) suggested that the description "serum-resistant" be restricted to bacterial strains that are totally refractory to inhibition by serum. These strains even show an increase in viable count when tested in early logarithmic phase in assays containing excess amounts of the components of the bactericidal system. "Serum-susceptible" strains were classified as those that displayed a significant reduction in viability, even after lengthy periods of incubation. Other studies have described "serum-resistant" organisms as those that are more refractory to serum than other strains within the same study (Binns etal, 1979). In a study by Taylor (1974) strains of E. coli not killed immediately on exposure to normal human serum were described as "delayed-serum-sensitive", as opposed to "promptly-serum-sensitive". Studies

of serum bactericidal activity have used a number of viable counting procedures to determine bacterial survival rates after exposure to serum-buffer mixtures. It is customary to estimate survival at a single point in time, usually in the one-to two-hour range. In the present study the end point for assessing the effect of serum on the test organism was defined as a <50% reduction of the original bacterial inoculum after two hours incubation in 50% PNHS.

Due to the clumping of H. ducreyi cells, the viable counts recorded at each step of the SBA was an inaccurate reflection of the number of bacteria that survived in the reaction mixture. This phenomenon also resulted in some difficulty in determining the bacterial inoculum size, which varied from 10^4 to 10^6 CFU/ml.

(5.4.2.2) Serum resistance and complement

The role of serum bactericidal activity as a system for host defence is determined by both host and microbial factors. Host factors include appropriate ("bactericidal") antibodies, which serve a recognition function, and complement, which serves as the effector system for the expression of serum bactericidal activity (Ross etal, 1985). The role of complement in this process has been reviewed in chapter

three.

The results of SBAs performed in the present study (FIGURES 5.1 to 5.11), demonstrate that the bactericidal activity of 50% PNHS against H. ducreyi is complement dependant. This observation was previously noted by Odumeru etal (1984).

The mechanism of complement activation by serum-susceptible H. ducreyi strains was also elucidated by these authors (Odumeru etal, 1985). Serum treated with Mg^{2+} -EGTA, which selectively inhibits the classical complement pathway, and serum treated with EDTA, which inhibits both the classical and alternative pathway, were nonbactericidal to serum-susceptible H. ducreyi strain A77. However, the organism was rapidly killed in untreated serum and in serum pretreated with inulin, or serum heated at 50°C for twenty minutes to deplete it of alternative pathway activity. These results suggest that the serum killing of H. ducreyi is mediated by the classical complement pathway. It is still not known whether serum-resistant H. ducreyi isolates prevent the membrane attack complex from incorporating into the bacterial membrane once bound, or whether these strains lack a surface-exposed receptor for firmly binding complement.

Results of serum absorption experiments with heat-killed H. ducreyi cells suggest that heat-stable components of the bacterial cell surface are involved in serum killing (Odumeru etal, 1985). Absorption of serum with whole cells of a serum-susceptible strain abolished the bactericidal effect of serum against the absorbing strain and other serum-susceptible strains. Absorption of serum with serum-resistant strains resulted in only a partial reduction in the bactericidal effect. (Odumeru etal, 1985).

In the present study it would have been interesting to preabsorb serum with a purified outer membrane protein preparation from serum-susceptible H. ducreyi reference strains prior to use in SBAs. This could have provided information on the potential role of OMPs in the serum-susceptibility of H. ducreyi.

(5.4.2.3) Serum resistance and bacterial virulence

The virulence of H. ducreyi has been studied by a number of investigators (Dienst, 1948 ; Feiner and Mortara, 1945 ; Hammond etal, 1978 ; Thayer etal, 1955). The single, generally accepted test of virulence is the rabbit intradermal test (Dienst, 1948 ; Feiner and Mortara, 1945). Dienst (1948) demonstrated that the majority of clinical

isolates of H. ducreyi produce a localised area of necrosis and eschar formation in rabbits and humans after intracutaneous inoculations with forty-eight hour old cultures. Strains exhibiting this property are regarded as virulent.

Odumeru etal (1984) further examined the basis for virulence in virulent, clinical and avirulent reference strains A75, A76 and A77. Virulent strains were found to be more resistant to the bactericidal action of normal human serum. These results are confirmed in the present study which demonstrates H. ducreyi reference strains A75, A76 and A77 to be extremely sensitive (FIGURES 5.1 to 5.3). Strain A76 appears to be the most serum-susceptible out of the above three reference strains.

H. ducreyi clinical strains FQ33, FQ61 and Y206 (FIGURES 5.5, 5.6 and 5.7, respectively) are resistant to the bactericidal action of 50% PNHS, whereas clinical strains Y137, D29 and Y56 (FIGURES 5.8, 5.9, and 5.10, respectively) are serum-susceptible. The reasons for this unexpected finding are unclear. One possibility is that repeated passage of these strains on artificial media could have resulted in the loss of a factor required for serum resistance (Ward etal, 1970).

Resistance of H. ducreyi to lysis by normal human serum may allow persistent multiplication of the organism within tissues thereby contributing to virulence (Odumeru etal, 1984). Clearly, a better definition of virulence is required for H. ducreyi. Until this is achieved, tests for other characteristics-which may in fact be epiphenomena-may continue to be paradoxical or non-reproducible.

(5.4.3) Induction of Serum Resistance

Both methods used to induce serum resistance in H. ducreyi reference strains A75, A76 and A77 were unsuccessful.

In the first method, H. ducreyi cells were repeatedly passaged through ten serum bactericidal assays.

In the second method, H. ducreyi cells were transferred from one broth suspension to the next thus exposing the organism to a stepwise increase in the concentration of PNHS.

Failure of the first method may have been partly due to the subculture of cells onto solid media lacking serum. This may have reduced the selection pressure on the bacterial cells, enabling serum-susceptible populations to re-emerge from each passage. In addition, the exposure of the bacterial cells to

PNHS for two hours may not have been sufficient to facilitate spontaneous mutation to serum resistance.

The second method was based on work reported by Schiller etal (1984). These authors isolated six serum-resistant P. aeruginosa mutant strains from six serum-susceptible parental strains by subculturing the sensitive strains in increasing concentrations of PNHS, ranging from 0.625% to 40%. Initially growth occurred only in those broths with low serum concentrations. These bacterial cells were used to inoculate a second series of broths yielding bacteria that appeared more serum-resistant. After the third subculture, bacteria grew in the presence of 40% PNHS. The fact that this method failed when using H. ducreyi suggests that a different induction mechanism which is more readily expressed, is present in P. aeruginosa.

It is noteworthy that Odumeru etal (1984) were able to obtain an isogenic serum-resistant mutant of H. ducreyi strain A77 by passaging the serum-susceptible strain A77 through increasing concentrations of PNHS. Unfortunately details of their methods are not mentioned in their report.

Clinical isolates described by Odumeru etal (1984) displayed

a greater degree of resistance to 50% PNHS than the clinical strains used in this study. Their strains showed a survival rate of greater than 50% over a two hour incubation period. The authors claimed that they used sixteen hour bacterial cultures from solid media as the inoculum in all their SBAs. Experiments conducted in the present study cast doubt on this report, as no visible growth of H. ducreyi on solid media was obtained following twenty-four hours of incubation. Furthermore, the present study shows that H. ducreyi enters a logarithmic growth phase only after twenty-four hours incubation. Thus the cultures used by Odumeru etal (1984) may not have been in logarithmic growth phase, possibly resulting in the resistance to killing by 50% PNHS.

(5.5) Conclusion

H. ducreyi reference strains A75, A76, A77 and ATCC 27722 are more serum-sensitive than the clinical strains included in this study. Clinical strains FQ33, FQ61 and Y206 appear to be most resistant to 50% PNHS. Nevertheless, there is a general trend amongst all the H. ducreyi bacterial strains towards decreased survival with an increased incubation time in 50% PNHS, with the reference strains being killed more rapidly.

It is difficult to implicitly link the serum resistance of clinical strains FQ33, FQ61 and Y206 with virulence, since clinical isolates Y56, D29 and Y137 were clearly serum-susceptible. This highlights the potential difficulties of reproducing in vivo conditions in vitro. In addition, the relationship between the virulence of H. ducreyi strains as assessed by means of the rabbit intradermal skin test, and their virulence in human beings remains to be fully established.

The serum bactericidal activity of 50% PNHS on reference and clinical H. ducreyi strains is complement dependant, involving the classical complement pathway.

Two different methods employed to induce a serum-resistant isogenic strain from a serum-susceptible reference strain proved unsuccessful.

The considerable interest for many years in the role of serum resistance as a virulence factor has not led to a suitably applicable mechanism of resistance for most bacteria. Clearly, future analysis of such mechanisms will entail a detailed study of the bacterial gene(s) responsible for this property, followed by in vivo and in vitro biochemical and

physiological investigations of the gene product(s). This process will require the isolation of isogenic mutants from serum-susceptible or serum-resistant parent strains. Although attempts to do this failed in the present study, it is likely that molecular genetic techniques may ultimately prove more successful (Moll etal, 1979 ; Mcshan etal, 1987).

CHAPTER SIX**OUTER MEMBRANE PROTEINS AND ANTIGENICITY OF H. DUCREYI****(6.1) Introduction**

The outer membrane of Gram-negative bacteria is a highly specialised structure that lies outside the cytoplasmic membrane and peptidoglycan layer and forms the interface between the cell and its external environment (Osborn and Wu, 1980). Thus, the outer membrane constitutes a physical and functional barrier between the cell body and its surroundings which effectively controls access of solutes and external agents to the cytoplasmic membrane (Osborn and Wu, 1980).

The specialised functions of the membrane are reflected in its composition, which includes the cellular LPS and a limited number of highly characteristic and unusual major proteins (Osborn and Wu, 1980).

Electron microscopical examination of ultrathin sections of H. ducreyi both in vitro (Kilian and Theilade, 1975) and in

biopsies of ulcers from patients with chancroid (Marsch etal, 1978), reveal the typical appearance of a Gram-negative cell wall with a cytoplasmic membrane surrounded by a rugose outer membrane. The OMP profile is also typical of Gram-negative bacteria, with one to several proteins predominating (Odumeru etal, 1983 ; Taylor etal, 1985 ; Saunders and Folds, 1986 ; Abeck and Johnson, 1987).

In 1983, Odumeru etal, characterised the proteins present in the outer membrane of H. ducreyi by means of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). All strains were shown to possess a major OMP with a molecular mass that varied between 39 and 40kD depending on the electrophoretic conditions. The mass of this protein is in the same range as OMPs that function as porins in other Gram-negative bacteria (Morse, 1989). In addition, Odumeru etal (1983) divided one hundred and five strains of H. ducreyi into at least seven different subtypes by comparing proteins in the 24 to 50kD molecular weight range. They suggested that typing strains according to their OMP profiles might prove useful for epidemiological studies. As an example, they showed that thirteen strains of H. ducreyi isolated during an outbreak of chancroid in Winnipeg, Manitoba Canada, all had identical OMP profiles (Odumeru

etal, 1983).

Two years later, Taylor etal (1985) also described the use of SDS-PAGE for analysing OMPs of H. ducreyi for epidemiological studies. These workers based their analysis on the interstrain variation of OMPs in the molecular weight range 27 to 60kD. They were able to group strains of H. ducreyi into seven types although the characteristic protein profiles of their seven types differed from those described previously by Odumeru etal (1983). The OMP profiles of H. ducreyi strains were reproducible from one gel preparation to the next, and the profiles of strains isolated from multiple ulcers on the same patient were identical.

In addition to having potential value as epidemiological markers, OMPs of H. ducreyi may also be important as determinants of antigenicity and virulence. Saunders and Folds (1986) undertook a study of the antigenicity of H. ducreyi using Western blotting with rabbit antisera. While isolates of H. ducreyi were heterogeneous in protein composition, isolates from single outbreaks appeared similar both in protein profiles and antigenic analysis. This study, however, was complicated by the reactivity of the pre-immunisation sera with some H. ducreyi antigens. A

modification of this investigation was carried out by Abeck etal (1988) using antisera produced in specific pathogen-free mice, and Finn by etal (1990) using murine monoclononal antibodies to increase the specificity of the reactions. In order to determine which OMPs were exposed at the cell surface, Abeck and Johnson (1987) treated intact H. ducreyi organisms with Iodogen to catalyse the iodination of surface-exposed proteins. The proteins labelled most efficiently with ^{125}I were the 39kD protein and a prominent protein within the range of 27 to 30.5kD. The authors could not confirm, however, that all the labelled proteins were surface exposed; previous investigators (Loeb and Smith, 1983) reported that the use of a similar surface-labelling technique had resulted in some iodination of inner membrane proteins of Haemophilus influenzae.

The present study contains an analysis of the OMPs of four serum-sensitive reference strains and eight clinical strains of H. ducreyi of ranging serum susceptibility, and four other Haemophilus species. The OMP profiles, antigenicity and surface exposure was determined by SDS-PAGE, Western blotting and radioiodination, respectively.

(6.2) Material and Methods

(6.2.1) Strains and Media

Three H. ducreyi reference strains A75, A76 and A77 were obtained from the Pasteur Institute in France. The fourth H. ducreyi reference strain ATCC 27722 was obtained from the American Type Culture Collection. The clinical isolates of H. ducreyi namely, Y206, D29, FQ61, FQ33, Y137, Y56, Y155 and FQ24 were isolated from patients with chancroid attending the Leslie Williams Memorial Hospital in Carletonville, South Africa. In addition, four clinical isolates of other Haemophilus species were included namely, H. aegyptius, H. aphrophilus, H. influenzae and H. parainfluenzae.

All H. ducreyi strains were cultured on Nsanze medium (APPENDIX 1). The four other Haemophilus species were cultured on chocolate agar (APPENDIX 2).

(6.2.2) OMP isolation

Forty-eight hour cultures of H. ducreyi were harvested from Nsanze agar plates into 5ml of a 0.01M Tris HCl buffer (pH 7.0), until a wet weight of approximately 1.0g was attained. The bacterial cells were then pelleted by centrifugation for twenty minutes at 10 000Xg in a Beckman JA14 rotor. The cells

were resuspended in 10.0ml of 0.01M HEPES buffer (pH 7.0; Merck; APPENDIX 5), and sonicated for six twenty-second bursts at fifteen microns while cooling in an ice bath. The whole cell sonicate was then centrifuged for twenty minutes at 10 000Xg to remove the intact cells, and the supernatant centrifuged at 100 000g at 4⁰C for one hour in a Beckman Ty65 rotor. The resultant pellet represented the total membrane preparation. The pellet was resuspended in 1.0ml of 0.01M HEPES buffer, using a glass rod as well as passing the pellet through a 0.45 mm gauge needle attached to a 1.0ml syringe (Microfine). One ml of 2% sodium lauryl sarcosinate (APPENDIX 5) was then added to the resuspended pellet, and the extraction allowed to continue for thirty minutes at room temperature. The sarcosinate insoluble fraction was then harvested by centrifuging at 100 000Xg at 4⁰C for one hour in a Beckman Ty65 rotor. The pellet was resuspended in 0.5ml of sterile distilled water by repeated passage through a 0.45mm gauge needle attached to a 1.0ml syringe. Twenty ul was removed to determine the protein concentration of the OMP fraction using the Bio-Rad standard protein assay kit. The remaining volume was stored at minus 70⁰C, and analysed using SDS-PAGE within four weeks after purification.

(6.2.3) SDS-PAGE

All OMP samples were separated using a modification of the gradient polyacrylamide gel system of Saunders and Folds (1986). The slab gels used consisted of a 5% stacking gel and an 8 to 20% gradient resolving gel (APPENDIX 6). A 60ug concentration of purified OMPs was prepared with solubilisation buffer (APPENDIX 6), and after boiling for five minutes, 100ul of the total mixture was loaded onto each well of a Hoeffer Instruments SE 600 vertical slab gel apparatus (APPENDIX 6). A low molecular weight standard (Pharmacia, APPENDIX 7) was loaded onto one of the wells in each gel to determine the molecular weights of the resulting OMP bands.

Each gel was run at a constant current of 30mA until the marker dye reached the bottom of the gel. The proteins were then fixed and stained overnight in Coomassie blue (Biorad), and destained in a methanol-acetic acid solution (APPENDIX 8). Protein profiles were analysed by visual inspection.

(6.2.4) Western Blot Analysis

Two 8 to 20% gradient gels were loaded with OMP samples of both H. ducreyi and other Haemophilus strains as described above. After the proteins were separated by SDS-PAGE, one of

the gels was stained with Coomassie blue and destained, while the second gel was incubated with constant agitation for one hour in ice cold transfer buffer (APPENDIX 9). This gel was then carefully laid onto nitrocellulose paper (Amersham International), together with blotting paper and scotchbrite pads. The "sandwich" thus created was placed into the blotting unit pre-filled with transfer buffer (APPENDIX 9). After the electrophoretic transfer was complete, the "sandwich" was disassembled with care being taken not to tear the nitrocellulose paper or the gel. The gel was placed into Coomassie blue in the usual manner to determine the efficiency of transfer. The nitrocellulose paper was quenched by incubation for one hour in a 3% gelatin solution (APPENDIX 9). It was then washed twice for twenty minutes in washing buffer (APPENDIX 9). The nitrocellulose paper was incubated for ninety minutes at room temperature with immune rabbit serum previously diluted 1:100 with tris buffered saline (TBS). After five washings of five minutes each in washing buffer, the nitrocellulose paper was incubated for one hour with gentle agitation with the conjugate probe (protein-A-horseradish peroxidase; Biorad; APPENDIX 9) previously diluted 1:600 with TBS for one hour. After the nitrocellulose paper was washed twice for twenty minutes in washing buffer, the HRP colour development solution

(4-chloro-1-naphol; Biorad) was added (APPENDIX 9), and the reaction stopped by the addition of distilled water upon full colour development of the protein bands.

(6.2.5) Radioiodonation of Surface-Exposed Proteins

Approximately fifteen H. ducreyi colonies were swabbed from forty-eight hour Nsanze agar plates, and resuspended in 1.5ml PBS (pH7.2; APPENDIX 4). The colonies were washed and centrifuged twice in PBS, and the final pellet resuspended in 25ul PBS containing 0.08% lactoperoxidase (Sigma; APPENDIX 10). To this was added 5ul of 10^{-5} M potassium iodide (Merck; APPENDIX 10) and 4ul of ^{125}I (50uCi/ul; Amersham International). At 0, 2.5, 5.0, 7.5 and 10 minute intervals, 5ul of fresh 0.3% hydrogen peroxide was added. At 12.5 minutes, the bacteria were pelleted by centrifugation and washed three times in PBS with 0.005M cysteine (Sigma; APPENDIX 10). Two hundred ul of radioiodonated bacteria was then combined with 200ul of solubilising buffer, and boiled for five minutes.

Fifty ul of solubilised radioiodonated bacteria was loaded onto each well of an 8 to 20% gradient gel, and electrophoresis carried out as mentioned above. Rainbow ^{14}C molecular weight markers (Amersham International; APPENDIX

10) were used to determine the molecular weights of the radio-labelled OMPs. Upon completion of electrophoresis, the gel was removed and fixed in a methanol-tricarboxylic acid solution (APPENDIX 8) for two hours. The gel was then dried for two hours on a slab gel drier (LKB 2003), applied to X-ray film (type X-D, 3M) and exposed with a trimax intensifying screen at minus 70⁰C in a metal X-ray cassette (Okamata). After an incubation period of forty-eight hours, the autoradiograph was developed (APPENDIX 10) and analysed by visual inspection.

(6.3) Results

(6.3.1) SDS-PAGE

Identification of the protein components of the outer membrane is dependent on separation of proteins using SDS-PAGE. The molecular weight of the proteins can also be determined by this method. Larger proteins migrate more slowly and settle out at the top of the gel; smaller molecules migrate towards the bottom.

Figures 6.1 and 6.2. represent the SDS-PAGE polypeptide profiles of the strains included in this study.

The banding pattern of H. ducreyi OMPs is relatively homogeneous, although differences in the protein profiles are evident based on their electrophoretic mobility and intensity of staining. These OMP profiles were reproducible from one gel preparation to the next.

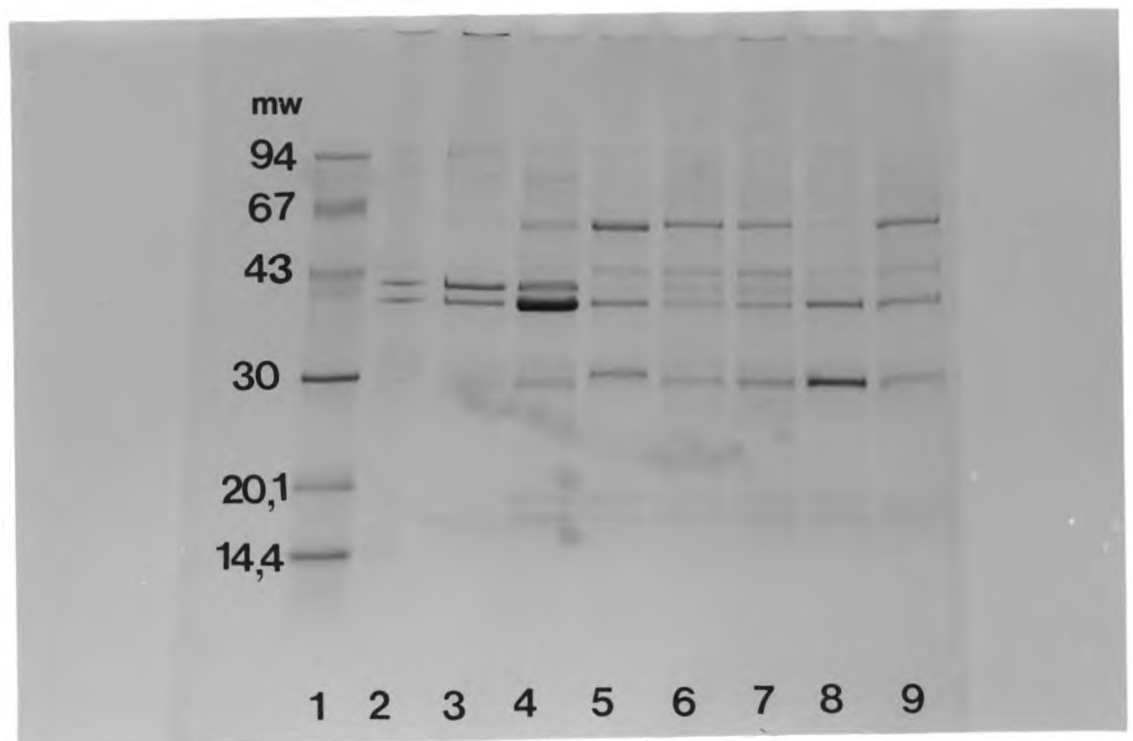


FIGURE 6.1 SDS-PAGE profiles of OMP preparations of H. ducreyi strains: Lane 1, low molecular eight standards ; lane 2, A75 ; lane 3, A76 ; lane 4, A77 ; lane 5, Y155 ; lane 6, Y137 ; lane 7, FQ24 ; lane 8, ATCC 27722; lane 9, D29.

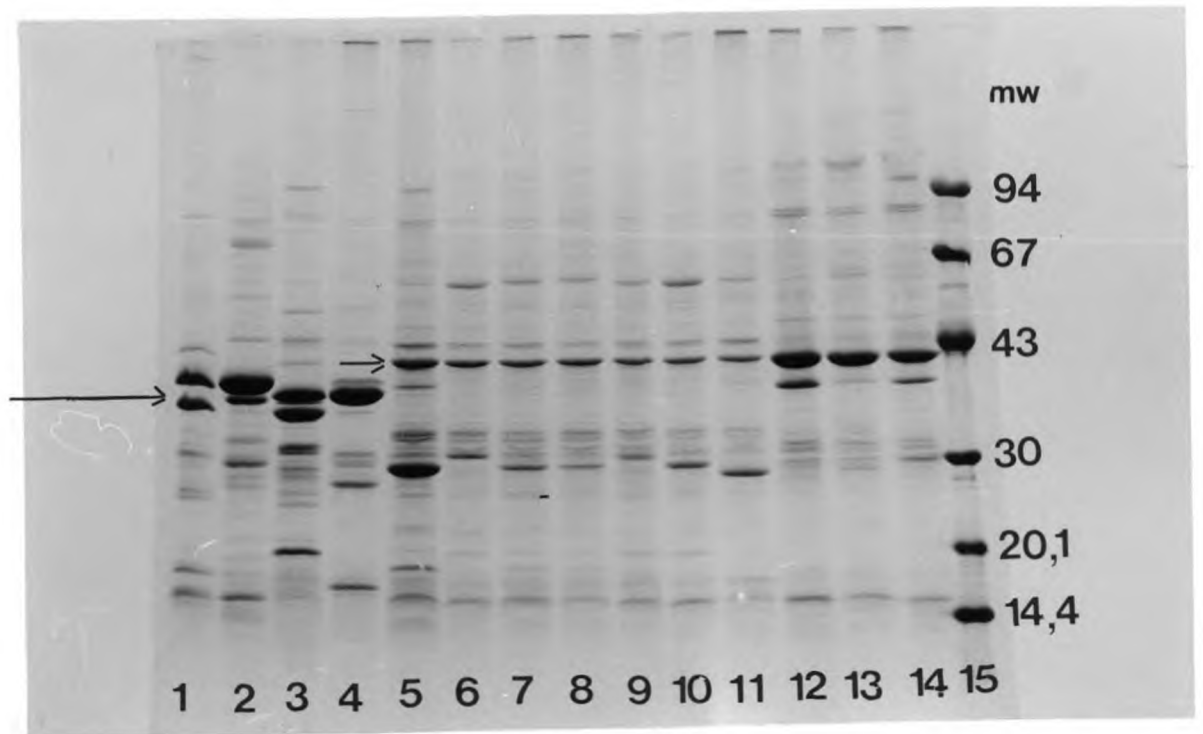


FIGURE 6.2 SDS-PAGE profiles of OMP preparations of *H. ducreyi* and other *Haemophilus* species : Lane 1, *H. aegyptius* ; lane 2, *H. influenzae* ; lane 3, *H. aphrophilus* ; lane 4, *H. parainfluenzae* ; lane 5, ATCC 27722; lane 6, Y56 ; lane 7, Y137 ; lane 8, FQ33 ; lane 9, FQ61 ; lane 10, D29 ; lane 11, Y206 ; lane 12, A77 ; lane 13, A76 ; lane 14, A75 ; lane 15, low molecular weight standards.

All H. ducreyi strains contained a number of OMPs ranging in molecular weight from approximately 16 to 98kD. Although some heterogeneity existed between the OMP profiles, at least seven to nine intensely staining protein bands of molecular weights 16, 30, 32, 33, 41, 43, 60, 83 and 96kD were consistently present (FIGURE 6.2). The 41kD OMP was the most intensely staining protein band displayed by all H. ducreyi strains (indicated by the short arrow in FIGURE 6.2).

Less homogeneity of OMP banding patterns was evident within the four other Haemophilus species. Differences also existed between the Haemophilus species and H. ducreyi strains. A protein band of molecular weight 38kD was common to H. ducreyi strains A75, A76, A77, ATCC 27722 and all four Haemophilus species (indicated by the long arrow in FIGURE 6.2). Although this band may be present in strain Y206, it was absent from the OMP profiles of the remaining clinical H. ducreyi isolates.

Various minor OMP bands, especially within the molecular weight range 25 to 35kD were present in both the H. ducreyi strains and the four other Haemophilus species. The greatest heterogeneity of OMP banding patterns amongst H. ducreyi strains occurred within the molecular weight range 29 to 45kD

(FIGURE 6.2).

(6.3.2) Western Blot Analysis

Western blot analysis of the OMPs of H. ducreyi and other Haemophilus species are depicted in FIGURES 6.3 to 6.8

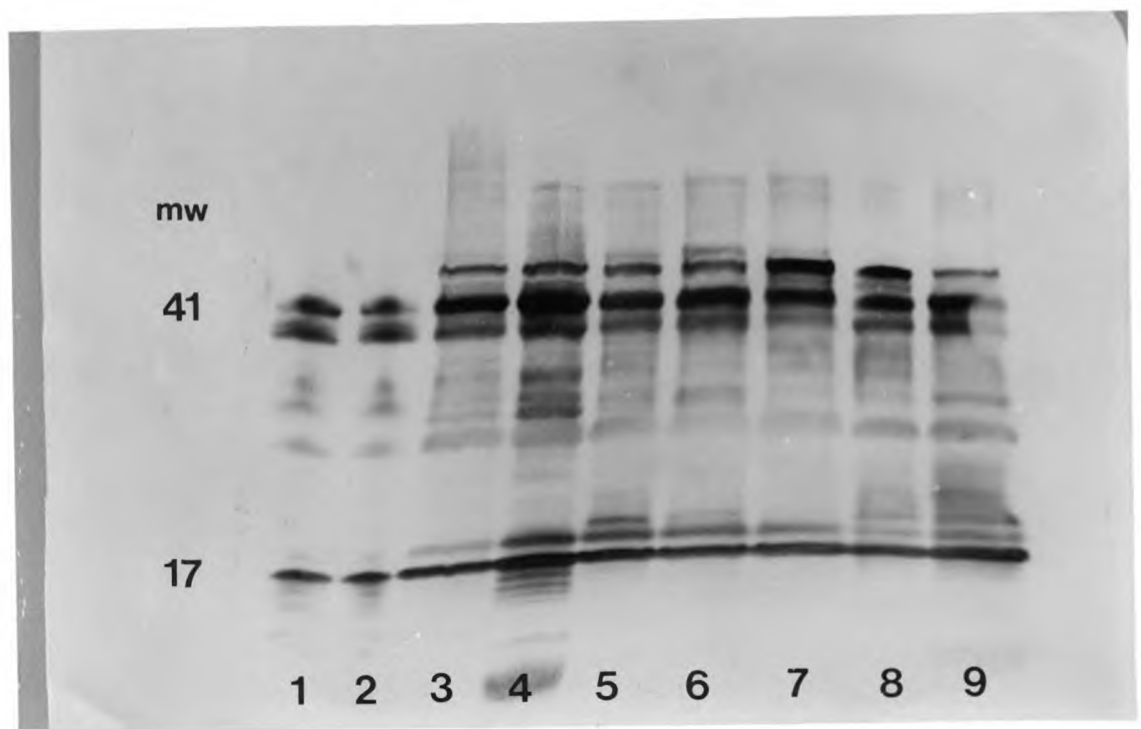


FIGURE 6.3 Western blot analysis of H. ducreyi OMPs using anti-A75 rabbit immune serum (diluted 1:100) :
Lane 1, A75 ; lane 2, A75 ; lane 3, A76 ; lane 4, A77 ; lane 5, Y155 ; lane 6, D29 ; lane 7, Y137 ; lane 8, FQ24 ; lane 9, ATCC 27722.

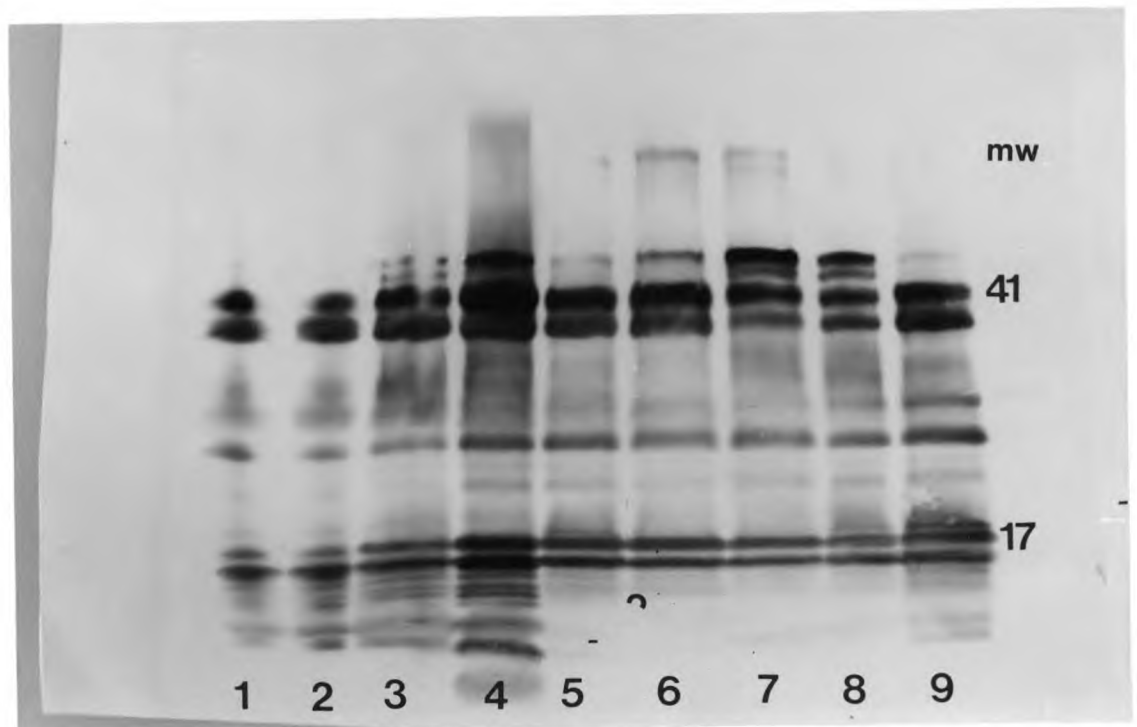


FIGURE 6.4 Western blot analysis of *H. ducreyi* OMPs using anti-A76 rabbit immune serum (diluted 1:100) :
Lane 1, A75 ; lane 2, A75 ; lane 3, A76 ; lane 4, A77 ; lane 5, Y155 ; lane 6, D29 ; lane 7, Y137 ; lane 8, FQ24 ; lane 9, ATCC 27722.

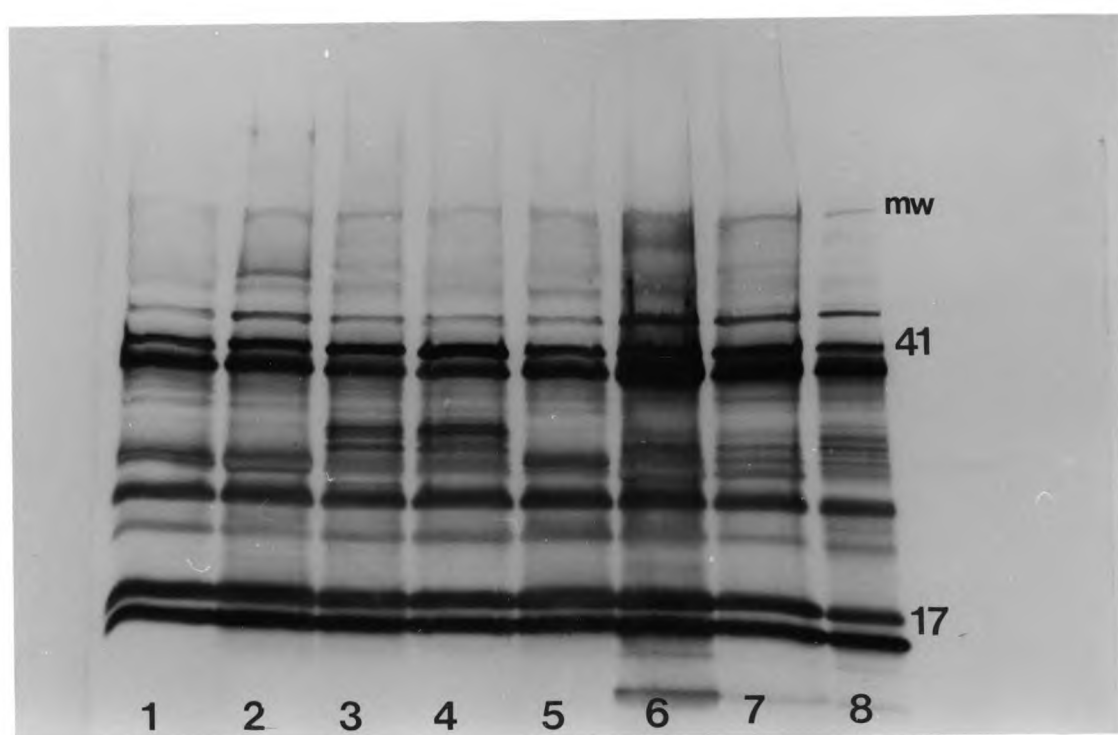


FIGURE 6.5 Western blot analysis of *H. ducreyi* OMPs using anti-A77 rabbit immune serum (diluted 1:100) :
Lane 1, D29 ; lane 2, ATCC 27722 ; lane 3, FQ24 ;
lane 4, Y137 ; lane 5, Y155 ; lane 6, A77 ; lane
7, A76 ; lane 8, A75

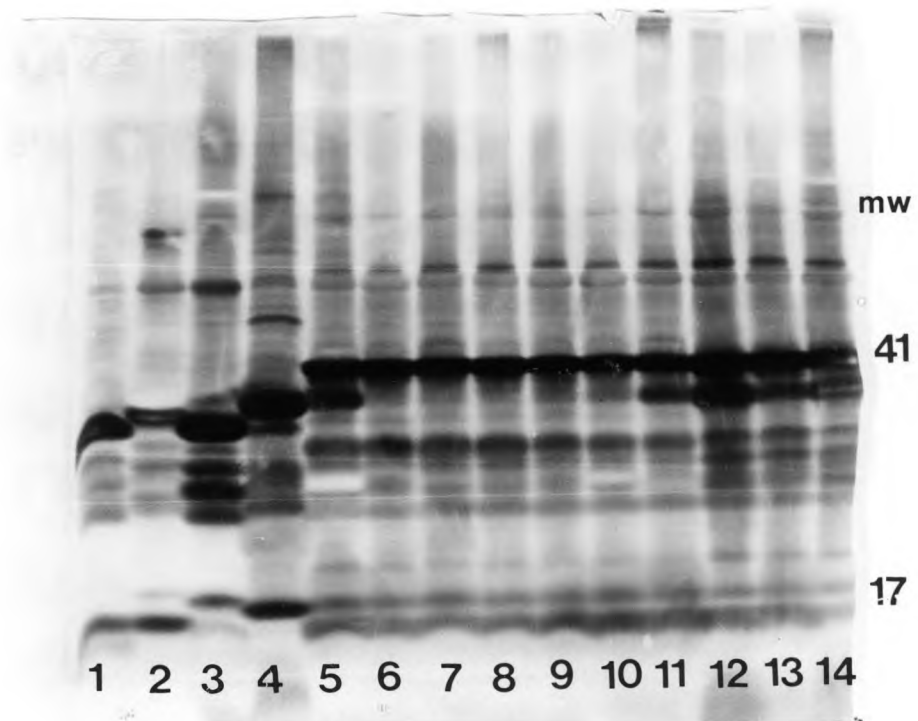


FIGURE 6.6 Western blot analysis of H. ducreyi and other Haemophilus species OMPs using anti-A75 rabbit immune serum (diluted 1:100) : Lane 1, H. aegyptius ; lane 2, H. influenzae ; lane 3, H. aphrophilus ; lane 4, H. parainfluenzae ; lane 5, ATCC 27722 ; lane 6, Y56 ; lane 7, Y137 ; lane 8, FQ33 ; lane 9, FQ61 ; lane 10, D29 ; lane 11, Y206 ; lane 12, A77 ; lane 13, A76 ; lane 14, A75.

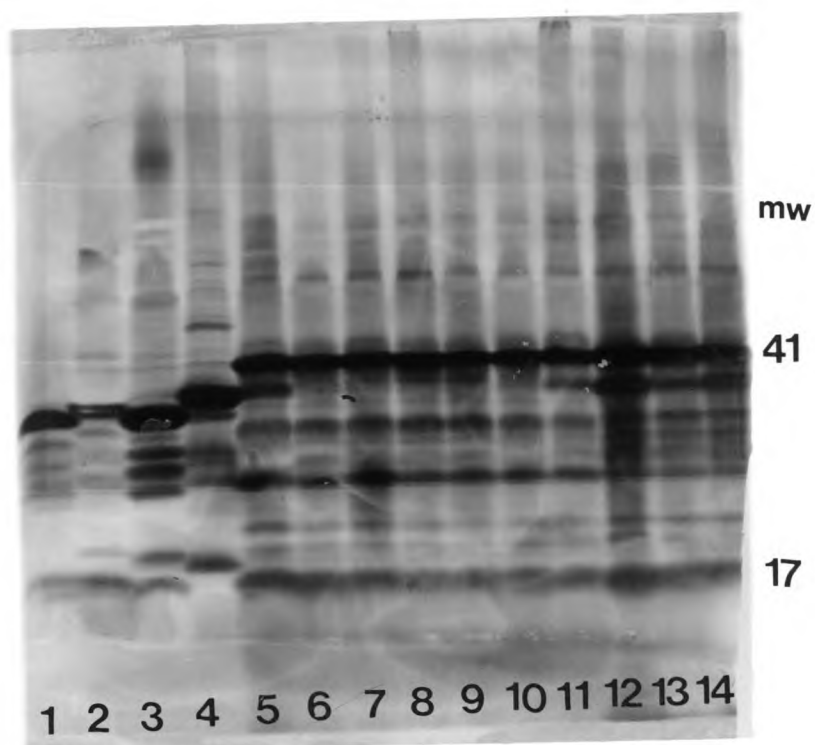


FIGURE 6.7 Western blot analysis of H. ducreyi and other Haemophilus species OMPs using anti-A76 rabbit immune serum (diluted 1:100) : Lane 1, H. aegyptius ; lane 2, H. influenzae ; lane 3, H. aphrophilus ; lane 4, H. parainfluenzae ; lane 5 ; ATCC 27722 ; lane 6, Y56 ; lane 7, Y137 ; lane 8, FQ33 ; lane 9, FQ61 ; lane 10, D29 ; lane 11, Y206 ; lane 12, A77 ; lane 13, A76 ; lane 14, A75.

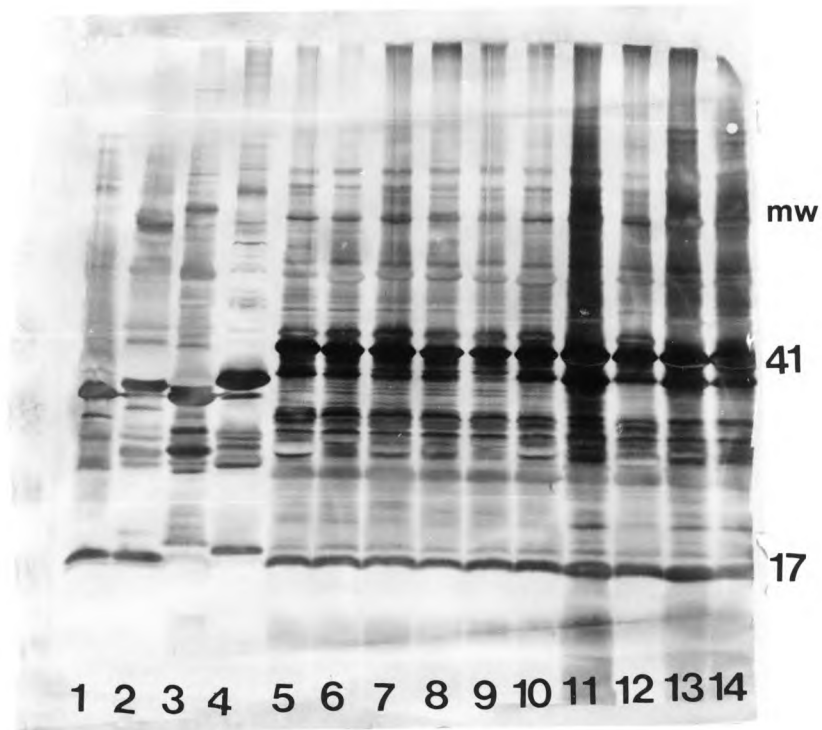


FIGURE 6.8 Western blot analysis of H. ducreyi and other Haemophilus species OMPs using anti-A77 rabbit immune serum (diluted 1:100) : Lane 1, H. aegyptius ; lane 2, H. influenzae ; lane 3, H. aphrophilis ; lane 4, H. parainfluenzae ; lane 5, ATCC 27722 ; lane 6, Y56 ; lane 7, Y137 ; lane 8, FQ33 ; lane 9, FQ61 ; lane 10, D29 ; lane 11, Y206 ; lane 12, A77 ; lane 13, A76 ; lane 14, A75.

(6.3.2.1) Analysis of H. ducreyi by Western blotting

The antigenicity of H. ducreyi OMPs was analysed by immunoblotting them using immune rabbit antisera. The optimal dilution of all antisera was found to be 1:100.

When electrophoretically separated OMPs of H. ducreyi were blotted with either the homologous or heterologous antiserum, strongly staining bands of molecular weights 17, 19, 23, 28, 31, 41, 43, 53, 60 and 83kD were observed (FIGURES 6.5 and 6.8). Anti-A77 rabbit immune serum was the most strongly reacting (FIGURES 6.5 and 6.8). The Western blot profiles of H. ducreyi OMPs were fairly homogeneous using all three immune rabbit sera. However, strains FQ24, Y137 and A77 (FIGURE 6.5) displayed additional stained protein bands. These were of molecular weights 32 and 33kD in the case of strains FQ24 and Y137, and 15kD in the case of strain A77. Additional faint bands were evident (FIGURES 6.3 to 6.8), which were detected when the HRP colour development reaction (APPENDIX 9) was prolonged.

Overall, the protein bands seen in the OMP profiles of reference strains reacted with homologous antisera were also present in the sarkosyl-insoluble outer membrane preparations of the clinical strains, indicating a close structural and

antigenic relationship.

(6.3.2.2) Antigenic cross-reactivity between OMPs of *H. ducreyi* and other *Haemophilus* species.

The reactivity of rabbit antiserum to OMPs of *H. ducreyi* strains A75, A76, and A77 and four other *Haemophilus* species was examined by Western blotting. *H. ducreyi* was found to cross-react with *H. aegyptius*, *H. influenzae*, *H. aphrophilis*, and *H. parainfluenzae*. Representative results are shown in FIGURES 6.6 to 6.8. Close homology was noticed amongst OMPs in the molecular weight range 20 to 38kD.

(6.3.3) Radioiodonation of Surface Exposed Proteins

¹²⁵I-Iodonated proteins of intact *H. ducreyi* cells are shown in FIGURES 6.9 and 6.10.

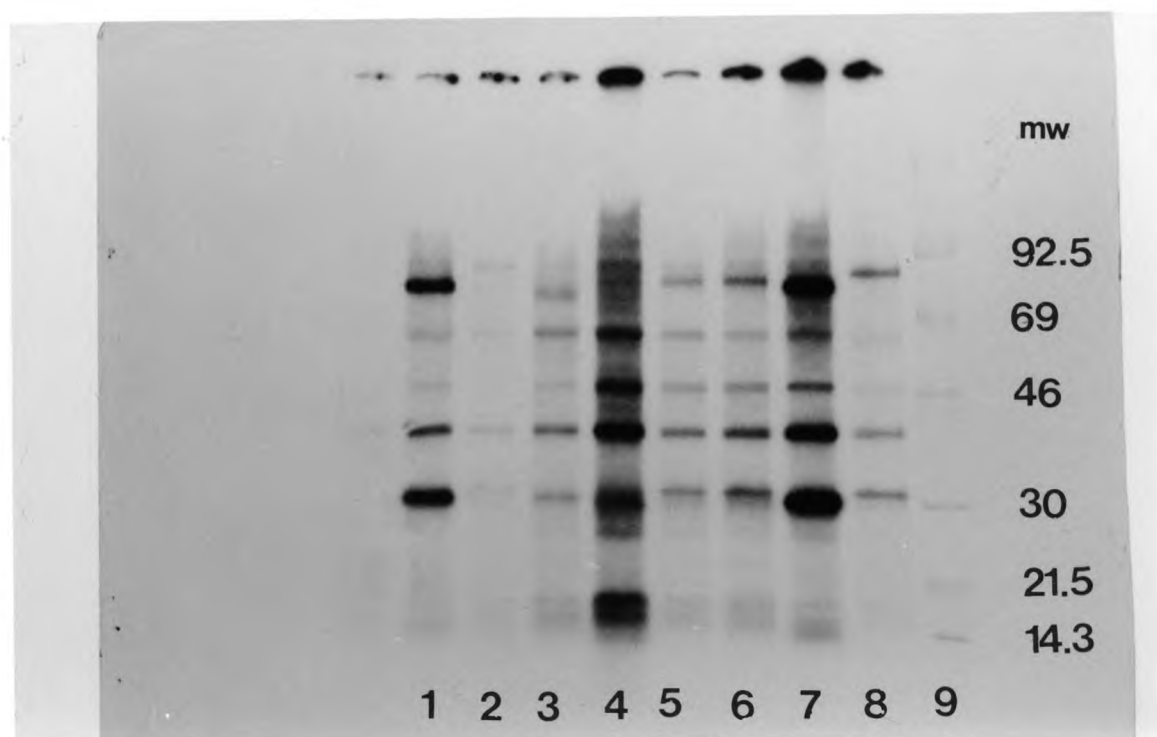


FIGURE 6.9 Autoradiograph of radioiodinated intact cells of *H. ducreyi* separated by SDS-PAGE : Lane 1, ATCC 27722 ; lane 2, Y56 ; lane 3, Y137 ; lane 4, FQ61 ; lane 5, FQ24 ; lane 6, FQ33 ; lane 7, D29 ; lane 8, Y206 ; lane 9, low molecular weight standards.

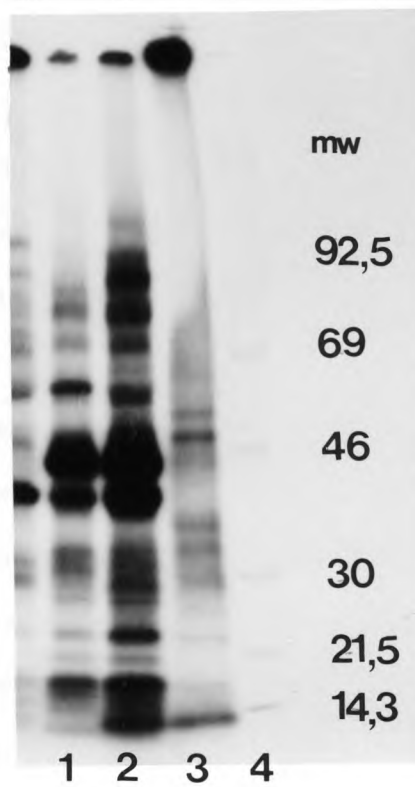


FIGURE 6.10 Autoradiograph of radioiodinated intact cells of H. ducreyi reference strains separated by SDS-PAGE: Lane 1, A77 ; lane 2, A76 ; lane 3, A75 and lane 4, low molecular weight standards.

FIGURES 6.9 and 6.10, demonstrate the homogeneous radioiodonation profiles of H. ducreyi OMPs.

The common major 41kD protein and other prominent proteins of molecular weights 30, 47, 60 and 83kD exhibited the strongest radioactive signals amongst all clinical strains. The protein of molecular weight 14 to 16kD appeared prominent in strain FQ61 (FIGURE 6.9 lane 4), but was less apparent in all other clinical strains.

The radioiodonated OMP pattern of H. ducreyi strain A76 and A77 (FIGURE 6.10) was quite homogeneous. The resolution of strain A75 was poor and difficult to compare with these two strains. Proteins of molecular weights 14 to 16, 30, 41, 60 and 83kD were common to clinical and reference strains. A protein band of 38kD was absent from clinical strains.

(6.4) Discussion

(6.4.1) OMP isolation

all OMP isolation techniques generally consist of a three-step extraction process, beginning with the mechanical or ultrasonic disruption of the bacterial cell. Thereafter the cytoplasmic and outer membranes are separated by

differential centrifugation, and finally the OMPs are rendered insoluble by treatment of isolated cell envelopes with certain detergents. It is accepted that treatment of isolated cell envelopes with certain detergents can selectively solubilise the cytoplasmic membrane while leaving the outer membrane proteins intact. (Lugtenberg and Van alphen, 1983).

The most widely used detergent, especially amongst E. coli cells is Triton X-100 (Barenkamp etal, 1981 ; Kelley and Parker, 1981 ; Loeb etal, 1981 ; Coulton and Wan, 1983 ; Blaser etal, 1984). The effect of Triton X-100 on the solubilisation of membrane fractions has been studied in detail (Schnaitman, 1971). Extraction of cell envelopes with Triton X-100 in the presence of magnesium results in complete solubilisation of the cytoplasmic membrane. The concentration of magnesium during the Triton X-100 extraction is critical (Schnaitman, 1971).

Morphologically the outer membrane appears to remain intact. However, chemical analysis has shown that this "Triton-insoluble cell wall" contains only about half of the LPS and one-third of the phospholipid of the outer membrane but that all the OMPs are still present (Lugtenberg and Van

alphen, 1983).

Sodium lauryl sarcosinate (Sarkosyl), the detergent used in the present study, has also been extensively used by other investigators (Barenkamp etal, 1981, 1982 ; Mills and Bradbury, 1984 ; Clark etal, 1987 ; Ward etal, 1987). In a study carried out by Filip etal (1973), in the absence of magnesium, treatment of E. coli cells with 0.5% sodium lauryl sarcosinate resulted in the solubilisation of the cytoplasmic membrane proteins and phospholipids. Their data suggested that treatment with sodium lauryl sarcosinate resulted in an increase of the bouyant density of the outer membrane, indicating a loss of phospholipid. However, in some cases they found the inner membrane of E. coli to be slightly resistant to solubilisation by sodium lauryl sarcosinate. The basis for this resistance was not known. Under conditions in which sodium lauryl sarcosinate appeared to solubilise protein of the inner membrane selectively, the detegent Triton X-100 solubilised all membrane protein. For this reason, Triton X-100 is most often used on E. coli cells.

Sodium lauryl sarcosinate has been the detergent most commonly selected for obtaining OMPs from Haemophilus species (Barenkamp etal, 1981, 1982 ; Odumeru etal, 1983 ; Taylor

etal, 1985), Pseudomonas (Ward etal, 1987), Campylobacter (Mills and Bradbury, 1984) and Neisseria (Clark etal, 1987).

The present study used sodium lauryl sarcosinate at the recommended concentration of 2% (Barenkamp etal, 1983 ; Odumeru etal, 1983). Differences in OMP profiles of H. ducreyi could thus be attributed to electrophoretic conditions and interstrain variations.

(6.4.2) SDS-PAGE

Analysis of cell proteins by PAGE has been used to characterise a wide variety of Gram-negative bacteria, including porcine strains of Haemophilus (Nicolet etal, 1980), H. influenzae strain Rd (Zoon and Scocca, 1975) and H.influenzae type b (Loeb and Smith, 1980 ; Loeb etal, 1981). Nicolet etal (1980) characterised the whole cell proteins of Haemophilus suis by SDS-PAGE and compared the proteins of these strains with those of H. influenzae type b and H. parahaemolyticus. The protein patterns were found to be species-specific.

Protein profiles from SDS-PAGE of sodium lauryl sarcosinate-insoluble OMPs of H. ducreyi reference strains A75, A76, A77 and ATCC 27722, six clinical isolates and four

other Haemophilus species revealed the species-specific nature of their OMPs. This phenomenon has also been observed by Odumeru etal, 1983). One OMP of molecular weight 38kD was, however, shared by all Haemophilus strains (FIGURE 6.2). The OMP profiles are typical of Gram-negative bacteria containing up to seven major and several more minor proteins (Loeb and Smith, 1980 ; Overbecke and Lugtenberg, 1980 ; Odumeru etal, 1983 ; Van alphen etal, 1983). Odumeru etal (1983) found that storage, serial passage and age of culture did not appear to result in significant variations in the OMP patterns. Abeck etal (1987) also found cultural conditions to have no effect on the total cellular protein profiles of H. ducreyi strains. Conversely, the expression of other bacterial surface components may be influenced by the enviroment in which they are grown (Brown and Williams, 1985). In a study by Ward etal (1987) of Pseudomonas aeruginosa isolates obtained directly, without subculture from infected wounds of two patients with burns, OMPs were investigated and compared with those of cells cultivated in the laboratory. The objective was to select defined growth conditions more closely resembling those found in vivo for examination of surface antigens. Several high molecular weight proteins expressed in the outer membranes of isolates from the patients could be phenotypically induced by cultivating the same isolate in

iron-depleted conditions in vitro.

Clark etal (1987) found that Neisseria gonorrhoeae strains expressed at least three proteins at much higher levels when grown anaerobically than were present in aerobic cultures. Conversely, at least five other proteins were found to be expressed at significantly higher levels in aerobically grown cells. Similar studies have not been carried out on H. ducreyi.

The OMP patterns of H. ducreyi has been previously examined by Odumeru etal (1983), Taylor etal (1985) and Saunders and Folds (1986). The common H. ducreyi OMP has been described as having a molecular mass of 40kD (Odumeru etal, 1983 ; Saunders and Folds, 1986) and 39.4kD (Taylor etal, 1985), very similar to the 41kD OMP reported in the present study. Saunders and Folds (1986) observed considerable heterogeneity amongst their protein profiles, although three common protein bands of molecular weights 39, 43 and 67kD occurred in all isolates. This could reflect the differing origins of these strains. Alternatively these differences could be due to the use of total membrane proteins by the authors as opposed to the use of OMP fractions described by other investigators. Another methodological source of variation in OMP banding

patterns is the gel concentration employed in SDS-PAGE. Odumeru etal (1983) used a 15% discontinuous gel, whereas Taylor etal (1985) used a 12% discontinuous gel. In the present study an 8 to 20% gradient separating gel was used which produces a higher resolution of the OMP banding patterns. Considerable homogeneity existed between the OMP banding patterns of the H. ducreyi strains in this study (FIGURES 6.1 and 6.2).

(6.4.3) Western Blot analysis

Protein immunoblotting has provided a powerful extension of electrophoresis technology. Polypeptides separated by gel electrophoresis are transferred to solid support matrices, most commonly to nitrocellulose, by the electrotransfer technique devised by Towbin etal (1979). Proteins thus immobilised on nitrocellulose sheets are used to detect their homologous antibodies. With radioactively-labelled or dye-conjugated antibodies the method is sensitive enough to detect small amounts of electrophoretically separated antigen. This simple procedure can therefore be used to show the presence of small amounts of antibody in a serum of low titre. Because the antigen is immobilised on a sheet, the antibody is not required to form a precipitate with the antigen. The blotting technique thus has the potential for

immuno-electrophoretic analysis of proteins by using binding of Fab fragments or binding of antibodies against a single determinant such as monoclonal antibodies produced by hybridomas.

The results of the present study indicate that H. ducreyi isolates are antigenically highly conserved. This is similar to findings of studies previously carried out on C. jejuni (Blaser etal, 1984) and P. aeruginosa (Murtheria etal, 1982), which found OMPs also to be highly conserved. Although this phenomenon could be due to a common geographical source of the strains used in this study, a number of antigens have been consistently found in H. ducreyi strains isolated in different parts of the world (Abeck etal, 1988).

The results of the present study agree in part with those reported by Saunders and Folds (1986) and Finn etal (1990) who used rabbit antisera, and Abeck etal (1988) who used antisera produced in pathogen-free mice to analyse the antigenicity of H. ducreyi.

Abeck etal (1988) described immunogenic protein bands of molecular weight 16, 22, 28, 41, 46 and 58kD. Saunders and Folds (1986) described protein bands of molecular weight 20,

22.5, 42, 45 and 67kD, and Finn etal (1990) showed a number of protein bands in the molecular weight range 25 to 65kD. In the present study, the immunologically reactive OMP bands of molecular weights 17, 23, 28, 41, 43 and 53kD correspond with the protein bands of molecular weight 20, 22.5, 42 and 45kD reported by Saunders and Folds (1986) and 16, 22, 28, 41, 46 and 58kD reported by Abeck etal (1988). These variations could be attributed to the technical differences mentioned in 6.4.2 above, or to variations in preparation of the OMP suspensions used for animal inoculation.

Saunders and Folds (1986) found that normal rabbit sera reacted with an antigen of molecular weight 67kD, common only to the H. ducreyi isolates, and a low molecular weight antigen common to other Haemophilus species. This band was not observed in the present study. It is possible, however, that some of the other reactive bands could be due to the presence of cross-reacting antibodies in non-immune rabbit sera.

It was previously reported by Abeck and Johnson (1987) that two proteins of molecular weight 39 and 27 to 30.5kD were the predominant protein antigens exposed at the surface of H. ducreyi. It is most likely that their 39kD antigen is the

same as the major common 41kD antigen described in the present study.

(6.4.4) Radioiodination of Surface-Exposed Proteins

The incorporation of radioiodine into protein molecules provides increased dimensions to the study of their interactions and subsequent fates. Most recently this technique has been used to investigate the arrangement of proteins in macromolecular structures such as membranes (Morrison, 1980). The incorporation of ^{125}I involves its covalent linkage to tyrosyl and histidine residues of proteins.

Radioiodination is the most widely used method for determining the antigenicity of bacterial surface components (Newell et al, 1984). In the present study, radioiodination of H. ducreyi OMPs was used to elucidate which of these proteins were surface-exposed. The common major OMP of molecular weight 41kD gave the strongest radioactive signal. This is in agreement with the study of Abeck and Johnson (1987). The radioiodinated OMP profile of clinical and reference H. ducreyi strains (FIGURES 6.9 and 6.10) were similar to the OMP patterns obtained by SDS-PAGE (FIGURES 6.1 and 6.2). The

intensity of radiolabelling corresponded to the intensity of staining with Coomassie blue. These results indicate that H. ducreyi strains have approximately six prominent surface exposed OMPs ranging in molecular weight from 14 to 83kD, with the reference strains A75, A76 and A77 exhibiting one additional surface exposed OMP of molecular weight 38kD. Other proteins exhibit a low degree of radioactive labelling probably denoting partial exposure at the cell surface (Abeck and Johnson, 1987).

The surface exposure in large quantity of the 41kD protein make it a potential candidate for further study of host-pathogen interactions.

(6.5) Conclusion

SDS-PAGE demonstrates sodium lauryl sarcosinate-insoluble OMPs of H. ducreyi reference and clinical strains and four other Haemophilus isolates as having a species-specific pattern. The OMP profiles are typical of Gram-negative bacteria.

All H. ducreyi strains contain intensely staining protein bands of molecular weights 16, 30, 32, 33, 41, 43, 60, 83 and

96kD. The common H. ducreyi major OMP was found to have a molecular mass of 41kD.

Considerable homogeneity exists between the OMP banding patterns of the reference and clinical H. ducreyi strains. Nevertheless, a typing system based on OMP profiles could become useful in epidemiological studies of chancroid. Western blot analysis demonstrates that H. ducreyi is highly antigenically conserved but shows some cross-reactivity with other Haemophilus species.

Radioiodination demonstrates that the common major OMP of molecular weight 41kD generates the strongest signal. All H. ducreyi strains have approximately six prominent surface-exposed OMPs ranging in molecular weight from 14 to 83kD, with the reference strains A75, A76 and A77 having one additional surface-exposed OMP of molecular weight 38kD. No interstrain variation was evident in the radioactively labelled OMPs of clinical isolates. This could reflect their localised geographical origin.

Studies with other pathogenic organisms have shown that surface antigens frequently act as determinants of pathogenicity, for example by promoting the adherence of

organisms to eukaryotic cells, or by mediating resistance to killing by host defences (Smith, 1977).

The role, if any, of the surface-exposed proteins in the pathogenesis of H. ducreyi infection is unknown.

CHAPTER SEVEN

CONCLUSIONS AND FUTURE CONSIDERATIONS**(7.1) Conclusions**

The mechanisms by which H. ducreyi causes genital ulcer disease are unknown. It seems reasonable to suppose that bacterial surface antigens contribute to the pathogenesis of chancroid. In addition, previous studies of clinical isolates of H. ducreyi have implicated serum resistance as an important virulence factor. The present study was undertaken to determine the role of surface-exposed proteins and serum resistance in the pathogenesis of H. ducreyi infection. A possible connection between these factors was also sought. The level of resistance to 50% PNHS amongst selected clinical and reference strains of H. ducreyi was determined. The profile, antigenicity and surface exposure of their OMPs was characterised. In order to correlate these features with serum resistance as a virulence factor, attempts were made to generate an isogenic serum-resistant mutant of H. ducreyi. Unfortunately, these experiments did not produce a truly

serum-resistant isogenic mutant.

The fastidious nature of H. ducreyi necessitated the use of complex growth media in all experiments. A formula consisting of gonococcal agar base, haemoglobin, IsoVitaleX, FCS and vancomycin adequately supported the growth of both clinical and reference strains of H. ducreyi.

In experiments to determine serum resistance of H. ducreyi survival of bacteria was measured at varying time intervals after exposure to 50% PNHS. While the results obtained were consistently reproducible, it is difficult to compare them with those of other investigators due to the marked variability in methods used by different authors.

The present study confirmed that reference strains A75, A76, A77, and ATCC 27722 were serum-sensitive, while clinical strains FQ33, FQ61 and Y206 were more serum-resistant. High levels of serum resistance could not, however, be documented.

The bactericidal activity of 50% PNHS was complement-dependant, since bacterial growth occurred in all experiments using heat-inactivated PNHS.

The OMP profiles of H. ducreyi clinical and reference strains, and other Haemophilus species were analysed by SDS-PAGE. All strains tested showed a pattern typical of that found in most Gram-negative bacteria. OMP profiles were similar amongst isolates of H. ducreyi but differed from those of other Haemophilus species. This homogeneous OMP banding pattern could reflect the common southern African origin of these isolates; previous studies have confirmed that strains isolated in different geographical regions have their own characteristic profile.

All H. ducreyi strains included in this study possessed a major OMP band of molecular weight 41kD. Radioiodination of H. ducreyi strains followed by SDS-PAGE and autoradiography demonstrated homogeneous radioactive banding patterns, with the strongest signal being emitted from this 41kD protein band. All strains possessed approximately six surface-exposed proteins. Western blots of OMPs using immune rabbit serum confirm the 41kD protein to be most immunogenic. Numerous other bands were consistently observed, including six OMPs which were shown to be surface exposed. Only minimal differences were noted, however, between serum-sensitive and serum-resistant strains or between reference and clinical

isolates. Studies of other pathogenic bacteria have shown that surface-exposed antigens frequently act as determinants of pathogenicity. The role, if any, of surface-exposed proteins in the pathogenesis of H. ducreyi infection is unknown. The results of the present study do not confirm or exclude a relationship between OMPs, including those which are surface-exposed, and serum resistance. Each may function as independent virulence factors, but further studies are needed to confirm this.

(7.2) Future Considerations

A number of aspects concerning chancroid and H. ducreyi remain unresolved and are fertile areas for research. Defining the virulence factors of H. ducreyi is perhaps the most important area for future research if an understanding of the pathogenesis of this organism is to be gained.

The first challenge would be to develop a definitive virulence assay in order to standardise pathogenesis investigations in different laboratories. The bacterial components and epitopes responsible for virulence could then be more clearly defined. Molecular cloning of genes encoding these structures and the use of monoclonal antibodies would

allow a more accurate assessment of their role in serum resistance and virulence of H. ducreyi. In addition, molecular techniques could be used to prepare large quantities of highly purified cellular components for further study.

The immune response in patients with chancroid has been inadequately studied, and it is unclear which membrane-exposed antigen(s) interact with lymphocytes and macrophages. Identification of putative cellular components involved in the virulence of H. ducreyi would allow more accurate delineation of the humoral and cellular immune mechanisms operative in patients with chancroid. An additional benefit accruing from further studies would be the advancement of diagnostic serological techniques using purified and/or cloned H. ducreyi-specific antigens, and the possible development of a vaccine.

LIST OF APPENDICES**APPENDIX 1****Nsanze agar / 1L**

(A) Gonococcal agar base (Gibco) 36g

Distilled water 600ml

Autoclave at 121⁰C for fifteen minutes

(B) Haemoglobin powder (Difco) 10g

Distilled water 400ml

Mix rapidly on magnetic stirrer

Autoclave at 121⁰C for fifteen minutes

Keep in waterbath

Final medium

Mix solutions (A) and (B). Cool to 50⁰C.

Add IsoVitaleX (BBL, Microbiological systems) 10ml

Add vancomycin (Lilly) 3mg

Add foetal calf serum (heated to 56⁰C for 30 minutes)
50ml

Note : If the haemoglobin forms a deposit, DO NOT

transfer the deposit.

Mix well and distribute into sterile petri dishes.

APPENDIX 2

Chocolate agar / 2L

(A) Columbia agar base (Oxoid) 80g

Autoclave at 121⁰C for fifteen minutes.

(B) Citrated horse blood 100ml

Cool (A) to 50⁰C and then add (B). Heat to 80⁰C. The suspension must be dark chocolate in colour. Cool to 50⁰C and distribute into sterile petri dishes.

APPENDIX 3

Brain Heart Infusion (BHI)-Haemin Broth / 1L

(A) BHI broth powder (Difco) 37g

Distilled water 1000ml

Autoclave at 121⁰C for fifteen minutes

(B) Haemin (Sigma) 5g

Histidine (Sigma) 5g

Triethanolamine (Merck) 10ml

Distilled water 250ml

Dispense into 10ml aliquots. Sterilise by heating at 60⁰C for thirty minutes. Store at minus 20⁰C until required.

Final medium :

Add to BHI broth (solution A) / L

Haemin stock solution (solution B) 5ml (0.5%)

IsoVitaleX (BBL, Microbiological systems) 10ml (1%)

Mix well

APPENDIX 4

Indirect Immunofluorescence Assay

The following solutions were required for indirect immunofluorescence assay :

(A) Phosphate buffered saline (PBS), pH 7.2

Na₂CO₃ (Merck) 1.59g

NaHCO₃ (Merck) 2.93g

NaN₃ (Merck) 0.20g

KCl (Merck) 0.20g

Dissolve in 500ml distilled water and adjust the pH to 7.2 with 5M NaOH. Make up to a final volume of 1000ml.

(B) Rhodamine stock solution

Two microlitres Bacto FA rhodamine (Difco) was added to 498ul PBS (pH7.2 ; a 1:50 dilution) and vortexed.

(C) Donkey anti-rabbit immunoglobulin conjugated with fluorescein (Amersham International)

A one-in-forty dilution was made using the rhodamine stock solution.

(D) Anti-A75,-A76 and-A77 rabbit serum

Doubling dilutions of the rabbit antiserum was made using PBS (pH7.2). The dilutions ranged from 1:2 to 1:2560. The antibody titre was the reciprocal of the highest dilution at which the full staining of the bacterial cell contour was retained.

(E) Mounting glycerol

A 10% (vol/vol) glycerol suspension was made by adding one part glycerol to nine parts PBS (pH7.2).

APPENDIX 5

Outer Membrane Protein Isolation

The following solutions were required for OMP isolation :

(A) 0.01M Tris-HCl buffer (pH7.0) / 100ml

Tris base (Associated Chemical Enterprises) 1.21g

Adjust to 90ml with distilled water. pH this solution to 7.0 with concentrated hydrochloric acid. Adjust the final volume to 100ml. Store at 4⁰C.

(B) 0.01M HEPES buffer (pH7.0) /100ml

HEPES (Merck) 2.38g

Adjust to 90ml with distilled water. pH this solution to 7.0 with concentrated hydrochloric acid. Adjust the final volume to 100ml. Store at 4⁰C.

(C) 0.01M HEPES buffer (pH7.4) / 200ml

HEPES (Merck) 4.76g

Adjust to 90ml with distilled water. pH this solution to 7.4 with concentrated hydrochloric acid. Adjust the final volume to 200ml. Store at 4⁰C.

(D) Sodium lauryl sarcosinate (Merck) (2%)

Twenty ul of sodium lauryl sarcosinate added to 980ul of 0.01M HEPES buffer (pH7.4) and the suspension vortexed.

(E) The Bio-Rad standard protein assay kit was used to determine the protein concentration of the OMP samples.

APPENDIX 6

Sodium dodecyl sulphate polyacrylamide gel electrophoresis
(SDS-PAGE)

The following solutions and buffers were required for SDS-PAGE :

(A) Acrylamide 30% w/v

Acrylamide (Isophore electrophoresis) 29.2g

Bis-acrylamide (Isophore electrophoresis) 0.8g

Dissolve acrylamide and bis-acrylamide in distilled water with stirring. Use a 100ml beaker. When the acrylamide is in solution adjust the final volume to 100ml. Store in an amber bottle at 4⁰C.

(B) Sodium Dodecyl Sulphate (SDS) 10% w/v

SDS (Holpro analytics) 10g

Dissolve in distilled water and adjust the final volume to 100ml. Store at room temperature.

(C) Upper (Hydroxymethyl) aminomethane (Tris) 0.5M (stacking gel tris)

Tris base (Associated Chemical Enterprises) 6.06g

10% SDS 4.0ml

Adjust to 90ml with distilled water. pH this solution with concentrated hydrochloric acid. Adjust the final volume to 100ml. Store at 4⁰C.

(D) Lower Tris 1.5M (separating gel tris)

Tris base 36.6g

10% SDS 8ml

Adjust volume to 180ml with distilled water. pH this solution to 8.8 with concentrated hydrochloric acid. Adjust the final volume to 200ml. Store at 4⁰C.

(E) Ammonium Persulphate 10% (made up fresh daily)

Ammonium persulphate (Isophore electrophoresis) 0.1g

Add 0.9ml distilled water. Use within four hours.

(F) N,N,N',N',-tetramethyl ethylene diamine (TEMED) (Isophore electrophoresis), use directly with no dilution.

(G) Solubilising buffer (Saunders and Folds, 1986)

SDS 0.2g

Mercaptoethanol (Biorad) 0.4ml

Glycerol 1ml

Tris buffer pH6.8 8.4ml

Bromothymol blue (Merck) Add until blue colour appears.

(H) Running buffer (Four litre volume)

Tris base 12.12g

Glycine (BDH Chemicals) 57.6g

SDS 4g

Adjust to final volume with distilled water.

The glass plates forming a sandwich were assembled as outlined in the electrophoresis manual (Hoeffer Instruments SE 600).

The following formulations were prepared for the 8-20% gradient separating gel (1.5mm) :

Compound	% Monomer	
	8	20
Lower Tris	5.0ml	5.0ml
Distilled water	9.6ml	0.0ml
30% acrylamide	5.4ml	13.4ml
Glycerol	0.0	2.0ml
Amm. persulphate	50ul	50ul
TEMED	15ul	15ul

The first four solutions were mixed with a magnetic stirrer, following which the ammonium persulphate and Temed were added.

Allow 1.0cm for the stacking gel on top of the gradient gel. Overlay with water and wait for the gel to polymerise, until a sharp band appears at the water-gel interface.

Remove the water by blotting with dry filter paper.

The stacking gel (5%) consisted of :

4.75ml upper tris buffer

2.63ml distilled water

1.26ml acrylamide
250ul ammonium persulphate
8ul TEMED

Just before pouring the solution, the ammonium persulphate and TEMED were added while stirring continuously. The comb was placed between the two glass plates, the stacking gel then poured on top of the resolving gel and polymerisation allowed to occur. The comb was then gently removed, and the wells filled with running buffer. The OMP samples (100ul) were then loaded into the wells with a Hamilton syringe (Dynatech Instruments), ensuring they remained at the bottom.

The upper buffer chamber was connected to the apparatus containing the gel sandwiched between the glass plates, and gently immersed into the lower buffer chamber which consisted of running buffer and the cooling unit. This entire unit was connected to the power supply.

Each gel was run at 30mA until the marker dye reached the bottom of the gel.

APPENDIX 7**Molecular weight markers for SDS-PAGE**

The low molecular weight calibration kit (Pharmacia) used contained the following protein mixture :

<u>Protein</u>	<u>Molecular weight</u>
Phosphorylase b	94
Albumin	67
Ovalbumin	43
Carbonic Anhydrase	30
Trypsin Inhibitor	20.1
Alpha-Lactalbumin	14.4

After resuspending with 100ul of solubilising buffer, 10ul was loaded onto the well.

APPENDIX 8

Coomassie blue stain for detecting proteins in polyacrylamide gels

Protein fixing and staining solution :

Coomassie brilliant blue R250 (Biorad) 0.5% (w/v)

Methanol 50% (v/v)

Tricarboxylic acid (TCA) (Merck) 10% (w/v)

Make up to a final volume of one litre with distilled water. Gels were placed in the above solution in a clean covered plastic container, and incubated overnight at room temperature.

Gel destaining solution :

Acetic acid 20% (v/v)

Methanol 20% (v/v)

Make up to a final volume of two litres with distilled water. Gels were destained, with frequent changes of the solution until all the background stain had disappeared. Gels were then stored in distilled water at 4°C.

APPENDIX 9

Western Blotting

The following solutions were required for Western blotting :

(A) Transfer buffer

0.02M Tris base 9.68g

0.1M Glycine 30g

Methanol 20% (v/v)

Make up to four litres with distilled water. Store at 4⁰C.

(B) Tris-Buffered Saline (TBS)

0.02M Tris base 2.42g

0.9% NaCl 9g

Add 200ml distilled water. pH to 7.5 with concentrated hydrochloric acid. Make up to a final volume of one litre with distilled water. Store at 4⁰C.

(C) Washing Buffer

Add 1.5ml Tween-20 to three litres TBS. Store at 4⁰C.

(D) Quench

Gelatin 15g

TBS 500ml

Combine and filter before use. Store at 4⁰C.

(E) Preparation of the HRP colour development solution

(1) TBS 100ml

(2) HRP colour development reagent (Biorad) 60mg (protect from light)

(3) Ice-cold methanol 20ml

(4) Ice-cold 30% hydrogen peroxide 60ul

(5) Combine 2 and 3. Protect from light. Dissolve immediately before use. Immediately prior to use, add 1 and 4. Mix with 5 at room temperature. Use instantly. Reaction stopped with addition of distilled water.

(F) Nitrocellulose paper (NCP, Amersham) preparation

Cut from roll, a piece of NCP larger than the gel

Wet the NCP by rolling onto distilled water. When completely moist, submerge for five to ten minutes.

Equilibrate the NCP by immersing in transfer buffer for ten minutes.

(G) Formation of the blot sandwich :

Soak scotch-brite pads, Whatman filter paper, and NCP in distilled water. The sandwich is made up as follows :

Bottom

Scotch-brite pad

Filter paper

NCP

Gel-make sure all air bubbles are removed

Filter paper

Scotch-brite pad

Top

Close the sandwich, and place into blotting unit (pre-filled with transfer buffer). The gel must be placed next the cathode, and the NCP next the anode. Complete transfer of protein occurs at 100mA, overnight at 4⁰C.

APPENDIX 10

Radioiodonation of surface exposed proteins

The following solutions were required for radioiodonation :

(A) Phosphate Buffered Saline (PBS) pH7.2

(B) Lactoperoxidase (Sigma) 0.08%

Lactoperoxidase 0.8mg

PBS 1.0ml

Combine and vortex vigorously until the lactoperoxidase is totally dissolved. Protect the suspension from light.

(C) 10^{-5} M Potassium iodide (KI, Merck) 1.6mg

Add this to 200ml PBS (pH7.2)

(D) 0.005M Cysteine (Sigma) 78.8mg

Add to this 100ml PBS (pH7.2)

(E) Solubilising buffer was the same as that used in SDS- PAGE

(F) Molecular weight markers

The rainbow ^{14}C protein molecular weight markers

(Amersham International) contained the following proteins:

Protein	Molecular weight (kD)	
Myosin	200	BLUE
Phosphorylase b	92.5	BROWN
Bovine serum albumin	69	RED
Ovalbumin	46	YELLOW
Carbonic anhydrase	30	ORANGE
Trypsin inhibitor	21.5	GREEN
Lysozyme	14.3	MAGENTA

Ten microlitres of the above mixture was added to 10ul solubilising buffer, boiled for one minute, and the total volume loaded onto the well.

(G) Autoradiograph development

Place the exposed X-ray plate in developer (Ilford phenisol developer) for five minutes. Rinse in running water for thirty seconds. Fix in fixer (Ilford) for five minutes. Rinse in running water for ten minutes, and then air dry.

LIST OF REFERENCES

Abeck D. and Johnson A.P. 1987. Identification of surface-exposed proteins in Haemophilus ducreyi. FEMS Microbiol. Lett. 44 : 49-51.

Abeck D., Johnson A.P., Ballard R.C., Dangor Y., Fontaine E.A. and Taylor-Robinson D. 1987. Effect of culture conditions on the protein and lipopolysaccharide profiles of Haemophilus ducreyi analysed by SDS-PAGE. FEMS Microbiol. Lett. 48 : 397-399.

Abeck D., Johnson A.P. and Taylor-Robinson D. 1988. Antigenic analysis of Haemophilus ducreyi by Western blotting. Epidemiol. Infect. 101 : 151-157.

Aguero M.E. and Cabello F.C. 1983. Relative contribution of colV plasmid and K1 antigen to the pathogenicity of Escherichia coli. Infect. Immun. 40 : 359-368.

Aguero M.E., Aron L., DeLuca A.G., Timmis K.N. and Cabello F.C. 1984. A plasmid-encoded outer membrane protein, Tra T, enhances resistance of Escherichia coli to phagocytosis.

Infect. Immun. 46 : 740-746.

Ajello G.W., Deacon W.E., Paul L. and Walls K.W. 1956. Nutritional studies of a virulent strain of Haemophilus ducreyi. J. Bacteriol. 72 : 802-808.

Albritton W.L., Maclean I.W., Bertram P.D. and Ronald A.R. 1981. Haemin requirements in Haemophilus with special reference to H. ducreyi. p. 75-82. In Haemophilus, Pasteurella and Actinbacillus. Eds. Kilian M, Fredericksen W. and Biberstein E.L. Academic Press Inc., New York.

Albritton W.L., Plummer F.A., Sottnek F.O. and Kraus S.J. 1985. Haemophilus ducreyi and Calymmatobacterium granulomatis. p. 869-873. In Manual of clinical microbiology, Fourth Edition Eds. Lennette E.H., Balows A., Hausler W.J. and Shadomy H.J. American Society for Microbiology, Washington D.C.

Albritton W.L. 1989. Biology of Haemophilus ducreyi. Microbiol. Rev. 53 : 377-389.

Amano T.K., Fujikawa T., Morioka T., Miyama A. and Ichikawa S. 1958. Quantitative studies on immune bacteriolysis. 1. A new method of quantitative estimation. Biken J. 1 : 13-25.

Anderson B., Albritton W.L., Bidlle J. and Johnson S.R. 1984. Common beta-lactamase-specifying plasmid in Haemophilus ducreyi and Neisseria gonorrhoeae. Antimicrob. Agents Chemother. 25 : 296-297.

Asin J. 1952. Chancroid : a report of 1402 cases. Am. J. Syph. Gonorrhea. Vener. Dis. 36 : 483-487.

Atkinson J.P. and Frank M.M. 1980. Complement. p. 219-271. In Clinical Immunology. Eds. Parker C.W. The W.B. Saunders Company, Philadelphia.

Ballard R.C., Fehler H.G. and Msikinya B.S. 1982. Subpreputial genital ulceration-diagnostic problems. S.A. J. Sex. Transm. Dis. 2 : 34-36.

Ballard R.C., Duncan M.O., Fehler H.G. and Dangor Y. 1989. Treating chancroid : summary of studies in southern Africa. Genitourin. Med. 65 : 54-57.

Barenkamp S.J., Munson Jnr. R.S., Granoff D.M. 1981. Subtyping isolates of Haemophilus influenzae type b by outer membrane profiles. J. Infec. Dis. 143 : 668-676.

Barenkamp S.J., Munson Jnr. R.S., Granoff D.M. 1982. Outer membrane proteins and biotype analysis of pathogenic nontypable Haemophilus influenzae. Infec. Immun. 36 : 535-540.

Bassereau P.I. 1852. Traite de affections de la peau symptomatiques de la syphilis. J.B. Bailliere, Paris.

Bassford P.J., Diedrich D.L., Schnaitman C.A. and Reeves P. 1977. Outer membrane proteins of Escherichia coli VI. Protein alteration in bacteriophage-resistant mutants. J. Bacteriol. 131 : 608-622.

Beeson P.B. 1946. Studies on chancroid. IV. The Ducrey bacillus : growth requirements and inhibition by antibiotic agents. Proc. Soc. Exp. Biol. Med. 61 : 81-85.

Beeson P.B. and Heyman A. 1945. Studies on chanroid. II. Efficiency of the cultural method of diagnosis. Am. J. Syph. Gonorrhea. Vener. Dis. 29 : 633-640.

Benge G.R. 1988. Bactericidal activity of human serum against strains of Klebsiella from different sources. J. Med. Microbiol. 27 : 11-15.

Biberstein E.L. 1963. Action of Haemophilus cultures on delta-amino-levulinic acid. J. Bacteriol. 86 : 814-819.

Biggar R.J. 1986. The AIDS problem in Africa. Lancet. 1 : 79-83.

Bilgeri Y.R., Ballard R.C., Duncan M.O., Mauff A.C. and Koornhof H.K. 1982. Antimicrobial susceptibility of 103 strains of Haemophilus ducreyi isolated in Johannesburg. Antimicrob. Agents Chemother. 22 : 686-688.

Binns M.M., Davis D.L. and Hardy K.G. 1979. Cloned fragments of the plasmid Col V, I-K94 specifying virulence and serum resistance. Nature (London) 279 : 778-781.

Bjorksten B., Bortolussi R., Gothefors L. and Quie P.G. 1976. Interaction of Escherichia coli strains with human serum : lack of relationship to K1 antigen. J. Pediatr. 89 : 892-897.

Bjorksten B. and Kaijser B. 1978. Interaction of human serum and neutrophils with Escherichia coli strains : differences between strains isolated from urine of patients with pyelonephritis or asymptomatic bacteriuria. Infec. Immun. 22 : 308-311.

Blackmore C.A., Limpakarnjanarat K., Rigau-Perez J.G., Albritton W.L. and Greenwood J.R. 1985. An outbreak of chancroid in Orange County California. J. Infect. Dis. 151 : 840-844.

Blaser M.J., Hopkins J.A., Berka R.M., Vasil M.L. and Wang W.L. 1983. Identification and characterisation of Campylobacter jejuni outer membrane proteins. Infec. Immun. 42 : 276-284.

Blaser M.J., Hopkins J. A. and Vasil M.L. 1984. Campylobacter jejuni outer membrane proteins are antigenic for humans. Infec. Immun. 43 : 986-993.

Blaser M.J., Smith P.F. and Kohler P.F. 1985. Susceptibility of Campylobacter isolates to the bactericidal activity of human serum. J. Infect. Dis. 151 : 227-235.

Blaser M.J., Smith P.F., Hopkins J.A., Heinzer I., Bryner J.H. and Wang W.L. 1987. Pathogenesis of Campylobacter fetus infections : serum resistance associated with high-molecular-weight surface proteins. J. Infect. Dis. 155 : 696-706.

Borchardt K.A. and Hoke A.W. 1970. Simplified laboratory technique for diagnosis of chancroid. Arch. Dermatol. 102 : 188-192.

Borowski R.S. and Schiller N.L. 1983. Examination of the bactericidal and opsonic activity of normal human serum for a mucoid and non-mucoid strain of Pseudomonas aeruginosa. Current Microbiol. 9 : 25-30.

Bortolussi R., Ferrieri P., Bjorksten B and Quie P.G. 1979. Capsular K1 polysaccharide of Escherichia coli : relationship to virulence in newborn rats and resistance to phagocytosis. Infect. Immun. 25 : 293-298.

Boustouller Y.L. and Johnson A.P. 1986. Resistance of Gardnerella vaginalis to the bactericidal action of human serum. Genitourin. Med. 62 : 380-383.

Bowmer M.I., Nsanze H., D'Costa L.J., Dylewski J., Fransen L., Piot P. and Ronald A.R. 1987. Single-dose ceftriaxone for chancroid. Antimicrob. Agents Chemother. 31 : 67-69.

Boyd A.S. 1989. Clinical efficacy of antimicrobial therapy in Haemophilus ducreyi infections. Arch. Dermatol. 125 : 1399-1403.

Braun V. and Rehn K. 1969. Chemical characterisation, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the Escherichia coli cell wall. Eur. J. Biochem. 10 : 426-438.

Brooks G.F. and Ingwer I. 1978. Studies on the relationships between serum bactericidal activity and uncomplicated genital infections due to Neisseria gonorrhoeae. J. Infect. Dis. 138 : 333-339.

Brooks G.F., Ingwer I. and Petersen B.H. 1978. Importance of serum bactericidal and opsonic activity and complement in Neisseria gonorrhoeae infections. p. 199-203. In immunobiology of Neisseria gonorrhoeae. Eds. Brooks G.F., Gotschlich E.C., Holmes K.K., Sawyer W.D. and Young F.E. American Society for Microbiology. Washington D.C.

Brown M.R.W. and Williams P. 1985. The influence of the environment on envelope properties affecting survival of bacteria in infections. Annual Rev. Microbiol. 39 : 527-556.

Brunton J.L., Maclean I., Ronald A.R. and Albritton W.L. 1979. Plasmid-mediated ampicillin resistance in Haemophilus ducreyi. Antimicrob. Agents Chemother. 15 : 294-299.

Brunton J., Bennett P. and Grinsted J. 1981. Molecular nature of a plasmid specifying beta-lactamase production in Haemophilus ducreyi. J. Bacteriol. 148 : 788-795.

Brunton J., Meier M., Ehrman N., Maclean I., Slaney L. and Albritton W.L. 1982. Molecular epidemiology of beta-lactamase-specifying plasmids of Haemophilus ducreyi. Antimicrob. Agents Chemother. 21 : 857-863.

Butler L.O. 1962. A defined medium for Haemophilus influenzae and Haemophilus parainfluenzae. J. Gen. Microbiol. 27 : 51-60.

Cabello F.C. 1979. Determinents of pathogenicity of Escherichia coli K1. p. 155-160. In plasmids of medical, enviromental and commercial importance. Eds. Timmis K.N. and Puhler A. Elsevier / North-Holland Publishing Co. Amsterdam.

Cagle G.D. 1975. Fine structure and distribution of extracellular polymer surrounding selected aerobic bacteria. Can. J. Microbiol. 21 : 395-407.

Cannon J.G., Lee T.J., Guymon L.F. and Sparling P.F. 1981. Genetics of serum resistance in Neisseria gonorrhoeae : the sac-1 genetic locus. Infec. and Immun. 32 : 547-552.

Carroll E.J. and Jasper D.E. 1977. Bactericidal activity of standard bovine serum against coliform bacteria isolated from udders and the enviroment of dairy cows. Am. J. Vet. Res. 38 : 2019-2022.

Carpenter J.L., Back A., Gehle D. and Oberhoffer T. 1981. Treatment of chancroid with erythromycin. Sex. Trans. Dis. 8 : 192-197.

Casciato D.A., Rosenblatt J.E., Bluestone R., Goldberg L.S. and Finegold S.M. 1979. Susceptibility of isolates of Bacteroides to the bactericidal activity of normal human serum. J. Infect. Dis. 140 : 109-113

Casin I., Grimont F., Grimont P.A.D. and Sanson-LePors M.J. 1985. Lack of deoxyribonucleic acid relatedness between Haemophilus ducreyi and other Haemophilus species. Int. J. Syst. Bacteriol. 35 : 23-25.

Cazarre A. and Barreto T.M. 1974. Haemophilus ducreyi : alguns aspectos metabolicos e morfologicos pesquisados pelo emprego de precursores radioactivos e microscopia electronica. Rev. Inst. Med. Trop. Sao Paulo 16 : 95-102.

CDC 1982. Sexually transmitted diseases - treatment guidelines. M.M.W.R. 31 (Suppl.) : 33-60.

Ceppellini R. and Landy M. 1963. Suppression of blood group agglutinability of human erythrocytes by certain bacterial polysaccharides. *J. Exp. Med.* 117 : 321-338.

Chai T.J. and Foulds J. 1977. Escherichia coli K-12 tolF mutants : alterations in protein composition of the outer membrane. *J. Bacteriol.* 130 : 781-786.

Chan A.C. and Atkinson J.P. 1985. The complement system : biology of the opsonic components. p. 4-10. In *Microbiology 1985*. Eds. Leive L. American Society for Microbiology. Washington D.C.

Chapel T.A., Brown W.J., Jeffries C. and Stewart J.A. 1977. How reliable is the morphological diagnosis of penile ulcerations? *Sex. Transm. Dis.* 4 : 150-152.

Chapel T.A., Brown W.J., Jeffries C. and Stewart J.A. 1978. The microbiological flora of penile ulcerations. *J. Infect. Dis.* 137 : 50-56.

Chernew I. and Braude A.I. 1962. Depression of phagocytosis by solutes in concentrations found in the kidney and urine. J. Clin. Invest. 41 : 1945-1953.

Chester I.R. and Meadow P.M. 1975. Heterogeneity of the lipopolysaccharide from Pseudomonas aeruginosa. Eur. J. Biochem. 58 : 273-282.

Chopra I. and Howe T.G.B. 1978. Bacterial resistance to the tetracyclines. Microbiol. Rev. 42 : 707-724.

Choudhary B.P., Kumari S., Bhati R. and Agarwal D.S. 1982. Bacteriological study of chancroid. Indian J. Med. Res. 76 : 379-385.

Clark V.L., Campbell L., Palermo D.A., Evans T.M. and Klimpel K. W. 1987. Induction and repression of outer membrane proteins by anaerobic growth of Neisseria gonorrhoeae. Infec. Immun. 55 : 1359-1364.

Cohen I.R. and Norins L.C. 1966. Natural human antibodies to Gram-negative bacteria : immunoglobulins G, A and M. Science. 152 : 1257-1259.

Collins F.M. 1964. The effect of the growth rate on the composition of Salmonella enteritidis cell walls. Aust. J. Exp. Biol. Med. Sci. 42 : 255-262.

Collins F.M. 1967. Serum mediated killing of three group D salmonellas. J. Gen. Microbiol. 46 : 247-253.

Cooper N.R. 1985. The classical complement pathway : activation and regulation of the first complement component. p. 42-52. In Advances in Immunology. Vol. 37. Eds. Dixon F.J. Academic Press. London.

Coovadia Y.M., Kharsany A. and Hoosen A. 1985. The microbial etiology of genital ulcers in black men in Durban, South Africa. Genitourin. Med. 61 : 266-269.

Coulton J.W. and Wan D.T.F. 1983. The outer membrane of Haemophilus influenzae type b : cell envelope associations of major proteins. Can. J. Microbiol. 29 : 280-287.

Crewe-Brown H.H., Krige F.K., Davel G.H., Barron C., Jansen van Vuuren J.A.M., Shipman S.O. and Roux J.G.H. 1982. Genital ulcerations in males at Ga-Rankuwa hospital, Pretoria. S. Afr. Med. J. 62 : 861-863.

Dangor Y. 1988(a). The etiology of sexually-acquired genital ulcerations in a population of mineworkers. MSc. Dissertation, University of the Witwatersrand, Johannesburg.

Dangor Y., Miller S.D., Exposto F. and Koornhof H.J. 1988(b). Antimicrobial susceptibilities of southern African isolates of Haemophilus ducreyi. Antimicrob. Agents Chemother. 32 : 1458-1460.

Dangor Y., Miller S.D. and Koornhof H.J. 1988(c). Chloramphenicol-resistant Haemophilus ducreyi in southern Africa. Lancet 2 : 344.

Dangor Y., Ballard R.C., Miller S.D. and Koornhof H.J. 1990. Antimicrobial susceptibility of Haemophilus ducreyi. Antimicrob. Agents Chemother. 34 : 1303-1307.

Darveau R.P., Charnetzky W.T. and Hurlbert R.E. 1980. Outer membrane protein composition of Yersinia pestis at different growth stages and incubation temperatures. J. Bacteriol. 143 : 942-949.

Datta D.B., Arden B. and Henning U. 1977. Major proteins of the E. coli outer cell envelope membrane as bacteriophage receptors. J. Bacteriol. 131 : 821-829.

Davis S.D. and Wedgewood R.J. 1965. Kinetics of the bactericidal action of normal serum on Gram-negative bacteria. J. Immunol. 95 : 75-79.

D'Costa L.J., Bowmer I., Nsanze H., Dylewski J., Fransen L., Plummer F.A., Piot P. and Ronald A.R. 1986. Advances in the diagnosis and management of chancroid. Sex. Transm. Dis. 13 : 189-191.

Deacon W.E., Albritton D.C., Olansky S. and Kaplan W. 1956. V.D.R.L. chancroid studies. I. A simple procedure for the isolation and identification of Haemophilus ducreyi. J. Invest. Dermatol. 26 : 399-406.

De Hormaeche R.D., Thornley M.J. and Glauert A.M. 1978. Demonstration by light and electron microscopy of capsules of gonococci recently grown in vivo. J. Gen. Microbiol. 106 : 81-91.

De Hormaeche R.D., Thornley M.J. and Holmes A. 1983. Surface antigens of gonococci : correlation with virulence and serum resistance. J. Gen. Microbiol. 129 : 1559-1567.

De Martini M., Inouye S. and Inouye M. 1976. Ultrastructure of paracrystals of a lipoprotein from the outer membrane of Escherichia coli. J. Bacteriol. 127 : 564-571.

De Matteo C.S., Hammer M.C., Baltch A.L., Smith R.P., Sutphen N.T. and Michelsen P.B. 1981. Susceptibility of Pseudomonas aeruginosa to serum bactericidal activity. J. Lab. Clin. Med. 98 : 511-518.

Denys G.A., Chapel T.A. and Jeffries C.D. 1978. An indirect fluorescent antibody technique for Haemophilus ducreyi. Health Lab. Sci. 15 : 128-132.

Dienst R.B. 1948. Virulence and antigenicity of Haemophilus ducreyi. Amer. J. Syph. Gonorrhea. Vener. Dis. 32 : 289-291.

DiRienzo J.M., Nakamura K. and Inouye M. 1978. The outer membrane proteins of Gram-negative bacteria : biosynthesis, assembly and functions. Ann. Rev. Biochem. 47 : 481-532.

Dlabac V. 1968. The sensitivity of smooth and rough mutants of Salmonella typhimurium to bactericidal and bacteriolytic action of serum, lysozyme and to phagocytosis. Folia Microbiol. (Prague) 13 : 439-449.

Duncan M.O., Bilgeri Y.R., Fehler H.G. and Ballard R.C. 1981. The diagnosis of sexually acquired genital ulcerations in black patients in Johannesburg. S. Afr. J. Sex. Transm. Dis. 1 : 20-23.

Duncan M.O., Bilgeri Y.R., Fehler H.G. and Ballard R.C. 1983. Treatment of chancroid with erythromycin. Br. J. Vener. Dis. 59 : 265-268.

Dylewski J., Nsanze H., Maitha G. and Ronald A. 1986. Laboratory diagnosis of Haemophilus ducreyi : sensitivity of culture media. Diagn. Microbiol. Infect. Dis. 4 : 241-245.

Eads M.E., Levy N.J. and Kasper D.L. 1982. Antibody-independant activation of C1 by type 1a group B streptococci. J. Infect. Dis. 146 : 665-672.

Eddy B.P. 1961. The Voges-Proskauer reaction and its significance : a review. J. Appl. Bacteriol. 24 : 27-41.

Eisenstein B.I., Lee T.J. and Sparling P.F. 1977. Penicillin sensitivity and serum resistance are independent attributes of strains of Neisseria gonorrhoeae causing disseminated gonococcal infection. *Infec. Immun.* 15 : 834-841.

Elgerfors B. and Olling S. 1978. The significance of serum-sensitive bacilli in Gram-negative bacteremia. *Scand. J. Infec. Dis.* 10 : 203-207.

Fast M.V., Nsanze H., D'Costa L.J., Karasira P., MacLean I.W., Piot P., Albritton W.L. and Ronald A.R. 1983(a). Antimicrobial therapy of chancroid : an evaluation of five treatment regimens correlated with in vitro sensitivity. *Sex. Transm. Dis.* 10 : 1-6.

Fast M.V., Nsanze H., Plummer F.A., D'Costa L.J., MacLean I.W. and Ronald A.R. 1983(b). Treatment of chancroid. A comparison of sulphamethoxale and trimethoprim-sulphamethoxazole. *Br. J. Vener. Dis.* 59 : 320-324.

Fearon D.T. 1978. Regulation by membrane sialic acid of beta1H-dependant decay-dissociation of amplification C3 convertase of the alternative complement pathway. Proc. Nat. Acad. Sci. USA 75 : 1971-1975.

Fearon D.T. and Austen K.F. 1977. Activation of the alternative complement pathway due to resistance of zymosan-bound amplification convertase to endogenous regulatory mechanisms. Proc. Nat. Acad. Sci. USA 74 : 1683-1687.

Feiner R.R. and Mortara F. 1945. Infectivity of Haemophilus ducreyi for the rabbit and the development of skin hypersensitivity. Am. J. Syph. Gonorrhoea. Vener. Dis. 29 : 71-79.

Feingold D.S., Goldman J.N. and Kuritz H.M. 1968. Locus of the action of serum and the role of lysozyme in the serum bactericidal reaction. J. Bacteriol. 96 : 2118-2126.

Feingold D.S., Goldman J.N. and Kuritz H.M. 1968. Locus of the lethal event in the serum bactericidal reaction. J. Bacteriol. 96 : 2127-2131.

Felman Y.M. and Nikitas J.A. 1983. Update on chancroid. *Cutis* 31 : 602-608.

Feltham S., Ronald A.R. and Albritton W.L. 1979. A comparison of the in vitro activity of rosamicin, erythromycin, clindamycin, metronidazole, and ornidazole against Haemophilus ducreyi, including beta-lactamase producing strains. *J. Antimicrob. Chemother.* 5 : 731-734.

Fierer J., Finley F. and Braude A.I. 1972. A plaque assay on agar for detection of Gram-negative bacilli sensitive to complement. *J. Immunol.* 109 : 1156-1158.

Fierer J., Finley F. and Braude A.I. 1974. Release of ⁵¹Cr-endotoxin from bacteria as an assay of serum bactericidal activity. *J. Immunol.* 112 : 2184-2192.

Filip C., Fletcher G., Wulff J.L. and earhart C.F. 1973. Solubilisation of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium-lauryl sarcosinate. *J. Bacteriol.* 115 : 717-722.

Finn G.Y., Karim Q.N. and Easmon C.S.F. 1990. The production and characterisation of rabbit antiserum and murine monoclonal antibodies to Haemophilus ducreyi. J. Med. Microbiol. 31 : 219-224.

Gaisen A. and Heaton C.L. 1975. Chancroid : alias the soft chancre. Int. J. Dermatol. 14 : 188-197..

Gemski P., Cross A.S. and Sadoff J.C. 1980. K1 antigen-associated resistance to the bactericidal activity of serum. FEMS Microbiol. Lett. 9 : 193-197.

Glynn A.A. 1969. The complement lysozyme sequence in immune bacteriolysis. Immunology. 16 : 463-471.

Glynn A.A. 1970. The sensitivity to complement of strains of Escherichia coli related to their K antigens. Immunology. 18 : 331-346.

Goldman R.C. and Leive L. 1980. Heterogeneity of antigenic-side-chain length in lipopolysaccharide from Escherichia coli 0111 and Salmonella typhimurium LT2. Eur. J. Biochem. 107 : 145-153.

Goldschneider I., Gotschlich E.C. and Artenstein M.S. 1969. Human immunity to the meningococcus 1. The role of humoral antibodies. J. Exp. Med. 129 : 1307-1326.

Gotze O. and Muller-Eberhard H.J. 1971. The C-3 activator system : an alternate pathway of complement activation. J. Exp. Med. Suppl. 134 : 90-108.

Gower P.E., Taylor P.W., Koutsaimanis K.G. and Roberts A.P. 1972. Serum bactericidal activity in patients with upper and lower urinary tract infections. Clin. Sci. 43 : 13-22.

Greenblatt R.B. and Sanderson E.S. 1938. The intradermal chancroid bacillary antigen test as an aid in the differential diagnosis of the venereal bubo. Am. J. Surg. 41 : 384-389.

Griffiths E. 1974. Metabolically controlled killing of Pasteurella septica by antibody and complement. Biochim. Biophys. Acta. 362 : 598-602.

Gudas L.J., James R. and Pardee A.B. 1976. Evidence for the involvement of an outer membrane protein in DNA initiation. J. Biol. Chem. 251 : 3470-3479.

Gulig P.A., McCracken G.H., Frisch C.F., Johnston K.H. and Jansen E.J. 1982. Antibody response of infants to cell-surface exposed outer membrane proteins of Haemophilus influenzae type b after systemic Haemophilus disease. Infec. Immun. 37 : 82-88.

Hafiz S., McEntergart M.G. and Kinghorn G.R. 1984. Sheffield medium for cultivation of Haemophilus ducreyi. Br. J. Vener. Dis. 60 : 196-198.

Hammond G.W., Lian C.J., Wilt J.C., Albritton W.L. and Ronald A.R. 1978. Determination of the haemin requirement of Haemophilus ducreyi : evaluation of the porphyrin test and media used in the satellite growth test. J. Clin. Microbiol. 7 : 243-246.

Hammond G.W., Lian C.J., Wilt J.C. and Ronald A.R. 1978. Antimicrobial susceptibility of Haemophilus ducreyi. Antimicrob. Agents Chemother. 13 : 608-612.

Hammond G.W., Lian C.J., Wilt J.C. and Ronald A.R. 1978. Comparison of specimen collection and laboratory techniques for isolation of Haemophilus ducreyi. J. Clin. Microbiol. 7 : 39-43.

Hammond G.W., Slutchuk M., Lian C.J., Wilt J.C. and Ronald A.R. 1979. The treatment of chancroid : comparison of one week of sulfisoxazole with single-dose doxycycline. J. Antimicrob. Chemother. 5 : 261-265.

Hammond G.W., Slutchuk M., Scatliff J., Sherman E., Wilt J.C. and Ronald A.R. 1980. Epidemiologic, clinical, laboratory, and therapeutic features of an urban outbreak of chancroid in North America. Rev. Infect. Dis. 2 : 867-879.

Hancock R.E.W. and Braun V. 1976. Nature of the energy requirement for the irreversable adsorption of bacteriophage T1 and O80 to Escherichia coli. J. Bacteriol. 125 : 409-415.

Hand W.L. and King N.L. 1977. Deficiency of serum bactericidal activity against Salmonella typhimurium in sickle-cell anaemia. Clin. Exp. Immunol. 30 : 262-270.

Handsfield H.H., Totten P.A., Fennel C.L., Falkow S. and Holmes K.K. 1981. Molecular epidemiology of Haemophilus ducreyi infections. Ann. Intern. Med. 95 : 315-318.

Hannah P. and Greenwood J.R. 1982. Isolation and rapid identification of Haemophilus ducreyi. J. Clin. Microbiol. 16 : 861-864.

Hanschell H.M. 1938. Sulfonamide in the treatment of chancroid. Lancet. i : 886-888.

Hansen E.J. and Loftus T.A. 1984. Monoclonal antibodies reactive with all strains of Haemophilus ducreyi. Infect. Immun. 44 : 196-198.

Harriman G.R., Podack E.R., Brauck A.I., Corbeil L.C., Esser A.F. and Curd J.G. 1982. Activation of complement by Neisseria gonorrhoeae. J. Exp. Med. 156 : 1235-1249.

Heckels J.E. 1977. The surface properties of Neisseria gonorrhoeae : isolation of the major components of the outer membrane. J. Gen Microbiol. 99 : 333-341.

Heckels J.E. 1978. The surface properties of Neisseria gonorrhoeae : topographical distribution of the outer membrane protein antigens. J. Gen. Microbiol. 108 : 213-219.

Hendley J.O., Powell K.R., Rodewald R., Holzgreffe H.H. and Lyles R. 1977. Demonstration of a capsule on Neisseria gonorrhoeae. N. Engl. J. Med. 296 : 608-611.

Herriott R.M., Meyer E.Y., Vogt M. and Modan M. 1970. Defined medium for growth of Haemophilus influenzae. J. Bacteriol. 101 : 513-516.

Heyman A., Beeson P.B. and Sheldon W.H. 1945. Diagnosis of chancroid. The relative efficiency of biopsies, cultures, smears, autoinoculations and skin tests. J. Amer. Med. Assoc. 129 : 935-938.

Hildebrandt J.F., Mayer L.W., Wang S.P. and Buchanan T.M. 1978. Neisseria gonorrhoeae acquire a new principal outer membrane protein when transformed to resistance to serum bactericidal activity. Infect. Immun. 20 : 267-273.

Hirsch R.L. 1982. The complement system : its importance in the host response to viral infection. Microbiol. reviews. 46 : 71-85.

Hollander R., Hess-Reihse A. and Mannheim W. 1981. Respiratory quinones in Haemophilus, Pasteurella and Actinobacillus : pattern, function and taxonomic evaluation. p. 83-97. In Haemophilus, Pasteurella and Actinobacillus. Eds. Kilian M., Frederiksen W. and Biberstein E.L. Academic Press Inc., New York.

Hughes C., Phillips R. and Roberts A.P. 1982. Serum resistance among Escherichia coli strains causing urinary tract infection in relation to O-type and the carriage of hemolysin, colicin and antibiotic resistance determinants. Infect. Immun. 35 : 270-275.

Inouye K. 1972. Immune bacteriolytic and bactericidal reactions. p. 177-222. In Research in immunochemistry and immunobiology. Vol. 1. Eds. Kwapinski J.B.G. University Park Press. Baltimore.

Inouye K., Takamizawa A., Kurimura T., and Yonemasu K. 1968. Studies on the immune bacteriolysis. XIII. Leakage of enzymes from Escherichia coli during immune bacteriolysis. Biken J. 11 : 193-201.

Inouye M. and Yee M. 1972. Specific removal of proteins from the envelope of Escherichia coli by protease treatments. J. Bacteriol. 112 : 585-592.

Inouye M. and Yee M. 1973. Homogeneity of envelope proteins of Escherichia coli separated by gel electrophoresis in SDS. J. Bacteriol. 113 : 304-312.

Inouye M. 1974. A three-dimensional assembly model of a lipoprotein from the Escherichia coli outer membrane. Proc. Nat. Acad. Sci. USA 71 : 2396-2400.

Jahn G., Laufs R., Kaulfers R.M. and Kolenda H. 1979. Molecular nature of two Haemophilus influenzae R-factors containing resistances and the multiple intergration of drug resistance transposons. J. Bacteriol. 138 : 584-597.

James J.F. and Swanson J. 1978. Studies on gonococcus infection XIII. Occurrence of colour-opacity colonial variants in clinical cultures. *Infect. Immun.* 19 : 332-340.

Jann B., Jann K., Schmidt G., Orskov I. and Orskov F. 1970. Immunochemical studies of polysaccharide surface antigens of Escherichia coli 0100:K?(B):H2. *Eur. J. Biochem.* 15 : 29-39.

Jann K. and Westphal O. 1975. Microbial polysaccharides. p. 1-125. In *The Antigens*. Vol. 3. Eds. Sela M. Academic Press Inc. New York.

Johnson A.P., Abeck D. and Davies H.A. 1988. The structure, pathogenicity and genetics of Haemophilus ducreyi. *J. Infect.* 17 : 99-106.

Johnson D.E., Calia F.M., Musher D.M. and Goree A. 1984. Resistance of Vibrio vulnificus to serum bactericidal and opsonising factors : relation to virulence in suckling mice and humans. *J. Infect. Dis.* 150 : 413-418.

Johnson S.R., Biddle J.W. and De Witt W.E. 1989. Detection and characterisation of the tetracycline-resistance determinant, Tet M, in Haemophilus ducreyi. Curr. Microbiol. 19 : 7-12.

Joiner K.A., Hammer C.H., Brown E.J., Cole R.T. and Frank M.M. 1982(a). Studies on the mechanisms of bacterial resistance to complement-mediated killing 1. Terminal complement components are deposited and released from Salmonella minnesota S218 without causing bacterial death. J. Exp. Med. 155 : 797-808.

Joiner K.A., Hammer C.H., Brown E.J. and Frank M.M. 1982 (b). Studies on the mechanisms of bacterial resistance to complement-mediated killing. II. C8 and C9 release C5b67 from the surface of Salmonella minnesota S218 because the terminal complex does not insert into the bacterial outer membrane. J. Exp. Med. 155 : 809-819.

Jones A.E. 1927. Antimony and potassium tartrate in chancroidal infections : results of intravenous treatment. JAMA 88 : 1699-1700.

Jones B.M., Hafiz S. and Duerden B.I. 1986. Susceptibility of Haemophilus ducreyi to ampicillin and sulbactam in vitro. Antimicrob. Agents Chemother. 29 : 1110-1112.

Judd R.C. 1982. ¹²⁵I-peptide mapping of protein III isolated from four strains of Neisseria gonorrhoeae. Infect. Immun. 37 : 622-631.

Kaijser B., Hanson L.A., Jodal U., Lidin-Jansen G. and Robbins J.B. 1977. Frequency of E. coli K antigens in urinary tract infections in children. Lancet 1 : 663-664.

Kampmeier R.H. 1982. The recognition of Haemophilus ducreyi as the cause of soft chancre. Sex. Trans. Dis. 9 : 212-213.

Kasper D.L. 1976. The polysaccharide capsule of Bacteroides fragilis subspecies fragilis : immunochemical and morphologic definition. J. Infect. Dis. 133 : 79-87.

Kato K. and Bito Y. 1978. Relationship between bactericidal action of complement and fluidity of cellular membranes. Infect. Immun. 19 : 12-17.

Kay W.W., Buckley J.T., Ishiguro E.E., Phipps B.M., Monette J.P.L. and Trust T.J. 1981. Purification and disposition of a surface protein associated with virulence of Aeromonas salmonicida. J. Bacteriol. 147 : 1077-1084.

Kelley J.T. and Parker C.D. 1981. Identification and preliminary characterisation of Vibrio cholerae outer membrane proteins. J. Bacteriol. 145 : 1018-1024.

Kerber R.E., Rowe C.E. and Gilbert K.R. 1969. Treatment of chancroid : a comparison of tetracycline and sulfisoxazole. Arch. Dermatol. 100 : 604-607.

Kilian M. 1974. A rapid method for the differentiation of Haemophilus strains. Acta. Pathol. Microbiol. Scand. 82 : 835-842.

Kilian M. 1976. A taxonomic study of the genus Haemophilus, with the proposal of a new species. J. Gen. Microbiol. 93 : 9-62.

Kilian M. and Theilade J. 1975. Cell wall ultrastructure of strains of Haemophilus ducreyi and Haemophilus piscium. Int. J. Syst. Bacteriol. 25 : 351-356.

Kilian M. and Biberstein E.L. 1984. Genus II. Haemophilus. p. 558-569. In Bergey's manual of systematic bacteriology, vol. 1. Eds. Krieg N.R. and Holt J.G. The Williams and Wilkins Co., Baltimore.

Kim K.S. and Anthony B.F. 1981. Importance of bacterial growth phase in determining minimal bactericidal concentrations of penicillin and methicillin. Antimicrob. Agents Chemother. 19 : 1075-1077.

Kimball H., Garcia M. and Petersdorf R.G. 1964. The epidemiology of nonenteric Escherichia coli infections. II. Relationship of prevalence of E. coli in urinary infections to the bactericidal effect of human serum. J. Lab. Clin. Med. 63 : 901-906.

Kimura A., Gulig P.A., McCracken G.H., Loftus T.A. and Hansen E.J. 1985. A minor high molecular weight outer membrane protein of Haemophilus influenzae type b is a protective antigen. Infec. Immun. 47 : 253-259.

King G.J. and Swanson J. 1978. Studies on gonococcus infection XV. Identification of surface proteins of Neisseria gonorrhoeae correlated with leukocyte association. *Infect. Immun.* 21 : 575-584.

King B.F., Biel M.L. and Wilkinson B.J. 1980. Facile penetration of the Staphylococcus aureus capsule by lysostaphin. *Infect. Immun.* 29 : 892-896.

Kinghorn G.R., Hafiz S. and McEntegart M.G. 1982. Pathogenic microbial flora of genital ulcers in Sheffield with particular reference to Herpes simplex virus and Haemophilus ducreyi. *Br. J. Vener. Dis.* 58 : 377-380.

Klein R.D. and Luginbuhl G.H. 1979. Simplified media for the growth of Haemophilus influenzae from clinical and normal flora sources. *J. Gen. Microbiol.* 113 : 409-411.

Koornhof H.J., Ballard R.C., Duncan M.O., Bilgeri Y.R. and Fehler H.G. 1981. A clinical and microbiological evaluation of the treatment of chancroid with co-trimoxazole. p. 937-938. In *Current Chemotherapy and Immunology. Proceedings of the 12th International Congress of Chemotherapy.* American Society for Microbiology, Washington D.C.

Kraus S.J., Kaufman H.W., Albritton W.L., Thornsberry C. and Biddle J.W. 1982. Chancroid therapy : a review of cases confirmed by culture. Rev. Infect. Dis. 4 (Suppl.) : 848-856.

Kreiss J.K., Koech D. and Plummer F.A. 1986. AIDS virus infection in Nairobi prostitutes : spread of the epidemic to East Africa. N. Engl. J. Med. 314 : 414-418.

Kunimoto D.Y., Slaney Y., Koss J., D'Costa L.J., Plummer F.A., Ndina-Achola J.O. and Ronald A.R. 1986. Field testing of modified Bieling media for the isolation of Haemophilus ducreyi in Kenya. Eur. J. Clin. Microbiol. 5 : 673-675.

Lambden P.R., Heckels J.E., James L.T. and Watt P.J. 1979. Variations in surface protein composition associated with virulence properties in opacity types of Neisseria gonorrhoeae. J. Gen. Microbiol. 114 : 305-312.

Landy M., Sanderson R.P. and Jackson A.L. 1965. Humoral and cellular aspects of the immune response to the somatic antigen of Salmonella enteritidis. J. Exp. Med. 122 : 483-504.

Lane B.C. and Hurlbert R.E. 1980. Isolation and partial characterisation of the major outer membrane protein of Chromatium vinosum. J. Bacteriol. 143 : 349-354.

Lapage S.P., Shelton J.E. and Mitchell T.G. 1970. Media for the maintenance and preservation of bacteria. p. 1-33. In Methods in microbiology, vol. 3A. Eds. Norris J.R. and Ribbons D.W. Academic Press Inc. London.

Latif A.S. 1982. Thiamphenicol in the treatment of chancroid in men. Br. J. Vener. Dis. 58 : 54-55.

Latif A.S., Crochiolo P.R. and Lencioni R. 1984. Thiamphenicol in the treatment of chancroid in men. Sex. Transm. Dis. 4 (Suppl.) : 445-455.

Laufs R., Kaulfers P.M., Jahn G. and Teschner V. 1979. Molecular characterisation of a small Haemophilus influenzae plasmid specifying beta-lactamase and its relationship to R factors from Neisseria gonorrhoeae. J. Gen. Microbiol. 111 : 223-231.

Loeb M.R. and Smith D.H. 1980. Outer membrane protein composition in disease isolates of Haemophilus influenzae : pathogenic and epidemiological implications. *Infec. Immun.* 30 : 709-717.

Loeb M.R., Zachary A.L. and Smith D.H. 1981. Isolation and partial characterisation of outer and inner membranes from encapsulated Haemophilus influenzae type b. *J. Bacteriol.* 145 : 596-604.

Loeb M.R. and Smith H.D. 1982. Human antibody response to individual outer membrane proteins of Haemophilus influenzae type b. *Infec. Immun.* 37 : 1032-1036.

Loeb M.R. and Smith D.H. 1983. Lactoperoxidase and Iodogen-catalyzed iodination labels inner and outer membrane proteins of Haemophilus influenzae. *J. Bacteriol.* 155 : 443-446.

Loos M., Bitter-Suermann D. and Dierich M. 1974. Interaction of the first (C1) , the second (C2) and the fourth (C4) component of complement with different preparations of bacterial lipopolysaccharides and with lipid A. J. Immunol. 112 : 935-940.

Loos M., Wellek B., Thesen R. and Opfer kuch W. 1978. Antibody-independent interaction of the first component of complement with Gram-negative bacteria. Infect. Immun. 22 : 5-9.

Luders G., Braun J., Pietzcker F. and Schule D. 1975. Neue therapeutische Gesichtspunkte beim ulcess Molle. Hautarzt 26 : 35-40.

Lugtenberg B. and Van Alphen L. 1983. Molecular architecture and functioning of the outer membrane of Escherichia coli and other Gram-negative bacteria. Biochim. et Biophys. Acta. 737 : 51-115.

Lwoff A. and Pirotsky I. 1937. Determination du facteur de croissance pour Haemophilus ducreyi. C.R. Soc. Biol. 124 : 1169-1171.

Lykke-Olsen L., Larsen L., Pederson T.G. and Gaarslev K. 1979. Epidemic of chancroid in Greenland. Lancet. 1 : 654-655.

Mabey D.C., Wall R.A. and Bello C.S. 1987. Etiology of genital ulceration in the Gambia. Genitourin. Med. 63 : 312-315.

Mackie E.B., Longenecker B.M., Rabin H.R., DiNinno V.L. and Bryan L.E. 1982. Immune response of the mouse to Gram-negative bacterial outer membrane extracts as assessed with monoclonal antibodies. J. Immunol. 129 : 829-832.

Madonna G.S. and Allen R.C. 1981. Shigella sonnei phase I and phase II : susceptibility to direct serum lysis and opsonic requirements necessary for stimulation of leukocyte redox metabolism and killing. Infect. Immun. 32 : 153-159.

Mallard R.H., Macaulay M.E., Riordan T., Chowdhury P.H., Chandio S. and Bhattacharyya M.N. 1983. Haemophilus ducreyi infection in Manchester. Lancet ii : 283.

Marcus R.L., Shin H.S. and Mayer M.M. 1971. An alternative complement pathway : C3-cleaving activity, not due to C4,2a, on endotoxic lipopolysaccharide after treatment with guinea-pig serum. Proc. Nat. Acad. Sci. USA 68 : 1351-1357.

Marmar J.L. 1972. The management of resistant chancroid in Vietnam. J. Urol. 107 : 807-808.

Marsch W.C., Haas N. and Stuttgen G. 1978. Ultrastructural detection of Haemophilus ducreyi in biopsies of chancroid. Arch. Dermatol. Res. 263 : 153-157.

Marshall B., Roberts M., Smith A. and Levy S.B. 1984. Homogeneity of transferable tetracycline-resistance determinants in Haemophilus species. J. Infect. Dis. 149 : 1028-1029.

Martin P.M.V., Patel P.V., Parsons N.J. and Smith H. 1982. Induction of phenotypically-determined resistance of Neisseria gonorrhoeae to human sera by serum from patients

with gonorrhoea. Br. J. Vener. Dis. 58 : 302-304.

Martines R.J. and Carroll S.F. 1980. Sequential metabolic expressions of the lethal process in human serum-treated Escherichia coli : role of lysozyme. Infect. Immun. 28 : 735-745.

Mayer M.M. 1978. Complement, past and present. Harvey Lectures. 72 : 139-150.

Mayer M.M. 1981. Membrane damage by complement. John Hopkins Med. J. 148 : 243-258.

McBride H.M., Lambden P.R., Heckels J.E. and Watt P.J. 1981. The role of outer membrane proteins in the survival of Neisseria gonorrhoeae P9 within guinea-pig subcutaneous chambers. J. Gen. Microbiol. 126 : 63-67.

McCabe W.R. and Jackson G.G. 1962. Gram-negative bacteremia. I. Etiology and ecology. Arch. Intern. Med. 110 : 847-855.

McCabe W.R., Kaijser B., Olling S., Uwaydah M. and Hanson L.A. 1978. Escherichia coli in bacteremia : K and O antigens and serum sensitivity of strains from adults and neonates. J. Infect. Dis. 138 : 33-41.

McConahey P.J. and Dixon F.J. 1980. Radioiodination of proteins by the use of the chloramine-T method. p. 210-213. In Methods in Enzymology. Vol. 70. Immunochemical techniques part A. Eds. Van Vunakis H. and Langone J.J. Academic Press Inc. New York.

McEntegart M.G., Hafiz S. and Kinghorn G.R. 1982. Haemophilus ducreyi infections - time for reappraisal. J. Hyg. 89 : 467-478.

McNicol P.J. and Ronald A.R. 1984. The plasmids of Haemophilus ducreyi. J. Antimicrob. Chemother. 14 : 561-564.

McShan W.M., Williams R.P. and Hull R.A. 1987. A recombinant molecule from a disseminating strain of Neisseria gonorrhoeae that confers serum bactericidal resistance. Infect. Immun. 55 : 3017-3022.

Meheus A., Van Dyck E., Ursi J.P., Ballard R.C. and Piot P. 1983. Etiology of genital ulcerations in Swasiland. Sex. Transm. Dis. 10 : 33-35.

Michael J.G. and Braun W. 1964. Analysis of sequential stages in serum bactericidal reactions. J. Bacteriol. 87 : 1067-1072.

Michael J.G. and Landy M. 1961. Endotoxic properties of Gram-negative bacteria and their susceptibility to the lethal effect of normal serum. J. Infect. Dis. 108 : 90 -94.

Michael J.G. and Rosen F.S. 1963. Association of "natural" antibodies to Gram-negative bacteria with the delta-macroglobulins. J. Exp. Med. 118 : 619-626.

Miles A.A. and Misra S.S. 1938. The estimation of the bactericidal power of the blood. J. Hyg. 38 : 732-738.

Miller T.E. and North J.D.K. 1974. Host response in urinary tract infections (editorial). Kidney Int. 5 : 179-186.

Mills S.D. and Bradbury W.C. 1984. Human antibody response to outer membrane proteins of Campylobacter jejuni during infection. *Infec. Immun.* 43 : 739-743.

Mittal K.R. and Ingram D.G. 1975. Factors involved in bactericidal activity of sheep serum. *Am. J. Vet. Res.* 36 : 1183-1187.

Moll A., Cabello F. and Timmis K.N. 1979. Rapid assay for the determination of bacterial resistance to the lethal activity of serum. *FEMS Microbiol. Lett.* 6 : 273-276.

Morrison D.C. and Kline L.F. 1977. Activation of the classical and properdin-pathways of complement by bacterial lipopolysaccharide. *J. Immunol.* 118 : 362-368.

Morrison M. 1980. Lactoperoxidase-catalysed iodination as a tool for investigation of proteins. p. 214-220. In *Methods in Enzymology*. Vol. 70. Immunochemical Techniques. Part A. Eds. Van Vunakis H. and Langone J.J. Academic Press Inc. New York.

Morse S.A. 1989. Chancroid and Haemophilus ducreyi. *Clin. Microbiol. Rev.* 2 : 137-157.

Mortara F., Feiner R.R. and Levenkron E. 1944. Activity of penicillin against Haemophilus ducreyi in vitro. Proc. Soc. Exp. Biol. Med. 56 : 163-166.

Mortara F.M. and Saito M.T. 1946. Sensitivity of Haemophilus ducreyi to antibiotic and other substances in vitro. Am. J. Syph. Gonorrhea. Vener. Dis. 30 : 352-360.

Muller-Eberhard H.J. and Schreiber R.D. 1980. Molecular biology and chemistry of the alternative pathway of complement. p. 2-46. In Advances in Immunology. Vol. 29. Eds. Kimkel H.G. and Dixon F.J. Academic Press. London.

Muller F. and Muller K.H. 1988. Immunoglobulin M and G antibody response in rabbits after experimental Haemophilus ducreyi infection. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe. A 268 : 238-244.

Munn C.B., Ishiguro E.E., Kay W.W. and Trust T.J. 1982. Role of surface components in the serum resistance of virulent Aeromonas salmonicida. Infec. Immun. 36 : 1069-1075.

Murtheria L.M., Nicas T.I. and Hancock R.E.W. 1982. Outer membrane proteins of Pseudomonas aeruginosa serotype strains. J. Infec. Dis. 146 : 770-779.

Muschel L.H. and Treffers H.P. 1956. Quantitative studies on the bactericidal actions of serum and complement. 1. A rapid photometric growth assay for bactericidal activity. J. Immunol. 76 : 1-10.

Muschel L.H. 1960. Serum bactericidal actions. Ann. N.Y. Acad. Sci. 88 : 1265-1272.

Muschel L.H. and Jackson J.E. 1966. The reactivity of serum against protoplasts and spheroplasts. J. Immunol. 97 : 46-51.

Muschel L.H. and Schmoker K. 1966. Activity of mitomycin C , other antibiotics and serum against lysogenic bacteria. J. Bacteriol. 92 : 967-971.

Muschel L.H., Ahl L.A. and Baron L.S. 1968. Effect of lysogeny on serum sensitivity. J. Bacteriol. 96 : 1912-1914.

Muschel L.H. and Larsen L.J. 1970. Effect of hypertonic sucrose upon the immune bactericidal reaction. Infect. Immune. 1 : 51-55.

Muschel L.H. and Larsen L.J. 1970. The sensitivity of smooth and rough Gram-negative bacteria to the immune bactericidal reaction. Proc. Soc. Exp. Biol. Med. 133 : 345-348.

Museyi K., Van Dyck E., Vervoort T., Taylor D., Hoge C. and Piot P. 1988. Use of an enzyme immunoassay to detect serum IgG antibodies to Haemophilus ducreyi. J. Infect. Dis. 157 : 1039-1048.

Myers D.E., Stocker B.A.D. and Roantree R.J. 1980. Mapping of genes determining penicillin-resistance and serum-sensitivity in Salmonella enteritidis. J. Gen. Microbiol. 118 : 367-376.

Naamara W., Plummer F., Greenblat R, D'Costa L.J., Ndinya-Achola J.O. and Ronald A.R. 1987. Treatment of chancroid with ciprofloxacin. Amer. J. Med. 825 : 317-320.

Nakae T. 1976. Outer membrane of Salmonella : Isolation of protein complex that produces transmembrane channels. J. Biol. Chem. 251 : 2176-2178.

Nakamura K. and Mizushima S. 1975. In vitro reassembly of the membranous vesicle from Escherichia coli outer membrane components : role of individual components and magnesium ions in reassembly. Biochim. Biophys. Acta. 413 : 371-393.

Nakano M., Kishimoto Y. and Saito K. 1968. Spot method in agar for the quantitative assay of anti-Salmonella antibody. Jpn. J. Microbiol. 12 : 364-366.

Nayyer K.C., Stolz E. and Michel M.F. 1979. Rising incidence of chancroid in Rotterdam. Br. J. Vener. Dis. 55 : 439-441.

Ndinya-Achola J.O., Nsanze H., Karasira P., Fransen L., D'Costa L.J., Piot P. and Ronald A.R. 1986. Three day oral course of augmentin to treat chancroid. Genitourin. Med. 62 : 202-204.

Nelson B.W. and Roantree R.J. 1967. Analyses of lipopolysaccharides extracted from penicillin-resistant, serum-sensitive Salmonella mutants. J. Gen. Microbiol. 48 : 179-188.

Newell D.G., McBride H. and Pearson A.D. 1984. The identification of outer membrane proteins and flagella of Campylobacter jejuni. J. Gen. Microbiol. 130 : 1201-1208.

Nicolet J., Paroz P. and Krawinkler M. 1980. Polyacrylamide gel electrophoresis of whole-cell proteins of porcine strains of Haemophilus. Int. J. System. Bacteriol. 30 : 69-76.

Nikaido H. and Vaara M. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49 : 1-32.

Noble R.C. and Schell S.C. 1978. Acrylamide gel electrophoresis of proteins of Neisseria gonorrhoeae as an epidemiological tool. Infect. Immun. 19 : 178-186.

Nobre G.N. 1982. Identification of Haemophilus ducreyi in the clinical laboratory. J. Med. Microbiol. 15 : 243-245.

Nsanze H., Fast M.V., D'Costa L.J., Tukei P., Curran J. and Ronald A. 1981. Genital ulcers in Kenya. Clinical and laboratory study. Br. J. Vener. Dis. 57 : 378-381.

Nsanze H., Plummer F.A., Maggwa A.B.N., Maitha G., Dylewski J., Piot P. and Ronald A.R. 1984. Comparison of media for the primary isolation of Haemophilus ducreyi. Sex. Trans. Dis. 11 : 6-9.

Oberhofer T.R. and Back A.E. 1982. Isolation and cultivation of Haemophilus ducreyi. J. Clin. Microbiol. 15 : 625-629.

O'Callaghan C.H., Morris A., Kirby S.M. and Stingler A.M. 1972. Novel method for detection of beta-lactamase by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. 1 : 283-288.

Odumeru J.A., Ronald A.R. and Albritton W.L. 1983. Characterisation of cell proteins of Haemophilus ducreyi by polyacrylamide gel electrophoresis. J. Infect. Dis. 148 : 710-714.

Odumeru J.A., Wiseman G.M. and Ronald A.R. 1984. Virulence factors of Haemophilus ducreyi. *Infect. Immun.* 43 : 607-611.

Odumeru J.A., Wiseman G.M. and Ronald A.R. 1985. Role of lipopolysaccharide and complement in susceptibility of Haemophilus ducreyi to human serum. *Infect. Immun.* 50 : 495-499.

Ogata R.T. and Levine R.P. 1980. Characterisation of complement resistance in Escherichia coli conferred by the antibiotic resistance plasmid R100. *J. Immunol.* 125 : 1494-1498.

Olling S. 1977. Sensitivity of Gram-negative bacilli to the serum bactericidal activity : a marker of the host parasite relationship in acute and persisting infections. *Scan. J. Infect. Dis. Suppl.* 10 : 1 -40.

Opal S., Cross A. and Gemski P. 1982. K antigen and serum sensitivity of rough Escherichia coli. *Infect. Immun.* 37 : 956-960.

Orskov I., Orskov F., Jann B. and Jann K. 1977. Serology, chemistry and genetics of O and K antigens of Escherichia coli. Bacteriol. Rev. 41 : 667-710.

Osborn M.J. and Wu H.C.P. 1980. Proteins of the outer membrane of Gram-negative bacteria. Ann. Rev. Microbiol. 34 : 369-422.

Ovchinnikov N.M., Delektorskij V.V., Tischenko L.D. and O.G. Omjelchenko O.G. 1976. Ultrastructure of the causative agent of soft chancre. Vestn. Dermatol. Venerol. 11 : 37-38.

Overbeeke N. and Lugtenberg B. 1980. Major outer membrane proteins of Escherichia coli strains of human origin. J. Gen. Microbiol. 121 : 373-380.

Page L.A. 1962. Haemophilus infections in chickens. I. Characteristics of 12 Haemophilus isolates recovered from diseased chickens. Am. J. Vet. Res. 23 : 85-95.

Pai C.H. and DeStephano L. 1982. Serum resistance associated with virulence in Yersinia enterocolitica. Infect. Immun. 35 : 605-611.

Parsons L.M., Shayegani M., Waring A.L. and Bopp L.H. 1989. DNA probes for the identification of Haemophilus ducreyi. J. Clin. Microbiol. 27 : 1441-1445.

Patel P.V., Martin P.M.V., Tan E.L., Nairn C.A., Parsons N.J., Goldner M. and Smith H. 1988. Protein changes associated with induced resistance of Neisseria gonorrhoeae to killing by human serum are relatively minor. J. Gen. Microbiol. 134 : 499-507.

Payne W.J., Marshall D.L., Shockley R.K. and Martin W.J. 1988. Clinical laboratory applications of monoclonal antibodies. Clin. Microbiol. Rev. 1 : 313-329.

Petersen B.H., Lee T.J., Snyderman R. and Broks G.F. 1979. Neisseria meningitidis and Neisseria gonorrhoeae bacteremia associated with C6, C7, or C8 deficiency. Ann. Intern. Med. 90 : 917-920.

Peterson L.R., Garding D.N., Hall W.H. and Schierl E.A. 1978. Medium-dependant variation in bactericidal activity of antibodies against susceptible Staphylococcus aureus. Antimicrob. Agents Chemother. 13 : 665-668.

Pien F.D. and Vosti K.L. 1974. Variation in performance of the serum bactericidal test. Antimicrob. Agents Chemother. 6 : 330-333.

Plummer F.A., D'Costa L.J., Nsanze H., Karasira P., MacLean I.W., Piot P. and Ronald A.R. 1983. Antimicrobial therapy of chancroid : effectiveness of erythromycin. J. Infect. Dis. 148 : 726-731.

Plummer F.A., Nsanze H., D'Costa L.J., Ndugga A.B., Maggwa Y., Girouard P., Karasira P., Albritton W.L. and Ronald A.R. 1983. Short-course and single-dose antimicrobial therapy for chancroid in Kenya : studies with rifampin alone and in combination with trimethoprim. Rev. Infect. Dis. 5 (suppl.) :565-572.

Poolman J.T., Hopman G.P. and Zanen H.C. 1983. Immunogenicity of meningococcal antigens as detected in patient sera. Infect. Immun. 40 : 398-406.

Quinn P.H., Crosson F.J., Winkelstein J.A. and Moxon A.R. 1977. Activation of the alternative complement pathway by Haemophilus influenzae type b. Infect. Immun. 16 : 400-402.

Rajan V.S. and Sng E.H. 1982. Streptomycin-resistant Haemophilus ducreyi. Lancet ii : 1043.

Rauschkolb J.E. 1939. Circumcision in treatment of chancroidal lesions of male genitalia. Arch. Dermatol. Syph. 39 : 319-325.

Reenstierna J. 1921. Chancre mou experimental ches le singe et le lapin. Acta. Dermat. Venereol. 2 : 1-7.

Reymann F. 1949. An investigation of the biochemical reactions of Haemophilus ducreyi. Acta. Pathol. Microbiol. Scand. 26 : 345-353.

Reynard A.M. and Beck M.E. 1976. Plasmid-mediated resistance to the bactericidal effects of normal rabbit serum. Infect. Immun. 14 : 848-850.

Reynolds B.L. and Rowley D. 1969. Sensitisation of complement-resistant bacterial strains. Nature. 221 : 1259-1261.

Rice P.A. and Kasper D.C. 1982. Roles of blocking antibody and gonococcal outer membrane proteins. J. Clin. Invest. 70 : 157-167.

Rittenberg S.C., Penn C.W., Parsons N.J., Veale D.R. and Smith H. 1977. Phenotypic changes in the resistance of Neisseria gonorrhoeae to killing by normal human serum. J. Gen. Microbiol. 103 : 69-75.

Roantree R.J. and Pappas N.C. 1960. The survival of strains of enteric bacilli in the bloodstream is related to their sensitivity to the bactericidal effect of serum. J. Clin. Invest. 39 : 82-88.

Roantree R.J. and Rantz L.A. 1960. A study of the relationship of the normal bactericidal activity of human serum to bacterial infection. J. Clin. Invest. 39 : 72-81.

Robbins J.B., McCracken G.H., Gotschlich E.C., Orskov F., Orskov I. and Hanson L.A. 1974. Escherichia coli K1 capsular polysaccharide associated with neonatal meningitis. N. Engl. J. Med. 290 : 1216-1220.

Roberts M.C., Actis L.A. and Crosa J.H. 1985. Molecular characterisation of chloramphenicol-resistant Haemophilus parainfluenzae and Haemophilus ducreyi. Antimicrob. Agents Chemother. 28 : 176-180.

Roberts M.C. 1989. Plasmid-mediated Tet M in Haemophilus ducreyi. Antimicrob. Agents Chemother. 33 : 1611-1613.

Robinson J.P., Schuffman S.S. and Sell S.H.W. 1972. Electron microscopic studies of complexes of Haemophilus influenzae, type b, with specific antibodies. Immunology 23 : 101-105.

Ronald A.R. and Albritton W.L. 1984. Chancroid and Haemophilus ducreyi. p. 385-393. In Sexually Transmitted Diseases. Eds. Holmes K.K., Mardh P.A., Sparling P.F. and Wiesner P.J. McGraw-Hill Book Co., New York.

Ronald A.R. 1986. Laboratory diagnosis of Haemophilus ducreyi infections. p. 59-69. In recent advances in sexually transmitted diseases. Vol. 3. Eds. Oriel J.D. and Harris J.R.W. Churchill Livingstone Publishers. Edinburgh.

Ronald A.R. and Plummer F. 1989. Chancroid : a newly important sexually transmitted disease. Arch. Dermatol. 125 : 1413-1414.

Rosenbusch J.P. 1974. Characterisation of the major envelope protein from Escherichia coli. J. Biol. Chem. 249 : 8019-8029.

Ross S.C., Berberich H.M. and Densen P. 1985. Natural serum bactericidal activity against Neisseria meningitidis isolates from disseminated infections in normal and complement-deficient hosts. J. Infect. Dis. 152 : 1332-1335.

Rowley D. 1956. Rapidly induced changes in the level of non-specific immunity in laboratory animals. Br. J. Exp. Pathol. 37 : 223-234.

Rowley D. 1968. Sensitivity of rough Gram-negative bacteria to the bactericidal action of serum. J. Bacteriol. 95 : 1647-1650.

Rowley D. 1973. Antibacterial action of antibody and complement. J. Infect. Dis. (suppl.) 128 : 170-175.

Rowley D. and Turner K.J. 1968. Passive sensitisation of Salmonella adelaide to the bactericidal action of antibody and complement. Nature (London) 217 : 657-658.

Rowley D. and Wardlaw A.C. 1958. Lysis of Gram-negative bacteria by serum. J. Gen. Microbiol. 18 : 529-533.

Saelhof C.C. 1924. Observations on chancroidal infection. J. Infect. Dis. 35 : 591-602.

Salzman R.S., Kraus S.J., Miller R.G., Sottnek F.O. and Kleris G.S. 1984. Chancroidal ulcers that are not chancroid. Arch. Dermatol. 120 : 636-639.

Sanson-Le Pors M.J., Casin I.M., Ortenberg M. and Perol Y. 1982. Detection of chloramphenicol acetyltransferase (CAT) activity in a strain of Haemophilus ducreyi. An. Microbiol. (Paris) 133B : 311-315.

Satulsky E.M. 1943. Management of chancroid in a tropical theatre : report of 1555 cases. JAMA 127 : 259.

Saunders J.M. and Folds J.D. 1986. Immunoblot analysis of antigens associated with Haemophilus ducreyi using serum from immunised rabbits. Genitourin. Med. 62 : 321-328.

Schalla W.O., Sanders L.L., Schmid G.P., Tam M.R. and Morse S.A. 1986. Use of dot-immunobinding and immunofluorescence assays to investigate clinically suspected cases of chancroid. J. Infect. Dis. 153 : 879-887.

Schiller N.L., Alasard M.J. and Borowski R.S. 1984. Serum sensitivity of a Pseudomonas aeruginosa mucoid strain. Infect. Immun. 45 : 748-755.

Schmid G.P. 1986. The treatment of chancroid. J. Am. Med. Assoc. 255 : 1757-1762.

Schmid G.P., Sanders L.L., Blount J.H. and Allexander E.R. 1987. Chancroid in the United States. Reestablishment of an old disease. J. Am. Med. Assoc. 258 : 3265-3268.

Schnaitman C.A. 1970. Protein composition of the cell wall and cytoplasmic membrane of Escherichia coli. J. Bacteriol. 104 : 890-901.

Schnaitman C.A. 1971. The effect of ethylenediaminetetracetic acid, Triton X-100 and lysozyme on the morphology and chemical composition of isolated cell walls of Escherichia coli. J. Bacteriol. 108 : 553-563.

Schneider H., Griffiss J.M., Williams G.D. and Pier G.B. 1982. Immunological basis of serum resistance of Neisseria gonorrhoeae. J. Gen. Microbiol. 128 : 13-22.

Schoolnik G.K., Buchanan T.M. and Holmes K.K. 1976. Gonococci causing disseminated gonococcal infection are resistant to the bactericidal action of human sera. J. Clin. Invest. 58 : 1163-1173

Schwab G.E. and Reeves P.R. 1966. Comparison of the bactericidal activity of different vertebrate sera. J. Bacteriol. 91 : 106-112.

Shafer W.M., Joiner K., Guymon L.F., Cohen M.S. and Sparling P.F. 1984. Serum sensitivity of Neisseria gonorrhoeae : the role of lipopolysaccharide. J. Infect. Dis. 149 : 175-183.

Shaw S., Smith A.L., Anderson P. and Smith D.H. 1976. The paradox of Haemophilus influenzae type b bacteremia in the presence of serum bactericidal activity. J. Clin. Invest. 58 : 1019-1029.

Shawar R., Sepulveda J. and Clarridge J.E. 1990. Use of the RapID-ANA system and sodium polyanetholesulphonate disc susceptibility testing in identifying Haemophilus ducreyi. J. Clin. Microbiol. 28 : 108-111.

Sheldon W.H. and Heyman A. 1946. Studies on chancroid : histology. Am. J. Pathol. 22 : 415-425.

Silver R.P., Finn C.W., Vann W.F., Aaronson W., Schneerson R., Kretschmer P.J. and Garon C.F. 1981. Molecular cloning of the K1 capsular polysaccharide genes of Escherichia coli. Nature (London) 289 : 696-698.

Simberkoff M.S., Ricupero I. and Rahal Jnr. J.J. 1976. Host resistance to Serratia marcescens infection : serum bactericidal activity and phagocytosis by normal blood leukocytes. J. Lab. Clin. Med. 87 : 206-217.

Singer S. and Deacon W.E. 1956. Effects of penicillin G in vitro on Haemophilus ducreyi. Public Health Rep. 71 : 1112-1114.

Slack J.M. and Snyder I.S. 1978. Bacteria and human disease. p. 23-39. Year Book Medical Publishers. Chicago.

Slootmans L., Vanden Berghe D.A. and Piot P. 1985. Typing Haemophilus ducreyi by indirect immunofluorescence assay. Genitourin. Med. 61 : 123-126.

Slootmans L., Vanden Berghe D.A., Van Dyck E. and Piot P. 1983. Susceptibility of 40 Haemophilus strains to 34 antimicrobial products. Antimicrob. Agents Chemother. 24 : 564-567.

Smith H. 1966. Leukocytes containing bacteria in plain blood films from patients with septicemia. Australas. Ann. Med. 15 : 210-221.

Smith H. 1977. Microbial surfaces in relation to pathogenicity. Bacteriol. Reviews. 41 : 475-500.

Sng E.H., Lim L.A., Rajan V.S. and Goh A.J. 1982. Characteristics of Haemophilus ducreyi : a study. Br. J. Vener. Dis. 58 : 239-242.

Sottnek F.O., Biddle J.W., Kraus S.J., Weaver R.E. and Stewart J.A. 1980. Isolation and identification of Haemophilus ducreyi in a clinical study. J. Clin. Microbiol. 12 : 170-174.

Stamm W.E., Handsfield H.H., Rompalo A.M., Ashley R.L., Roberts P.L. and Corey L. 1988. The association between genital ulcer disease and acquisition of HIV infection in homosexual men. JAMA 260 : 1429-1433.

Steven A.C., Ten Heggeler B., Muller R., Kistler J. and Rosenbusch J.P. 1977. Ultrastructure of a periodic protein layer in the outer membrane of Escherichia coli. J. Cell. Biol. 72 : 292-301.

Strakosch E.A., Kendell H.W., Craig R.M. and Schwemlein G.X. 1945. Clinical and laboratory investigation of 370 cases of chancroid. J. Invest. Dermatol. 6 : 95-107.

Sturm A.W. and Zanen H.C. 1984. Characteristics of Haemophilus ducreyi in culture. J. Clin. Microbiol. 19 : 672-674.

Stratton C.W. and Reller L.B. 1977. Serum dilution test for bactericidal activity. 1. Selection of a physiological diluent. J. Infect. Dis. 136 : 187-195.

Stratton C.W. 1983. Susceptibility testing revisited. Prog. Clin. Pathol. 9 : 47-100.

Stratton C.W. 1988. Serum bactericidal test. Clin. Microbiol. Rev. 1 : 19-26.

Sturm A.W. and Zanen H.C. 1984. Characteristics of Haemophilus ducreyi in culture. J. Clin. Microbiol. 19 : 672-674.

Sturm A.W., Zanen H.C., Stolting G.T. and Cormane R.H. 1987. Clinical and microbiological evaluation of 46 episodes of genital ulceration. Genitourin. Med. 63 : 98-101.

Sullivan M. 1940. Chancroid. Am. J. Syph. Gonorrhea. Vener. Dis. 24 : 482-521.

Sutherland I.W. 1979. Microbial exopolysaccharides : control of synthesis and acylation. p. 1-34. In Microbial polysaccharides and polysaccharases. Eds. Berkeley R.C.W., Gooday G.W. and Ellwood C.C. Academic Press Inc. London.

Sutton A, Schneerson R., Morris S.K. and Robbins J.B. 1982. Differential complement resistance mediates virulence of Haemophilus influenzae type b. Infect. Immun. 35 : 95-104.

Swanson J. 1981. Surface-exposed protein antigens of the gonococcal outer membrane. Infect. Immun. 34 : 804-816.

Tachibana D.K. 1965. Characterisation of the bactericidal action of precolostral piglet and calf sera against Gram-negative enteric bacteria in rough phase. Folia. Microbiol. Prague. 10 : 145-155.

Tan T., Rajan V.S., Koe S.L., Tan N.J., Tan B.H. and Goh A.J. 1977. Chancroid : a study of 500 cases. Asian J. Infect. Dis. 1 : 27-28.

Taylor D.N., Duangmani C., Suvongse C., O'Connor R., Pitarangsi C., Panikabutra K. and Echeverria P. 1984. The role of Haemophilus ducreyi in penile ulcerations in Bangkok, Thailand. Sex. Transm. Dis. 11 : 148-151.

Taylor D.N., Escheverria P., Hanchalay S., Pitarangsi C., Slootmans L. and Piot P. 1985 (a). Antimicrobial susceptibility and characterisation of outer membrane proteins of Haemophilus ducreyi isolated in Thailand. J. Clin. Microbiol. 21 : 442-444.

Taylor D.N., Pitarangsi C., Echeverria P., Panikabutra K. and Suvongse C. 1985 (b). Comparative study of ceftriaxone and trimethoprim-sulfamethoxazole for the treatment of chancroid in Thailand. J. Infect. Dis. 152 : 1002-1006.

Taylor P.C., Schoenknecht F.D., Sherris J.C. and Linner E.C. 1983. Determination of minimal bactericidal concentrations of oxacillin for Staphylococcus aureus : influence and significance of technical factors. Antimicrob. Agents Chemother. 23 : 142-150.

Taylor P.W., Roberts A.P. and Gower P.E. 1972. Evaluation of a technique for the estimation of serum bactericidal activity against Gram-negative organisms. Med. Lab. Technol. 29 : 272-279.

Taylor P.W. 1974. Sensitivity of some smooth strains of Escherichia coli to the bactericidal action of normal human serum. J. Clin. Pathol. 27 : 626-629.

Taylor P.W. 1975. Genetical studies of serum resistance in Escherichia coli. J. Gen. Microbiol. 89 : 57-66.

Taylor P.W. 1976. Immunochemical investigations on lipopolysaccharides and acidic exopolysaccharides from serum-sensitive and serum-resistant strains of Escherichia coli isolated from urinary tract infections. J. Med. Microbiol. 9 : 405-421.

Taylor P.W. and Parton R. 1977. A protein factor associated with serum resistance in Escherichia coli. J. Med. Microbiol. 10 : 225-232.

Taylor P.W. 1978. The effect of the growth environment on the sensitivity of some urinary Escherichia coli strains. FEMS Microbiol. Lett. 3 : 119-122.

Taylor P.W. and Hughes C. 1978. Plasmid carriage and the serum sensitivity of enterobacteria. Infect. Immun. 22 : 10-17.

Taylor P.W. and Robinson M.K. 1980. Determinants that increase the serum resistance of Escherichia coli. Infect. Immun. 29 : 278-280.

Taylor P.W., Gaunt H. and Unger F.M. 1981. Effect of subinhibitory concentrations of mecillinam on the serum susceptibility of Escherichia coli strains. Antimicrob. Agents Chemother. 19 : 786-788.

Taylor P.W., Messner P. and Parton R. 1981. Effect of the growth environment on cell envelope components of Escherichia coli in relation to sensitivity to human serum. J. Med. Microbiol. 14 : 9-19.

Taylor P.W. 1983. Bactericidal and bacteriolytic activity of serum against Gram-negative bacteria. Microbiol. Rev. 47 : 46-83.

Taylor P.W. and Kroll H.P. 1983. Killing of an encapsulated strain of Escherichia coli by human serum. Infect Immun. 39 :122-131.

Teague O. and Deibert O. 1920. The value of the culture method in the diagnosis of chancroid. J. Urol. 4 : 543-550.

Teague O. and Deibert O. 1922. Some observations on the bacillus of Unna-Ducrey. J. Med. Res. 43 : 61-75.

Tempest D.W. and Ellwood D.C. 1969. The influence of growth conditions on the composition of some cell wall components of Aerobacter aerogenes. Biotechnol. Bioeng. 11 : 775-783.

Thayer J.D., Field F.W. and Perry M.I. 1955. In vitro sensitivity of Haemophilus ducreyi to several antibiotics. Antibiotic. Chemother. (Washington D.C.) 5 : 132-134.

Thomson J.A. and Bilgeri Y.R. 1982. Plasmid-coded ampicillin resistance in Haemophilus ducreyi. Antimicrob. Agents Chemother. 22 : 689-692.

Totten P.A., Handsfield H.H., Peters D., Holmes K.K. and Falkow S. 1982. Characterisation of ampericillin resistance plasmids from Haemophilus ducreyi. Antimicrob. Agents Chemother. 21 : 622-627.

Towbin H., Staehelin T. and Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets : procedure and some applications. Proc. Nat. Aca. Sci. USA. 76 : 4350-4354.

Tramont E.C., Sadoff J.C. and Artenstein M.S. 1974. Cross-reactivity of Neisseria gonorrhoeae and Neisseria meningitidis and the nature of antigens involved in the bactericidal reaction. J. Infect. Dis. 130 : 240-247.

Tramont E.C., Sadoff J.C. and Wilson C. 1977. Variability of the lytic susceptibility of Neisseria gonorrhoeae to human sera. J. Immunol. 118 : 1843-1851.

Traub W.H. and Kleber I. 1976. Selective activation of classical and alternative pathways of human complement by "promptly serum-sensitive" and "delayed serum-sensitive" strains of Serratia marcescens. Infect. Immun. 13 : 1343-1346.

Tuomanen E., Cozens R., Tosch W., Zak O. and Tomasz A. 1986. The rate of killing of Escherichia coli by beta-lactam antibodies is strictly proportional to the rate of bacterial growth. J. Gen. Microbiol. 132 : 1297-1304.

Van Alphen L., Havekes L. and Lugtenberg B. 1977. Major outer membrane protein d of Escherichia coli K-12. FEBS Lett. 75 : 285-290.

Van Alphen L., Riemens T., Polman J. and Zanen H.C. 1983. Characteristics of major outer membrane proteins of Haemophilus influenzae. J. Bacteriol. 155 : 878-885.

Van den Berghe D.A. 1987. Selenium and the growth of Haemophilus ducreyi. J. Clin. Pathol. 40 : 1174-1177.

Vosti K.L. and Randall E. 1970. Sensitivity of serologically classified strains of Escherichia coli of human origin to the serum bactericidal system. Am. J. Med. Sci. 259 : 114-119.

Waisbren B.A. and Brown I. 1966. A factor in the serum of patients with persisting infections that inhibits the bactericidal activity of normal serum against the organism that is causing the infection. J. Immunol. 97 : 431-437.

Wall R.A., Mabey D.C.W. and Bello C.S.S. 1985. The comparative in vitro activity of twelve 4-quinolone antimicrobials against Haemophilus ducreyi. J. Antimicrob. Chemother. 16 : 165-168.

War 1968. Spot method in agar for the quantitative assay of anti-Salmonella antibody. Jpn. J. Microbiol. 12 : 364-366.

Wardlaw A.C. 1963. The complement-dependent bacteriolytic activity of normal human serum. II. Cell wall composition of sensitive and resistant strains. *Can. J. Microbiol.* 9 : 41-52.

Wardlaw A.C. 1964. Endotoxin and complement substrate. p. 81-88. In *Bacterial Endotoxins*. Eds. Landy M. and Braun W. Rutgers State University Press, New Brunswick, N.J.

Watanabe S. 1939. Studies concerning cultivation and antigenicity of Ducrey's bacillus. *Kitasato Arch. Exp. Med.* 16 : 1-5.

Watt P.J., Glynn A.A. and Ward M.E. 1972. Maintenance of virulent gonococci in laboratory culture. *Nature New Biology.* 236 : 186-191.

Westphal O. and Jann K. 1965. Bacterial lipopolysaccharides : extraction with phenol-water and further application of the procedure. p. 83-91. In *Methods in carbohydrate chemistry*, Vol. 5. Eds. Whistler R.L. Academic Press Inc. London.

Whaley K., Schur P.H., McCabe W.R. and Ruddy S. 1980. Modulation of the alternative complement pathway in patients with Gram-negative bacteremia. *Parasite Immunol.* (Oxford) 2 : 29-37.

Wilkinson S.G. 1977. Composition and structure of bacterial lipopolysaccharides. p. 97-175. In *Surface carbohydrates of the prokaryotic cell*. Eds. Sutherland I. Academic Press Inc. London.

Willcox R.R. 1963. The treatment of chancroid. *Br. J. Clin.Pract.* 17 : 455-460.

Williams Smith H. 1974. A search for transmissible pathogenic characters in invasive strains of Escherichia coli : the discovery of a plasmid-controlled toxin and a plasmid - controlled lethal character closely associated, or identical, with colicin V. *J. Gen. Microbiol.* 83 : 95-111.

Willson P.J., Deneer H.G., Potter A. and Albritton W. 1989. Characterisation of a streptomycin-sulphonamide resistance plasmid from Actinobacillus pleuropneumoniae. *Antimicrob. Agents Chemother.* 33 : 235-238.

Wilson M.E., Jonak-Urbanczyk J.T., Bronson P.M., Dudas K.C., Apicella M.A. and Genco R.J. 1987. Capnocytophaga species : increased resistance of clinical isolates to serum bactericidal action. J. Infect. Dis. 156 : 99-106.

Winkelstein J.A. 1983. Complement and natural immunity. Clinics in immunology and allergy. 3 : 421-439.

Wright S.D. and Levine R.P. 1981(a). How complement kills E. coli. 1. Location of the lethal lesion. J. Immunol. 127 : 1146-1151.

Wright S.D. and Levine R.P. 1981(b). How complement kills E. coli. 2. The apparent two hit nature of the lethal event. J. Immunol. 127 : 1152-1156.

Young L.S. and Armstrong D. 1972. Human immunity to Pseudomonas aeruginosa 1. In vitro interaction of bacteria, polymorphonuclear leukocytes, and serum factors. J. Infec. Dis. 126 : 257-276.

Zinner S.H. and McCabe W.R. 1976. Effects of IgM and IgG antibodies in patients with bacteraemia due to Gram-negative bacilli. J. Infect. Dis. 133 : 37-45.

Zoon K.C. and Scocca J.J. 1975. Constitution of the cell envelope of Haemophilus influenzae in relation to competence for genetic transformation. J. Bacteriol. 123 : 666-77.